IMMUNIZATION VIA THE COLONIC MUCOSA

USING ADENOVIRAL VECTORS

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ABSTRACT

Sexually-transmitted diseases (STDs) are among the most common causes of illness in the world. The annual incidence of STDs is rising, from 250 million in 1990 to 340 million in 2002. Viral infection is a frequent cause of STDs and the mucosal surfaces are the natural sites of transmission of viruses. Both genital and rectal tracts are involved in transmission of viral STDs. To infect a host, a virus has to penetrate mucosal immunologic barriers, including lumenal immunoglobulin A (IgA) intervention, epithelial defenses, and lamina propria (LP) lymphocyte-mediated protection. The breach of the mucosal immune system can lead to virus spreading to the rest of the body, thereby causing life-threatening disease. Induction of effective mucosal immunity is essential for the host to control local viral infection and prevent STD development.

Immune responses are initiated when pathogen-derived antigen (e.g., viral antigen) is encountered and taken by antigen-presenting cells (APCs), especially dendritic cells (DCs). After antigen processing, DCs present immunogenic determinants to and activate naïve lymphocytes in mucosal lymphoid tissues. Activated lymphocytes leave the lymphoid tissue and, via the bloodstream, migrate to the LP. These effector cells exert a series of immune functions, such as cytokine production, cytotoxicity and antibody secretion. Accordingly, the mucosal immune system is divided into the inductive and effector site. Gut-associated lymphoid tissues (GALT) represent the inductive site of the gastrointestinal (GI) system. The GALT within the rectal mucosa

mainly consists of iliac lymph nodes (ILN), Peyer's patch-like aggregated lymphoid follicles (ALF) and isolated lymphoid follicles (ILF). The ILN have been identified as the principal inductive site, whereas the role for mucosally located lymphoid follicles remains poorly understood.

To elicit specific mucosal immune responses against virus infection, a viral antigen must be introduced into the mucosal immune system, importantly the inductive site. Efficient antigen delivery ensures the success of inducing effective mucosal immunity. Both the mucus layer and the integral epithelial monolayers form barriers against the passage of proteins and particulate matter across the epithelium. Previous studies demonstrated that these barriers could be overcome by mucus removal with ethanol treatment followed by utilizing viral vectors such as replication-deficient adenoviral vectors (Adv) engineered to encode heterologous antigen genes. Adv can infect a broad range of mammalian cells, including human and mouse epithelial cells, and has proved to be efficient in transferring genes to the colonic mucosa.

It has been discovered that mucosal immunity can be induced at multiple mucosal sites, but rarely via a systemic route. This phenomenon is largely due to the mechanism of the common mucosal immunologic system (CMIS), within which activated mucosal lymphocytes migrate from one mucosal site (e.g., the upper respiratory tract mucosa) to another (e.g. the genital tract). Thus, the concept of CMIS is used as a guiding principle for mucosal vaccine design. However, emerging results have suggested that distant

mucosal immunization is less effective than local immunization and that CMIS might comprise several anatomical-based grouped networks having different lymphocyte homing mechanisms. In this context, the vaginal (local mucosal) immunization regimen might improve local protection against genital STDs. Following the similar logic, rectally-induced immune responses might have potential to combat rectal STD infections. As both genital and rectal mucosae are drained by the ILNs, the putative genito-rectal associated lymphoid tissue, rectal immunization might be an alternative solution to genital immunization, especially to deal with the problem of immunization in the male genitourinary tract. The major goal of this study was to evaluate the effects of Adv-based local mucosal immunization via the rectum of mice in the induction of mucosal immunity against virus infection at the rectal as well as genital mucosa.

A non-invasive intrarectal (IR) delivery method (pipetting and Dermabond® following a ethanol enema) for Adv was developed in the present study to provide better gene transfer for induction of mucosal immune responses. The first approach was to re-investigate gene transfer to the mouse colon after intrarectal (IR) administration. The transgene was found highly expressed at days 1-3 and mainly confined to the colon. Gene expression was not only identified within the epithelium but also immediately beneath the epithelium, probably due to the penetration of Adv through epithelial cells. Mucosal immune responses were examined in an antigen model using ovalbumin (OVA). After IR immunization with Adv encoding OVA (AdOVA), the frequency of LP interferon (IFN)-γ secreting cells was detected as early as day 4, and continually progressed upward. The

appearance of ILN IFN-γ-secreting cells was transient, and this was mirrored by the ILN cytolytic activities. CD8$^+$ T cells were stimulated to produce IFN-γ, and cytolytic activities depended largely on CD8. Also, IR immunization with Adv induced Th1 T-cell responses and local production of specific IgA. When challenged with recombinant vaccinia virus expressing OVA, immunized mice completely controlled local viral infection and prevented virus dissemination from the rectal tissue. Thus, an infectious mouse model of herpes simplex virus type 2 (HSV-2) given via the rectum was developed in the study and used to further validate the IR immunization regimen. Mice immunized with Adv encoding glycoprotein B (AdgB) were protected from rectal challenge of HSV-2 at absolute lethal doses. Clinical pathology and virus replication were remarkably reduced and virus-shedding period was significantly lessened. CD8$^+$ T cells, IFN-γ and interleukin (IL)-12 appeared to play an essential role in such protective immunity. Protection of mice against HSV-2 challenge in the vaginal tract was also achieved, thus indicating that rectal immunization could also confer protective genital immunity. In comparison with the intranasal (distant mucosal) and subcutaneous (systemic non-mucosal) immunization, rectal immunization proved to be more effective in the induction of rectal immune responses including the frequency of IFN-γ secreting cells and IgA production, and to provide better protection against rectal or vaginal HSV-2 challenge. All these results underscored the importance of applying local mucosal immunization to induce mucosal immunity at both rectal and genital tracts.

In conclusion, IR administration with Adv by the new delivery method efficiently

transferred antigen genes into the rectal mucosa and elicited protective local immunity to virus challenge. The present study provided evidence that rectal (local mucosal) immunization regimen was a better vaccination strategy than distant mucosal and systemic non-mucosal immunization to provide both rectal and genital protection against viral infection. Thus, the present approach supports the view that route is a critical determinant of vaccination and, furthermore, represents fertile ground for future studies of mucosal vaccination via the rectal mucosa.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

xii

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AB/PAS	Alcian blue and periodic acid-Schiff's
AdgB	adenoviral vector expressing glycoprotein B
AdLuc	adenoviral vector expressing luciferase
AdOVA	adenoviral vector expressing ovalbumin
Adv	adenoviral vector(s)
Adβ-gal	adenoviral vector expressing beta-galactosidase
AIDS	acquired immunodeficiency syndrome
ALF	aggregated lymphoid follicle
APC	antigen-presenting cell
β-gal	beta-galactosidase
BALT	bronchial-associated lymphoid tissue
BM	bone marrow
BSA	bovine serum albumin
CALT	colon-associated lymphoid tissue
CAR	coxsackievirus and adenovirus receptor
CMIS	common mucosal immunologic system
CP	cryptopatch
CT	cholera toxin
CTB	B subunit of cholera toxin
CTL	cytotoxic T cell

DC	dendritic cell
dIgA	dimeric immunoglobulin A
DLN	draining lymph node
DNA	deoxyribonucleic acid
E.coli	*Escherichia coli*
E1	early region 1 of adenovirus
E3	early region 3 of adenovirus
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbant assay
ELISPOT	enzyme-linked immunospot assay
EOS	eosinophil
FAE	follicle-associated epithelium
FasL	Fas ligand
FBS	fetal bovine serum
FcRn	neonatal Fc receptor
FDC	follicular dendritic cell
FITC	fluorescein isothiocyanate
GAG	glycosaminoglycan
GALT	gut-associated lymphoid tissue
gB	glycoprotein B of herpes simplex virus
GI	gastrointestinal
GU	genitourinary

HBSS	Hanks' balanced salt solution
HEV	high endothelial venule
HIV	human immunodeficiency virus
HSV	herpes simplex virus
ICCS	intracellular cytokine staining
IEC	intestinal epithelial cell
IEL	intraepithelial lymphocyte
IFN	interferon
IFR	interfollicular region
Ig	Immunoglobulin
IG	intragastric
IL	interleukin
ILF	isolated lymphoid follicle
ILN	Iliac lymph node
IM	intramuscular
IN	intranasal
IR	intrarectal
IVAG	intravaginal
LD_{100}	absolute (100%) lethal dose
LN	lymph node
LP	lamina propria
LPL	lamina propria lymphocyte

LT   heat labile toxin of *Escherichia coli*

mAb   monoclonal antibody

MadCAM-1   mucosal addressin cell adhesion molecule-1

MHC   major histocompatibility

MIP   macrophage inflammatory protein

MLN   mesenteric lymph node

NALT   nasal associated lymphoid tissue

NK   natural killer cell

OVA   ovalbumin

P&D   pipetting and Dermabond®

PBS   phosphate buffered saline

PBST   PBS containing Tween-20

PE   phycoerythrin

PFU   plaque-forming unit

pIg   polymeric immunoglobulin

pIg-R   polymeric-immunoglobulin receptor

PP   Peyer's patch

RALT   rectal-associated lymphoid tissue

RNA   ribonucleic acid

SALT   salivary-associated lymphoid tissue

sc   secretory component

SC   subcutaneous

SCID	severe combined immunodeficient
SED	subepithelial dome
SHIV	simian/human immunodeficiency virus
SIg	secretory immunoglobulin
sIg	surface immunoglobulin
SIgA	secretory IgA
SIV	simian immunodeficiency virus
STD	sexually-transmitted disease
TCR	T-cell receptor
TGF	transforming growth factor
Th	T helper cell
TILN	targeted iliac lymph node
TK	thymidine kinase
TNF	tumor necrosis factor
Tr1	T regulatory cell
tw	tissue weight
v/v	volume/volume
VAM	vaccinia virus Ankara strain
VV	vaccinia virus
VV-OVA	vaccinia virus expressing ovalbumin
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactoside

CHAPTER 1   INTRODUCTION

1.1   An overview

There are combined effects of non-immunologic and immunologic components of host defense that prevent pathogens from invasion via the intestinal mucosal surfaces. Since it is accessible to the external environment, the colon can harbor microorganisms, many of which are potentially pathogenic. The colonic epithelium, made up of monolayer epithelial cells, physically isolates lumenal pathogens from the underlying lamina propria milieu and represents the major non-immunological barrier. However, invasive pathogens can enter the mucosa by trans-epithelial transcytosis after having infected epithelial cells or in an abraded or lacerated condition especially during sexual practices. When the epithelial layer is unable to halt pathogen entry, infection can occur. In this case, The mucosal immunological barrier functions take place to provide further resistance [Lehner *et al.*, 1999].

The mucosal immunological barrier against pathogenic invasion can be established after pathogen-derived antigens have been exposed to the mucosal immune system. The mucosal lymphoid tissues and peripheral lymphoid organs share common architectural features and operate on similar principles in terms of antigen presentation

and the induction of antigen-specific immune responses [Bienenstock *et al.*, 1999]. Antigen-presenting cells (APCs) beneath the epithelium can acquire pathogen-derived antigens [Neutra, 1999] and present them to migratory mucosal lymphocytes, which are stimulated to become effector cells specific for the antigen [Reyes *et al.*, 1997]. By this means, intestinal immune cells are able to survey gut antigens and mount specific immune responses to eliminate invading pathogens. Therefore, in order to prepare the mucosal immune system for specific responses, antigens must be introduced to the mucosal immune system.

The successful introduction of antigens into the mucosa depends on the antigen delivery system and the route of administration. With a potent antigen delivery system, the mucosal immune system can be manipulated to function more efficiently than after natural infection of wild pathogen species [Cripps *et al.*, 2001; Ogra *et al.*, 2001]. Delivery of a vaccine via an appropriate route leads to activation and homing of lymphocytes that provide immune protection at certain mucosal sites [Ryan *et al.*, 2001]. It has been suggested that local mucosal immunization (at the mucosal site where immune protection is desired) induces better local immunity than distant mucosal immunization (at a mucosal site which is anatomically distant from the mucosal site for protection and where protection might not be required), while systemic immunization, applied at a non-mucosal site, is least effective [Gallichan *et al.*, 1993; Gallichan and Rosenthal, 1995; Kantele *et al.*, 1997; Kozlowski *et al.*, 1997; Belyakov *et al.*, 1998b; Ferko *et al.*, 1998; Shen *et al.*, 2000; Kozlowski *et al.*, 1999; Belyakov *et al.*, 2001;

Vajdy *et al.*, 2001]. Thus, both vaccine tools and the route of vaccine delivery are critical determinants for induction of mucosal immune protection.

Local mucosal immunization to induce colonic immune responses against sexually transmitted infections via the rectal route has recently been a topic in mucosal vaccination. Compared to the upper respiratory tract, lung, oral-gastric tract, small intestine, and genitourinary (GU) tract, the rectum is the least studied mucosal route for vaccination. Few studies have successfully induced colonic immune responses against rectally delivered infectious agents. One of the reasons for the failure to achieve immunity via this route probably results from insufficient antigen delivery. A possible solution to this problem is the use of a viable non-pathogenic intracellular vector capable of expressing ample heterologous antigens. Replication-deficient recombinant adenoviral vectors (Adv) have been used to transfer sufficient amounts of antigen to induce robust immune responses and protection against virus infection both systemically [Shiver *et al.*, 2002; Fitzgerald *et al.*, 2003; Sullivan *et al.*, 2003] and mucosally [McDermott *et al.*, 1989b; Gallichan *et al.*, 1993; Lubeck *et al.*, 1994; Juillard *et al.*, 1995; Van Ginkel *et al.*, 1995; Buge *et al.*, 1997; Van Ginkel *et al.*, 1997; Shanley and Wu, 2003]. Another reason might be associated with utilizing inefficient vaccine delivery methods which are not able to prevent leakage of delivered reagents from the colon as a result of the bowel movement. Therefore, for vaccination at the colonic mucosa, there is a need of developing a suitable immunization method for rectal delivery of Adv and induction of local protective immunity.

3

Evaluation of immunological outcomes is a critical step to validate a vaccine candidate. In order to do so, vaccine-induced cellular and humoral immune responses and, more importantly, protection from a subsequent challenge of pathogens should be examined. Also, an appreciation of relevant immunologic mechanisms provides critical clues on how vaccination strategies might be optimized to potentiate host defenses [Letvin *et al.*, 2002; Nabel, 2002].

In the present study, a new method for vaccine delivery at the rectal mucosa was developed and was found to be efficient in transfer of heterologous genes into the colon, resulting in significant gene expression. As compared to systemic and distant mucosal routes, a single immunization with Adv encoding antigen genes by the rectal route induced stronger antigen-specific local mucosal immune responses and provided better rectal as well as genital protection against virus challenge at lethal doses.

1.2   Mucosal immune system

1.2.1   The inductive and effector sites

The mucosal immune system includes immune cells and organized mucosal lymphoid tissues, such as Peyer's patches (PPs) and bronchial associated lymphoid tissue

(BALT) and solitary lymphoid aggregates, encompassing the epithelium and the lamina propria (LP), as well as mucosally-associated draining lymph nodes (DLNs). The role of the mucosal immune system is to provide immune protection against pathogens on one hand and tolerance to food antigens and natural flora on the other hand [Mowat, 2003]. Like the systemic compartment, mucosal immune responses are generally thought to be induced in organized mucosal lymphoid tissues and then possibly exert their effects at the local and distant mucosal surfaces such as in the LP. Accordingly, the mucosal immune system is functionally classified into two distinct sites, namely inductive and effector, which are spatially separated [Brandtzaeg et al., 1999a; Mowat, 2003]. In the gut, organized lymphoid follicles located within intestinal mucosa (for example PPs) and DLNs (for example mesenteric lymph nodes, MLNs) represent the inductive site (Figure 1.1a), while the epithelium and LP are regarded as the effector site (Figure 1.1b). It has been recently suggested that the inductive and effector sites might be structurally overlapped to a certain extant, e.g., immune responses might be induced in the LP [Kelsall and Strober, 1999; Fagarasan and Honjo, 2003]. Memory T cells specific to viral infections could be detected in both PPs [Belyakov et al., 1998b; Rayevskaya and Frankel, 2001] and MLNs [Arnaud-Battandier et al., 1978; Issekutz, 1984a; Issekutz, 1984b; Chen et al., 2001]. Moreover, uncommitted B cells were found to be activated to undergo isotype switching and differentiate into IgA-producing plasma cells in the LP [Fagarasan et al., 2001]. Thus, there might be a dual function at each mucosal immune anatomical structure in terms of immune induction and effector activities.

Figure 1.1 Schematic representation of the intestinal immune system. (a) The inductive

site comprises organized intestinal lymphoid tissues and draining lymph nodes (DLNs).

①: Antigens from the intestinal lumen can be taken by the epithelial cells. ②: Induction

of intestinal immune responses involves variety of organized lymphoid follicles and

immune cells distributed throughout the intestinal mucosa. ③: Antigen loading dendritic

cells (DCs) can migrate to the DLNs for antigen presentation and lymphocyte activation.

After activation, effector lymphocytes including IgA-producing cells and T cells leave

the DLNs and enter the bloodstream. (b) The effector site consists of the epithelial layer

and LP. ①: Effector lymphocytes migrate into local or a distant mucosal site from the

blood circulation and exert immune functions. ②: IgA-producing cells become plasma

cells and secrete immunoglobulin A (IgA), most of which are dimeric IgA (dIgA). dIgA

can be transcytosed by epithelial cells into the intestinal lumen and functions to

neutralize antigens or microorganisms. ③: Effector T cells can recognize and kill cells

infected with intracellular pathogens such that pathogens can be eliminated. ④:

Systemically primed lymphocytes rarely migrate to the mucosa and thus have limited

contribution to mucosal immunity. This figure was adapted from Nagler-Anderson

[2001], Trapani and Smyth [2002], Young et al. [2002], Fagarasan et al. [2002], Finn

[2003], Mowat [2003], and Phalipon and Corhesy [2003].
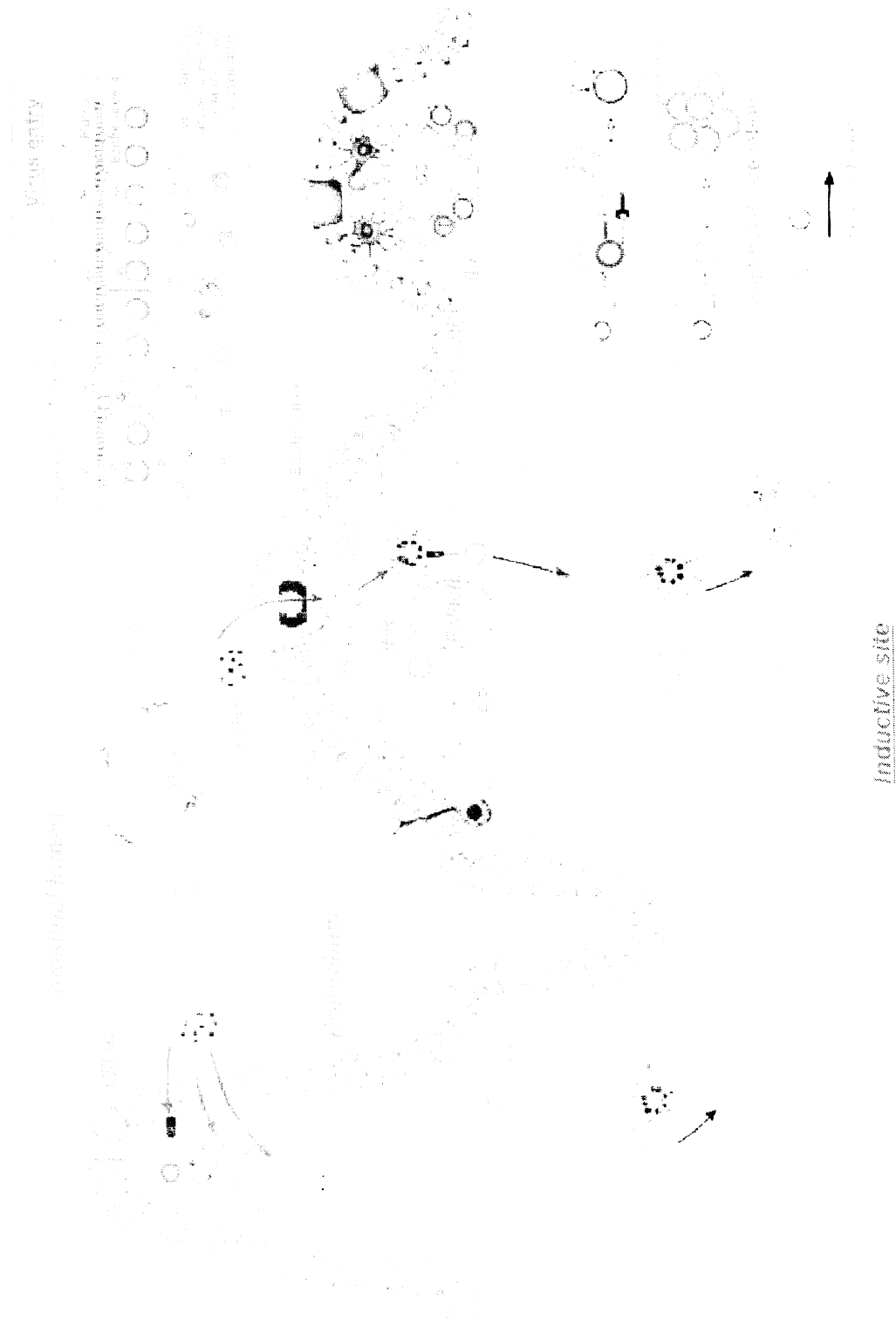
(Figure 1.1a)
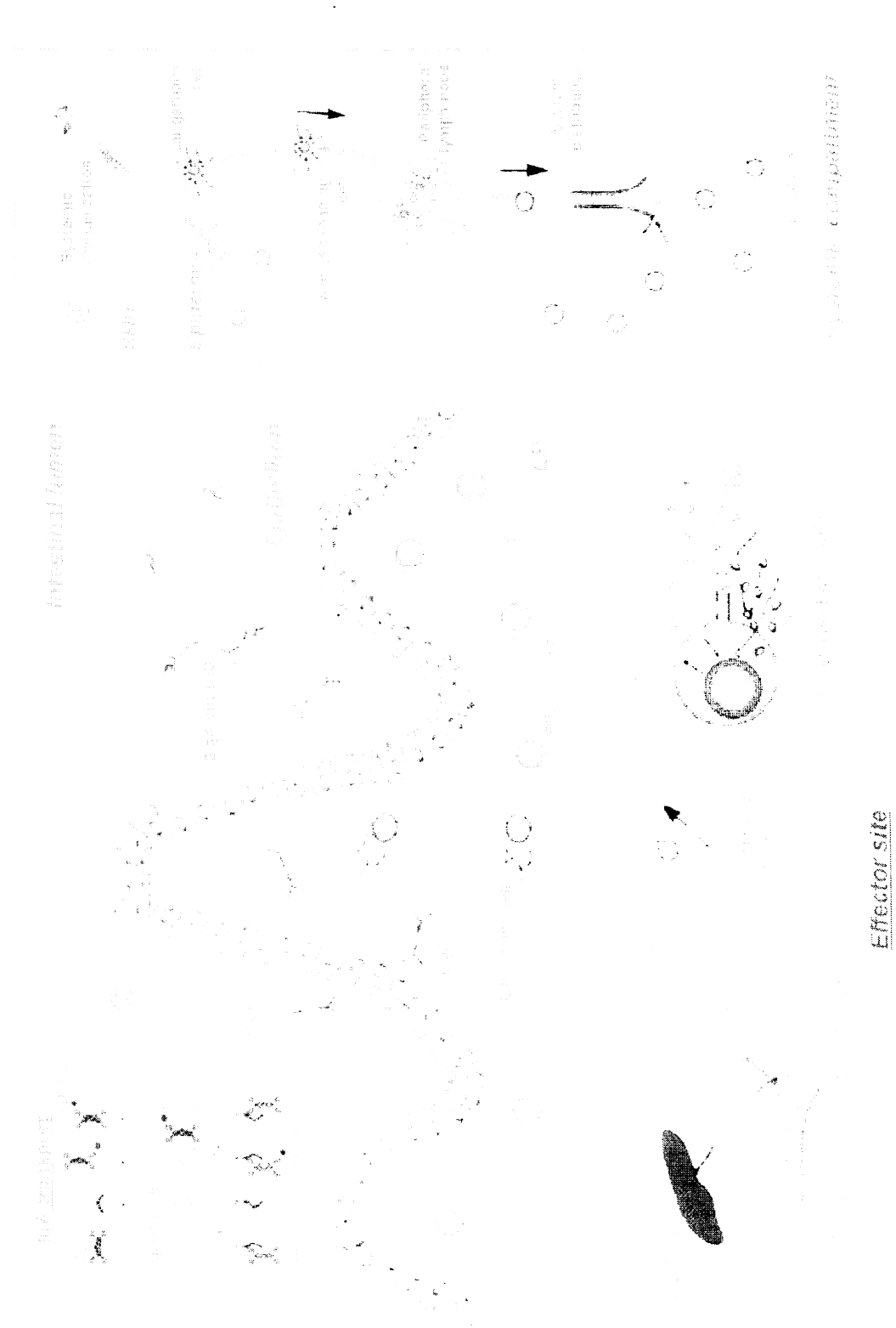
(Figure 1.1b)

1.2.2   Mucosal lymphoid tissues

Mucosal lymphoid tissues refer to all immune cells within LP or organized lymphoid follicles in the mucosal tissue aligned along the length of respiratory, gastrointestinal (GI) tract and female genital tract [Brandtzaeg, 1997; Bienenstock *et al.*, 1999; McGhee *et al.*, 1999], which act as surveillance sites for the mucosal immune system. They are termed mucosal-associated lymphoid tissue (MALT) [McDermott and Bienenstock, 1979; Bienenstock *et al.*, 1999]. MALT includes salivary-associated lymphoid tissues (SALT) in the oral cavity, nasal- and bronchus-associated lymphoid tissues (NALT and BALT) in the respiratory system, gut-associated lymphoid tissues (GALT) in the aeroalimentary and GI tract, and genital-associated lymphoid tissues in genitourinary tract. The GALT comprises mucosal lymphoid follicles (palatine tonsils, appendix and PPs formed in different anatomic GI segments), DLNs (MLNs and iliac lymph nodes or ILNs) and LP [Kelsall and Strober, 1999; Nagler-Anderson, 2001]. Mucosal lymphoid follicles within mucosal tissues and associated lymph nodes (LNs) are important inductive sites of mucosal immunity. Compared to LNs such as MLNs, lymphoid follicles are less organized and have no afferent lymphatics. In general, these follicles contain specialized epithelial cells termed the microfold or M cells scattered within the overlying epithelial layer. Mucosal follicles also have organized B-cell follicles with germinal centers and adjacent T-cell regions. There are efferent lymphatics that link these follicles for cell trafficking from mucosa to DLNs but there are no afferent lymphatics for cell recruitment. By expressing the adhesion molecule L-selectin, mucosal

9

follicles recruit naïve lymphocytes from the blood circulation through high endothelial venules (HEV), the specialized postcapillary venules that provide L-selectin ligand only present in LNs (e.g., MLNs) and lymphoid follicles (e.g., PPs) but not in LP [Butcher, 1999]. LP postcapillary venules serve as a homing site for integrin $\alpha4\beta7$-expressing activated lymphocytes by presenting mucosal addressin cell adhesion molecule (MAdCAM)-1. This preferentially allows naïve lymphocytes to transmigrate through HEV endothelial cells and enter lymphoid follicles for priming, and lymphocytes are selectively prohibited from entering LP unless they are activated [Butcher, 1999].

PPs are the most representative and studied GALT [Neutra *et al.*, 2001; Mebius, 2003]. PPs are distributed throughout the entire small intestine and, due to their large size, can be identified often by the unaided eye, especially in rodents, as tiny nodules that disperse underneath the epithelium of the antimesenteric wall of the bowel. PPs comprise follicle-associated epithelium (FAE), subepithelial dome (SED), B-cell follicles, and interfollicular regions (IFR). FAE, formed by conventional epithelial cells and scattered specialized M cells, overlies the SED with the B-cell follicles that contains a germinal center. IFRs are rich in T cells and contain HEV for cell recruitment. There are efferent lymphatics for cell emigration to DLNs. In the large intestines, including cecum, colon and rectum, there are M-cells overlain aggregated lymphoid follicles (ALFs) which resemble PPs. ALFs are localized in the crypts and found in humans [Langman and Rowland, 1986; O'Leary and Sweeney, 1986] and mice (only BALB/c mice have been investigated and difficulties in identification in some strains such as SJL) [Perry and

Sharp, 1988; Owen *et al.*, 1991;. Although functions of ALFs in the large intestine have not been fully examined, these mucosal structures are thought to play a role similar to their counterparts PPs. Whether ALFs are associated with follicular lymphoid hyperplasia, which increase in size in colitis of humans [Haque *et al.*, 1993; Yeung *et al.*, 2000] and mice [Dohi *et al.*, 1999; Dohi *et al.*, 2000], is unclear.

In addition to PP and ALF, there are a larger number of similarly structured but considerably smaller follicles that are termed isolated lymphoid follicles (ILFs). ILFs were found in the small intestine and ileum in humans [Moghaddami *et al.*, 1998], rabbits [Keren *et al.*, 1978] and guinea pigs [Rosner and Keren, 1984]. Mouse ILFs were recently identified both in small (~100-200 follicles in crypts and villi) and large (~50 follicles) intestines of both BALB/c and C57BL/6 mice [Hamada *et al.*, 2002]. Compared to PPs, ILFs generally contain a single B-cell follicle but lack T-cell enriched IFRs because T cells are interspersed in a relatively small numbers within the follicle [Hamada *et al.*, 2002]. ILFs are thought to function similarly to PPs and regarded as a failsafe system to PPs; PP^{null} mice (lacking PPs but with normally developed ILFs) still generate a strong IgA response [Yamamoto *et al.*, 2000; Lorenz *et al.*, 2003].

A tremendous number of tiny follicles termed cryptopatches (CPs) within the intestinal mucosa are structurally different from the aforementioned follicles. CPs are well distributed in both small (~1500 follicles) and large intestines of mice and do not contain germinal centers [Kanamori *et al.*, 1996]. CPs play an indispensable role in the

11

generation of extrathymic subset of CD8αα intraepithelial cells [Suzuki *et al.*, 2000; Oida *et al.*, 2000]. Therefore, the functions of CP differ from those of PPs or ALFs and ILFs in mucosal immune induction.

DLNs also play a crucial role in the induction of mucosal immunity and tolerance, serving, to some extent, as the crossroads between local and systemic pathways [Mowat, 2003]. Naïve lymphocytes can be activated in intestinal LNs by newly arrived APCs after being loaded with antigen in lymphoid follicles or LP [MacPherson and Liu, 1999] and activated lymphocytes leave LNs and enter the bloodstream via the thoracic duct [Karrer *et al.*, 1997; Nagler-Anderson, 2001; Mowat, 2003]. MLNs are the largest lymph nodes in the body that drain lymph from the small intestine, while ILNs are situated bilaterally along the iliac arteries and veins in the pelvis, in close proximity to the bifurcation of the vessels, and drain lymph from both large intestine and genitourinary tract [Lehner *et al.*, 1999]. Thus, Lehner [2003] proposed ILNs as genito-rectal associated lymphoid tissue for the induction of both colonic and genital immune responses.

Although ILNs play an inductive role, understanding to the rest of the rectal- or colon-associated lymphoid tissue (RALT or CALT) is still poor. ALF and ILF are less characterized for their functions probably due to the technical difficulties in isolating lymphoid cells from these follicles. However, structures of these lymphoid tissues might suggest the likelihood of their participation in immune induction of the lower GI tract.

1.2.3   Mucosal immune cells

1.2.3.a   Intestinal epithelial cells

Intestinal epithelial cells (IECs) are closely opposed to one another via tight junctions and form epithelial monolayers as a physical barrier. IECs physiologically control the selective movement of macromolecules between the lumen and the LP and minimize fluid and electrolyte loss into the lumen such that nutrients are absorbed and intestinal homeostasis is maintained. IECs secrete microbicidal and antiviral agents, such as complement and defensins, into the lumen and protect mucosa in a non-antigen-specific way [Ganz, 2003]. The structural integrity of epithelium separates harsh fecal contents and invasive microorganisms in the lumen from the underlining mucosa comprising internal milieu [Bhalla and Owen, 1982]. Therefore, IECs represent the first line of host defense against invasion of pathogens from the external environment of the gut lumen [Pitman and Blumberg, 2000] (Figure 1.1).

Epithelial-based protection is enhanced by the mucus layer covering the apical surface of epithelium. Other than lubricating fecal passage, the presence of mucus enhances the host resistance to the hostile attachment of pathogens or toxins [Cone, 1999]. The chief constituent of mucus is mucin, which is a group of mucoproteins present in both secreted and membranes-associated forms. The latter has a highly glycosylated extracellular domain that can extend up to several hundred nanometers from the cell

surface [Corfield *et al.*, 2000].

A number of mucus-mediated protection mechanisms have been proposed. The viscous quality of mucin enhances the depth of unstirred layer overlying the epithelial surface and reduces the diffusion of molecules larger than nutrition molecules. The carbohydrate moieties act as competitors for binding of lumenal proteins and microorganisms to the epithelial cells as they are analogous to the glycoprotein and glycolipid receptors that exist on IECs. These prevent the adherence of bacteria and viruses to epithelial cell surfaces by forming into mucus-microorganism complexes which are subsequently propelled down the intestinal tract [Sanderson and Walker, 1999]. The most concentrated glycoprotein layer in the mucosal surface is glycocalyx, being part of non-secreted mucin and forms a 10~500 nm-thick glycoprotein layer. The glycocalyx is the final layer that prevents pathogens from adhering to epithelial cells. Twenty-nanometer thickness of glycocalyx is sufficient to prevent access of 1 μm of microparticles to glycolipid receptors [Frey *et al.*, 1996]. Along the colonic epithelium, mucus is increasingly produced to be thicker and continuously covers the colon because of increasing numbers of goblet cells as compared to the small intestine [Szentkuti and Lorenz, 1995; Matsuo *et al.*, 1997; Atuma *et al.*, 2001]. Therefore anti-invasive epithelial function in the colon is enhanced.

IECs are able to transport dimeric IgA (dIgA) into the lumen and prevent pathogen attack. This is mediated by polymeric-immunoglobulin receptor (pIgR), a

membrane bound molecule present on epithelial cells of secretory mucosa [Rojas and

Apodaca, 2002; Phalipon and Corthesy, 2003]. The pIgR is synthesized within epithelial

cells and expressed on the basolateral membrane to capture free as well as antigen-bound

dIgA [Underdown *et al.*, 1977; Garcia-Pardo *et al.*, 1981]. Cytokines such as interleukin

(IL)-4 and interferon (IFN)-γ can upregulate pIgR expression and enhance IgA transport

capacity via the receptors [Phillips *et al.*, 1990]. When a dIgA binds specifically to pIgR,

the pIgR-dIgA complex is endocytosed and then transcytosed through the cytoplasm by

an endosome to the apical surface of IECs. Interaction between pIgR and dIgA represents

a ten-fold higher association constant compared to the antigen-antibody binding. The

dissociation of binding is significantly slower than the time required for transport,

ensuring that the complex has sufficient time to travel across the epithelial cell and be

presented on the apical surface [Nagura *et al.*, 1979]. After transcytosis, dIgA is secreted

on the cell surface as a secretory IgA (SIgA) which is the dIgA that binds to a secretory

component (sc), a polypeptide generated by cleavage from pIgR [Mostov and Deitcher,

1986]. dIgA bound to pathogens or antigens derived from LP can also be transported by

pIgR across adjacent epithelial cells in the same manner as free dIgA [Sixbey and Yao,

1992; Gan *et al.*, 1997; Robinson *et al.*, 2001]. IECs thus exert particular protective

immune functions by secreting IgA to prevent attack from lumen pathogens or expel

pathogens which gained entry into epithelial cells.

IECs are defined as playing an important role also in both innate and adaptive

immune responses by allowing antigen to enter LP via the transcellular pathway across

the epithelial cells, especially M cells, in addition to the paracellular pathway directly

through the tight junction [Sanderson and Walker, 1999]. M cells are specialized IECs,

scattered among conventional epithelial cells in follicular associated epithelium (FAE),

which covers lymphoid tissues of respiratory and gut mucosae including large intestines

[Langman and Rowland, 1986; O'Leary and Sweeney, 1986; Jacob *et al.*, 1987; Owen *et

al.*, 1991; Gullberg *et al.*, 2000]. M cells function to initiate the antigen sampling at the

apical surface by non-specific binding of macromolecules or microorganisms present in

the lumen and transporting these across the epithelium to the basolateral membrane

[Neutra *et al.*, 1996]. Due to the specific interaction of M cells with some viruses and

bacteria and their ability to transport these microorganisms, prototype vaccines have been

developed by targeting the M cell [Forrest *et al.*, 1990; Andino *et al.*, 1994; Phalipon and

Sansonetti, 1999]. In contrast to M cells, conventional IECs mainly mediate the passage

of soluble antigens by pinocytosis and the transport of IgG-bound antigen through Fc

receptors [Hussain *et al.*, 1991; Blumberg *et al.*, 1995]. Adult human IECs express major

histocompatibility (MHC) class I-like neonatal Fc receptors (FcRn), which exhibit

specificity for monomeric IgG. FcRn might serve to bind lumenal-derived immune

complexes composed of IgG and foreign antigens, thereby transferring them into the LP

across the epithelial barrier [Dickinson *et al.*, 1999; Spiekermann *et al.*, 2002]. From this

location, antigens can be internalized and processed by professional APCs such as

dendritic cells and presented to lymphocytes beneath the epithelium [Pitman and

Blumberg, 2000]. IECs also process and transport antigens via their MHC class II

molecules [Hershberg *et al.*, 1998; Hershberg and Mayer, 2000]. This is however

believed to be highly associated with antigen presentation [Mayer and Blumberg, 1999;

Pitman and Blumberg, 2000; Zimmer *et al.*, 2000]. Therefore, IECs are important in

antigens uptake and transport to the LP as well as antigen presentation to mucosal

lymphocytes.

1.2.3.b   Mucosal dendritic cells

As the most potent professional APC, dendritic cells (DCs) have been well

documented for their critical role in the induction of the adaptive immune response and

immune regulation [Steinman, 1991; Matzinger, 1994; Banchereau *et al.*, 2000; Heath

and Carbone, 2001b; Liu et al., 2001b; Shortman and Liu, 2002; Steinman *et al.*, 2003].

DCs originate from pluripotent stem cell precursors in the bone marrow (BM). Blood-

borne DC precursors enter the peripheral tissues and maintain their immature stage until

they encounter invading pathogens or antigens [Sallusto and Lanzavecchia, 1999; del

Hoyo *et al.*, 2002; Ardavin, 2003]. Except for normal brain parenchyma, DCs are present

in all tissues, including intestinal mucosae in which they are strategically distributed in

PP, LP and, as recently identified, within the epithelial layer [Maric *et al.*, 1996;

Rescigno *et al.*, 2001] (Figure 1.1a).

For the immune surveillance of pathogenic invasion, DCs have two distinct

functional stages, immature and mature, linked by a transitional phase [Cella *et al.*,

1997]. During their immature stage, DCs act as sentinel cells by acquiring and processing

antigens at non-lymphoid sites. Immature DCs capture antigens efficiently through various pathways, including phagocytosis (virus, bacteria and apoptotic cells) [Inaba *et al.*, 1993; Reis e Sousa *et al.*, 1993; Svensson *et al.*, 1997; Albert *et al.*, 1998a; Albert *et al.*, 1998b], receptor-mediated endocytosis (immune complexes) [Jiang *et al.*, 1995; Sallusto *et al.*, 1995; Arnold-Schild *et al.*, 1999] and macropinocytosis (soluble antigens, microbial fragments) [Crowley *et al.*, 1990; Sallusto and Lanzavecchia, 1994; Inaba *et al.*, 1998]. After processing antigen, DCs enter a mature stage and are no longer able to capture antigens but gain the ability to migrate to regional LNs and participate in eliciting antigen-specific immune responses through interactions with T and B cells [Steinman, 1991; Flores-Romo, 2001].

DCs utilize different organelles to process captured antigens. For endogenously synthesized cytosolic proteins of intracellular pathogens, such as virus or certain bacteria, antigenic peptides are generated by proteosomes and transported via transporter associated with antigen processing (TAP) into the endoplasmic reticulum (ER), where newly synthesized major histocompatibility complex (MHC) class I molecules uploaded with peptides are delivered onto the cell surface for the presentation to CD8$^+$ T cells [Koopmann *et al.*, 1997]. Infection of DCs by intracellular pathogens is not required for this pathway because they can acquire antigens also from other infected cells and cross-prime activate CD8$^+$ T cells by cross-presentation of processed antigens [Heath and Carbone, 2001a; Heath and Carbone, 2001b; Prasad *et al.*, 2001]. For soluble foreign proteins and microbial fragments, DCs are able to degrade these in endosomes and,

through the classical MHC class II pathway, transport the resultant peptides onto the cell surface for presentation to CD4$^+$ T cells, which are important to stimulate antibody responses against extracellular antigens [Sallusto and Lanzavecchia, 1994; Svensson *et al.*, 1997; Inaba *et al.*, 1998]. Therefore, DCs can process antigens through MHC class either I or II pathway to suit the needs of immune responses.

DCs in the intestine also have the ability to acquire antigens from the lumen and elicit immune responses. DCs isolated from the subepithelial dome (SED) of PPs stimulated naïve T-cell proliferation after ovalbumin (OVA) feeding in mice [Kelsall and Strober, 1996] and were able to produce IL-4 and IL-10 in such responses [Iwasaki and Kelsall, 1999a]. Freshly isolated immature DCs from PPs presented antigen to and stimulated resting PP T cells when cultured in the presence of granulocyte macrophage-colony stimulating factor (GM-CSF) and tumor necrosis factor (TNF)-α [Ruedl *et al.*, 1996; Ruedl and Hubele, 1997]. Antigen-bearing PP DCs in the SED could migrate to the MLN or within PP [Iwasaki and Kelsall, 1999b]. Lamina propria dendritic cells (LP DCs, diffuse in LP rather than within lymphoid tissues) of the lung, which were loaded with bacterial antigens, were found in the afferent intestinal lymph toward DLNs [McWilliam *et al.*, 1994]. It was demonstrated that DCs recovered from the lymphatics were able to induce antigen-specific T-cell responses both *in vitro* and *in vivo* [Liu and MacPherson, 1991; Liu and MacPherson, 1993]. Although there exists the possibility of LP DCs priming lymphocytes within the intestinal LP, DCs are thought to preferentially migrate to DLNs for immune induction [MacPherson and Liu, 1999; Mowat, 2003].

Recent data showed that there might be three phenotypically distinct subtypes of PP DCs localized in different regions of PPs [Iwasaki and Kelsall, 2000]. $CD8\alpha^-CD11b^+CD11c^+$ DCs were identified in SED and $CD8\alpha^+CD11c^+$ DCs in IFR, whereas $CD8\alpha^-CD11b^-CD11c^+$ DCs (double negative for both CD8α and CD11b) were found in both sites. DCs were able to migrate from SED into IFR after microbial stimulation probably due to the upregulated expression of the chemokine receptor CCR7 [Iwasaki and Kelsall, 2000]. These PP DCs might migrate reciprocally toward chemoattractant macrophage inflammatory protein (MIP)-3α and MIP-3β, which were found to be present in the SED and IFR, respectively [Iwasaki and Kelsall, 2001]. By resembling their splenic counterparts, these $CD8\alpha^-$ and $CD8\alpha^+$ PP DCs susbsets were shown to also have the ability to produce Th1 and Th2 cytokines, respectively [Iwasaki and Kelsall, 2001]. Thus, each identified subset might reflect of functional and maturational status of DCs.

Colonic DCs are distributed in both humans [Pavli *et al.*, 1993, Pavli *et al.*, 1996; Bell *et al.*, 2001] and mice [Hamada *et al.*, 2002; Krajina *et al.*, 2003]. DC accumulation in the colonic LP was found to be associated with the development of T-cell mediated colitis [Leithauser *et al.*, 2001, Krajina *et al.*, 2003]. Although DCs have been identified within ILFs, whether and how DCs are disposed in all colonic lymphoid tissues is unknown. Additionally, the role for colonic DCs in the induction of local mucosal immune responses is not yet understood.

1.2.3.c   Intraepithelial lymphocytes

The intestinal epithelium contains T lymphocytes termed intraepithelial lymphocytes (IELs). IELs are interspersed throughout the small and the large intestines, situated between the basolateral surface of IEC and the tight junction (Figure 1.1a). Intestinal IELs are a heterogeneous population made up of T-cell receptor (TCR) $\alpha\beta$ and $\gamma\delta$, CD3-expressing T cells. Remarkably, compared to systemic T cells, IELs contain a higher percentage of $\gamma\delta$ T cells mainly expressing CD8$\alpha\alpha$ and there are greater than 70% of CD8$^+$ T cells in the $\alpha\beta$ IELs subpopulation [Lefrançois and Puddington, 1999; Hayday et al., 2001]. In the small intestine, 90% of IELs are CD8$^+$, the majority of which express $\gamma\delta$, while in large intestine of humans and mice IELs are primarily of CD8$^-$CD4$^-$ double negative or CD4$^+$ natural killer T cells (NKT) expressing either $\alpha\beta$ or $\gamma\delta$ TCR [Lefrançois and Puddington, 1999; Pitman and Blumberg, 2000]. IELs provide protective function to control pathogenic invasion by eliminating infected epithelial cells [Goodman and Lefrancois, 1988; Hayday et al., 2001]. Both $\alpha\beta$ and $\gamma\delta$ IELs have NK-like non-specific cytotoxicity and contribute to the surveillance of epithelium [Kawaguchi et al., 1993]. TCR$\alpha\beta$ IELs can be primed against virally- and bacterially-infected epithelial cells in adaptive immune responses, while $\gamma\delta$ IEL exhibit cytolytic activity and produce IFN-γ mainly in a non-specific way [Hayday et al., 2001].

$\gamma\delta$ T cells perform critical physiological and immunological functions in the intestine. The presence of $\gamma\delta$ T cells might be important for the development and

immunologic functions of IEC as they produce keratinocyte growth factor (KGF), an epithelial cell growth factor [Boismenu and Havran, 1994]. γδ T cells play a stimulatory role in antibody production. Transfer of γδ IEL can restore impaired IgA synthesis in γδ-knockout mice [Fujihashi *et al.*, 1996]. Various lines of evidence have shown that γδ T cells are crucial to the induction of mucosal tolerance. The presence of γδ T cells correlated with the induction of oral tolerance and removal of these cells either by administration of monoclonal antibodies (mAbs) or targeted gene deletion completely abrogated or failed to maintain oral tolerance [Mengel *et al.*, 1995; Ke *et al.*, 1997; Wildner *et al.*, 1996]. γδ T cell-mediated tolerance via inhalation of aerosolized antigen has been also found in respiratory mucosa [McMenamin *et al.*, 1994; Harrison *et al.*, 1996]. The negative role of IELs might efficiently limit unwanted immune responses mediated through epithelial cells which express MHC and co-stimulatory molecules during inflammation. Overall, γδ T cells display more negative regulation than positive roles such as protection against pathogens [Hayday, 2000]. Comparatively, the number of γδ IELs becomes progressively less from the small intestine to the colon; approximately 39% of small intestine IECs versus only 3% of colonic IECs, while αβ IEL are the predominant lymphocyte type in the colon [Floochi, 1990; Hayday *et al.*, 2001]. Such differences in cell components as related to anatomic location are very likely to be due to the necessity for adjustment of immune response type in different segments of the GI tract, i.e. an adaptive antimicrobial response to intracellular pathogens, to which the large intestine is more frequently exposed than the small intestine [James and Graeff, 1985; Poccia *et al.*, 2001].

1.2.3.d   Lamina propria T cells

Within the LP, there exists a large quantity of T lymphocytes termed lamina propria T cells (LP T cells, Figure 1.1). Like peripheral T cells, LP T cells represent a heterogeneous group (CD4$^+$ and CD8$^+$) of functional lymphocytes. LP T cells might be substantially different from peripheral T cells due to the evidence of activated phenotypes and high potentials for cytokine release upon exposure to antigens. LP T cells have an equal distribution of CD4$^+$ and CD8$^+$ [Selby et al., 1983; James et al., 1986] and possess cytotoxic activity as they express markers such as Fas ligand (FasL) [De Maria et al., 1996]. The vast majority of them represent a specialized memory T cell subset, characterized by the phenotype of CD45RO^{hi} (a memory cell surface marker), which is in contrast to peripheral T cells that do not normally express this marker [Schieferdecker et al., 1990]. LP T cells are highly differentiated effectors with a great ability to produce cytokines such as IFN-γ, IL-2, IL-4 and IL-5 [Taguchi et al., 1990; Saparov et al., 1997]. As compared to their peripheral counterparts, however, LP T cells have poor proliferative responsiveness to lumenal antigens [James et al., 1987; Khoo et al., 1997] or anti-CD3 stimulation [Qian et al., 1991]. This might suggest that highly active LP T cells usually keep potential infections under control and use a raised threshold mechanism to avoid unnecessary expansion in response to the antigen-rich mucosal environment [Kelsall and Strober, 1999].

1.2.3.e   Lamina propria B cell

Lamina propria B (LP B) cells are composed of IgM$^+$/IgD$^+$ B cells and IgA

plasma cells (Figure 1.1), the latter of which are the main population and are derived

from lymphoid tissues such as PP and BALT. It is generally believed that PP-derived

plasma cells are primarily IgA-secreting cells, while resident IgM$^+$/IgD$^+$ B cells

presumably differentiate into only IgM- and IgG-secreting plasma cells as they do when

stimulated *in vitro* [Sanderson and Walker, 1999]. Recent studies showed that

uncommitted LP B cells performed isotype switching in the LP and served as a source of

IgA-producing plasma cells [Fagarasan *et al.*, 2001]. This might suggest that intestinal

LP also provides milieu for antibody isotype switching to IgA, although confirmation is

warranted in prospective studies. Other than secreting antibodies, B cells also have

antigen-presenting functions. B cells can take up soluble antigens through their cell-

surface immunoglobulins and present peptides via constitutively produced MHC class II

compartments. In association with co-stimulatory molecules, antigen-bearing B cells are

able to activate T cells. However, it is generally believed that B cells cannot efficiently

activate naïve T cells as compared to DCs [Banchereau *et al.*, 2000].

1.2.3.f   Other mucosal effector immune cells   ,

A large number of intestinal macrophages reside beneath the epithelial layer of

the small and large intestine. Macrophages undergo local activation in response to microbial and inflammatory stimuli and are able to phagocytose microorganisms or infected cells (through the engagement of their receptors on the surface), and fulfill their scavenging functions by producing various mediators and cytokines/chemokines [Celada and Nathan, 1994; Aderem and Underhill, 1999; Murray and Nathan, 1999; Savill *et al.*, 2002]. Most macrophages express undetectable to low levels of MHC class II, and their role in antigen presentation and T-cell activation proved to be ineffective [Pavli *et al.*, 1990; Pavli *et al.*, 1993]. Macrophages interact with activated T cells [Underhill *et al.*, 1999], and their low expression of MHC class II depends on T-cell derived Th1 and Th2 type cytokines, including IFN-γ, IL-4 and IL-13 [Dalton *et al.*, 1993; Boehm *et al.*, 1997; Gordon, 2003].

Eosinophils (EOS) reside in the stomach and intestinal mucosae in large quantity and play a critical role in response to parasitic infection as well as the regulation of gastrointestinal allergy [Dombrowicz and Capron, 2001]. EOS are localized not only in LP but also in PPs and their localization is critically regulated by IL-5 over-expression during Th2 responses as well as by the chemokine eotaxin which is constitutively expressed in the intestine [Rothenberg *et al.*, 2001]. Recently, attention has been drawn to intestinal EOS and studies found that EOS exhibit potentials in regulatory and inflammatory immune responses in the GI tract, for example their contribution to proinflammatory cytokine production and the development of colitis [Makiyama *et al.*, 1995; Garcia-Zepeda *et al.*, 1996; Nishitani *et al.*, 1998; Woerly *et al.*, 1999; Hogan *et*

al., 2001]. Natural killer (NK) cells contain preformed perforin and granzymes and exert

cytotoxic activity in the innate immune system [Biron *et al.*, 1999]. NK cells also play an

important role in regulating the functions of DCs, including promoting DC maturation by

producing tumor necrosis factor (TNF)-α or kill DCs lacking MHC class I [Moretta,

2002].

1.2.4 The common mucosal immunologic system

The concept of the common mucosal immunologic system (CMIS) was proposed

by McDermott and Bienenstock [1979] on the basis of their earlier observations that

transferred MLN IgA$^+$ B blasts, but not peripheral LN blasts, selectively repopulated (or

"homed to") the mucosal sites, such as gut, cervix and vagina, uterus, and mammary

glands, while only 8% did so in peripheral LNs. Importantly, only a small proportion of

cells isolated from peripheral LNs entered mucosal sites. This established the concept

that immune cells primed at one mucosal site might travel to a distant site to serve as

effectors, and systemic boosting might be only used to elevate mucosal immune response

but could not prime naïve mucosal cells because of the lack of preference mucosal

homing of systemic lymphocytes [Mestecky, 1987; Crotty *et al.*, 1999] This application

of CMIS was later confirmed by other studies in both animals [Gallichan and Rosenthal,

1995; Gallichan and Rosenthal, 1996b; Balmelli *et al.*, 1998] and humans [Kantele *et al.*,

1997; Kozlowski *et al.*, 1997; Kantele *et al.*, 1998]. Therefore, the CMIS might represent

an immunological cross-communization circuit that involves both inductive and effector

sites (Figure 1.1). Recent findings further suggest that the possession of the specific homing receptor pairs, such as the interaction of $\alpha 4\beta 7$ and mucosal addressin cell adhesion molecule (MAdCAM)-1 on the endothelium of mucosal venules, in combination with chemoattraction, might be the key mechanism by which the lymphocytes are able to undertake their tissue-selective trafficking to the gut [Berlin *et al.*, 1993; Butcher and Picker, 1996; Baggiolini, 1998; Kunkel and Butcher, 2003].

CMIS also comprises compartmentalized immunologic networks [Brandtzaeg *et al.*, 1999b]. First, McDermott and Bienenstock [1979] demonstrated that lymphocytes derived from mediastinal (bronchial) LNs migrated to the lungs but not to the intestine. A similar pattern was observed later in another study which showed that intratracheal immunization only led to the presence of antibody-forming cells in the lung and not in the gut [van der Brugge-Gamelkoorn *et al.*, 1986]. Other studies also demonstrated that immunization at one site induced immune response at a certain distant sites but not all mucosal sites [Kozlowski *et al.*, 1997; Kantele *et al.*, 1998; Kozlowski *et al.*, 1999; Kozlowski *et al.*, 2002]. Second, it was observed that activated lymphocytes tended to home preferentially to the mucosal site where they were primed and induced better local immunity as compared to at distant mucosal sites [Hopkins *et al.*, 1995; Kozlowski *et al.*, 1997; Brandtzaeg *et al.*, 1999b]. Therefore, CMIS reveals a site-dependent mechanism for mucosal lymphocyte homing probably associated with regional networks that might be anatomically and functionally grouped.

1.3   Mucosal immune response

1.3.1   Induction of adaptive immune response

Induction of adaptive immune responses specific for pathogen-derived antigens is the key to control infections without being harmful to immunologic self-antigen and self-antigen-expressing cells [Zinkernagel and Hengartner, 2001]. The adaptive immune response is the response of clonally-selected, antigen-specific lymphocytes and the generation of immunological memory of these lymphocytes. Both clonal selection and antigen specificity are the features that distinguish the adaptive immune response from the innate immune response. Adaptive immune responses are induced generally in the secondary lymphoid organs such as LNs and the spleen rather than peripheral tissues where pathogens or antigens are encountered [Karrer *et al.*, 1997; Picker and Siegelman, 1999]. DCs capture antigens often near the site of infection or in regional LNs and migrate to the lymphoid organs to activate naïve lymphocytes cells recruited from the bloodstream [Lanzavecchia and Sallusto, 2000a; Guermonprez *et al.*, 2002]. Adaptive immune responses are initiated when naïve T cells for the first time encounter antigen on the surface of DCs through the interactions of TCR with antigenic peptides bound to the MHC molecule presented by APCs (termed Signal 1) in association with the engagement of co-stimulatory molecules (termed Signal 2, involving primarily B7/CD28) [Robey and Allison, 1995] (Figure 1.1a). This leads the T cells to undergo clonal expansion and become antigen-specific, armed effector cells in significant numbers to combat infected

(target) cells [Medzhitov and Janeway, Jr., 1998]. With help from DCs and activated antigen-specific T helper (Th) cells, B cells are stimulated to undergo isotype switching and become antibody-producing cells [Banchereau *et al.*, 2000; McHeyzer-Williams *et al.*, 2000]. Activated lymphocytes can generate immunological memory and provide a rapid and efficient protection from subsequent challenge by the antigen encountered during the priming phase [Lanzavecchia and Sallusto, 2000b]. Therefore, induction of adaptive immune response is critical in the generating an antigen-specific, long-lasting immunological barrier against pathogen invasion.

1.3.2   Induction of mucosal immune responses

1.3.2.a   Immune responses induced by epithelial cells

Mucosal immune responses usually begin with antigen uptake by intestinal epithelial cells (IECs). Although the M cells express MHC class II molecules, the fairly low numbers and scattered distribution of such specialized cells might mean that their capacity is too limited to be main APCs in PPs and other lymphoid follicles compared to the role of conventional epithelial cells and DCs [Kelsall and Strober, 1999]. Conventional IECs express both class I and class II MHC molecules and function as non-professional APCs to interact with immune cells on the basolateral side through direct contact [Mayer and Blumberg, 1999]. Although IECs express MHC class I molecules,

few studies have shown the ability of IECs to directly prime MHC class I-restricted CD8$^+$ effector T beneath the intestinal epithelium. However, IECs might be more efficient to interact with CD4$^+$ T cells through the MHC class II molecules which are preferentially expressed on the basolateral membrane along the small and large intestines [Hershberg *et al.*, 1998]. This polarized expression is associated with antigen presentation to the LP immune cells [Zimmer *et al.*, 2000]. In contrast to M cells that take up particulate matter, IECs generally capture soluble antigens at the apical surface by pinocytosis. Antigens are processed within the endosomes and transcytosed and finally delivered onto the basolateral side in association with MHC class II molecules [Hershberg and Mayer, 2000; Pitman and Blumberg, 2000] (Figure 1.1a). In the absence of inflammation, co-stimulatory molecules CD80 and CD86 are normally absent on the surface of IECs in the small and large intestines [Hershberg and Mayer, 2000]. It is conceivable that lack of signal 2 might contribute to the generation of anergic or tolerant CD4$^+$ T cells [Sanderson *et al.*, 1993]. When inflammation is present, co-stimulatory molecules are profoundly expressed by IECs which become possible to play a role in stimulating immune responses [Ye *et al.*, 1997; Pitman and Blumberg, 2000]. Therefore, IECs function as a non-professional APC to regulate or induce mucosal immune responses under certain circumstances.

1.3.2.b   Immune responses induced in mucosal lymphoid

follicles

Induction of antibody responses is believed to occur within mucosal lymphoid

follicles such as PPs, ILFs and possibly ALFs. PPs have been the most studied lymphoid

tissue as an inductive site of IgA-producing cells, the dominant antibody-producing cells

in the mucosal surfaces when local, but not systemic, immunization or infection occurs

[Craig and Cebra, 1971, Weinstein and Cebra, 1991] (Figure 1.1). Lumenal antigens,

such as microbes or soluble antigens, gain access to the PP across the FAE and are

trapped in the SED by immature DCs [Neutra *et al.*, 2001]. During processing invading

antigens, DCs differentiate into their mature stage with particular markers and up-

regulated co-stimulatory molecules and, in association with MHC class I and class II

compartments, present antigens to T and B cells within the PP [Neutra *et al.*, 2001].

Uncommitted naïve IgM-bearing B cells recruited from the blood encounter antigens and

interact with activated PP CD4$^+$ Th cells via cognate interaction within the PP. The B-cell

follicle and CD4$^+$ T-cells enriched areas in the IFR are overlapped and form a mixed

zone, where T-B-interactions are proposed [MacLennan *et al.*, 1997]. The interaction

between the CD40 and the CD40L combined with the regulatory effect from cytokines,

such as transforming growth factor (TGF)-β, produced by the CD4$^+$ T cells results in

preferential isotype switching of the surface IgM (sIgM) to sIgA, rather than sIgG

[Weinstein and Cebra, 1991; Xu-Amano *et al.*, 1993]. In association with follicular

dendritic cells (FDC) in the germinal center of the B-cell follicle, B cells undergo affinity

maturation and differentiation into sIgA$^+$ B cells. Once having entered effector sites, these cells undergo terminal differentiation to become mature IgA-secreting cells [McIntyre and Strober, 1999].

In the interfollicular region (IFR) of PP, antigen-bearing mature DCs are able to stimulate resting CD4$^+$ and CD8$^+$ T cells recruited from the circulation by crossing the HEV located in IFR. Some DCs move out through the efferent lymphatics into the MLNs and activate T cells therein. If DCs are less differentiated or remain immature during antigen processing, it might lead to the generation of regulatory CD4$^+$ T cells that produce TGF-β and IL-10 to induce or maintain tolerance to antigens from the lumen [Mowat, 2003]. Through the lymphatic system and the blood circulation, activated T cells then home in the gut or other mucosal tissues and carry out their final effector functions [McIntyre and Strober, 1999].

Whether lymphocytes can be primed in the LP away from mucosal lymphoid follicles remains unclear. A recent study suggested that isotype switching of B220$^+$IgM$^+$ B cells to IgA-producing cells might occur in the LP [Fagarasan *et al.*, 2001]. It was then questioned about the origin of studied B cells and the microenvironment to support isotype switching because of the possible contamination of already-switched sIgA$^+$ cells often dispersed in the LP [Brandtzaeg *et al.*, 1999c]. One possibility for the origin of these B cells might be the isolated lymphoid follicle (ILF) diffuse in the LP with large numbers [Fagarasan *et al.*, 2002]. These post-witched ILF B cells probably enter the LP

interstitium and act as effectors.

1.3.2.c Immune responses induced by lamina propria dendritic cells

LP DCs are believed to migrate through the subepithelial mucosa and take up antigens that have penetrated through the epithelium (Figure 1.1a). The migration of intestinal DCs from mucosal tissues to regional LNs has been demonstrated to be associated with the initiation of immune responses in early studies [Pugh *et al.*, 1983; Mayrhofer *et al.*, 1986; MacPherson and Liu, 1999; Mowat, 2003]. MacPherson and colleagues [1995] showed that freshly isolated LP as well as PP DCs were as potent as Langerhans' cells in stimulation of mixed lymphocyte reaction (MLR, a primary T-cell response generated *in vitro* against allogeneic stimulators). Further, they found that LP DCs were also heterogeneous and similar to PP and MLN DCs with respect to phenotypes [Liu *et al.*, 1998]. They defined two subsets of LP DCs obtained from rats that were functionally distinct from each other in T-cell activation; one subset double positive for CD4 and CD172 were effective inducing T-cell responses while the double negative cells were not [Liu *et al.*, 1998]. Whether such classification correlates with maturational status or antigen processing and presentation is unclear. It has been proposed that LP DCs process and present antigens from the intestinal lumen directly to LP T cells rather than necessarily migrating into the DLNs. However, there has been no evidence to support this claim. In addition, it remains to be determined whether antigen-

loading DCs migrate through the interstitial tissue into widely distributed lymphoid follicles (PP, ALF or ILF) to activate T cells.

DCs highly expressing CD11c and MHC class II molecules can be identified also within the intestinal follicle-associated epithelium (FAE) and the conventional epithelium [Mayrhofer *et al.*, 1983; Maric *et al.*, 1996; Kelsall and Strober, 1997]. Bienenstock and his colleagues [1996] immunohistochemically localized MHC class II-expressing rat intraepithelial DCs in both jejunum and colon, thus suggesting that DCs might browse incoming antigens within the epithelium. Recent findings showed that DCs might actually pass through tight junctions and reach the apical surface to sample lumenal environment [Rescigno *et al.*, 2001]. It is likely that the previously identified intraepithelial DCs were those LP DCs that were able to migrate to the interepithelial space.

1.3.3 Humoral immune responses

Antibody responses develop such that plasma cells have the capacity to secrete a large amount of antibodies, particularly mucosal IgA, in response to antigen present at the intestinal mucosal surfaces which are constantly challenged by the presence of various pathogens [Lamm, 1997; Corthesy and Kraehenbuhl, 1999] (Figure 1.1b). Intestinally-produced IgA has some distinguishing features compared to its counterparts derived from systemic plasma cells [Mestecky *et al.*, 1999]. Other than PPs, most of LP

plasma cells locally produced IgA class [Benson and Strober, 1988; Fagarasan and Honjo, 2003], resulting in predominance of IgA over other classes of immunoglobulins. Ninety-five percent of intestinal IgA is polymeric, rather than monomeric form found in the serum. Most intestinal IgA molecules are dIgA, polymerized in plasma cells and secreted as a polymeric form [Parkhouse *et al.*, 1971; Yoo *et al.*, 1999] (Figure 1.1b). Two monomeric IgA units of a dIgA are linked by the J chain which is important in the interaction with polymeric-immunoglobulin receptors (pIgR) for IgA trans-epithelial transport [Johansen *et al.*, 2001]. Polymerization of immunoglobulin substantially slows down the dissociation of antibodies from their antigenic epitopes, thereby increasing the avidity and enhancing the capability of recognizing repetitive epitopes expressed by pathogens [Brandtzaeg and Farstad, 1999; Mostov and Kaetzel, 1999]. On the epithelial surface, dIgA bound to a secretory component (sc) cleaved from pIgR is secreted into the lumen as the secretory IgA (SIgA), a form that is resistant to proteolytic degradation [Crottet and Corthesy, 1998].

Mucosal IgA provides important local defense functions to prevent invading pathogens [Lamm, 1997; Corthesy and Kraehenbuhl, 1999; Rojas and Apodaca, 2002] (Figure 1.1b). SIgA can interact with lumenal antigen and pathogens and neutralize their ability to penetrate the epithelium [Bukawa *et al.*, 1995; Hocini and Bomsel, 1999; Alfsen *et al.*, 2001; Silvey *et al.*, 2001]. During IgA transcytosis, pIgR-bound IgA can also interact with invading viruses and newly synthesized proteins inside epithelial cells and, hence, intracellularly neutralize virus infection and prevent assembly of new viruses

[Mazanec *et al.*, 1992; Burns *et al.*, 1996; Fujioka *et al.*, 1998]. Beneath the epithelium, dIgA molecules also form immune complexes with antigens or pathogens that either have entered or have been produced in the LP and use the pIgR pathway to transport them to the lumen and expulse in mucus secretions [Taylor and Dimmock, 1985; Kaetzel *et al.*, 1991c; Kaetzel *et al.*, 1994; Robinson *et al.*, 2001].

IgM is the first antibody isotype to be produced by plasma cells. Elevated levels of antigen-specific serum IgM are indicative of a recent antigen exposure. Polymeric IgM (pIgM) can be mucosally detected and provide a measure of mucosal defense, albeit the production is very low compared to that of IgA as a result of a paucity of IgM-producing cells in the mucosa. Similar to dIgA, pIgM can also be transcytosed by pIgR and secreted at the apical surface of epithelial cells as a form of secretory IgM (SIgM) associated with sc [Rojas and Apodaca, 2002; Phalipon and Corthesy, 2003]. SIgM is however not transported as efficiently as SIgA due to its higher molecular weight [Lamm, 1997].

IgG predominates over other isotypes in the systemic compartment, including the blood, breast milk, peritoneal and lymphatic fluid. In the mucosal secretions, levels of IgG are generally similar to that of IgM, but higher concentrations can be found in the genitourinary (GU) tract and secretory fluids such as semen, urine and vaginal secretions, with tears [Kruze *et al.*, 1989; McDermott *et al.*, 1990; Sirigu *et al.*, 1997]. Although local IgG-producing cells were also found, the major contribution comes from serum IgG entering GU compartments by transudation [Brandtzaeg, 1997; Sirigu *et al.*, 1997;

36

Anderson and Pudney, 1999; Mestecky and Fultz, 1999]. Instead of being transported by pIgR, antigen-bound IgG was found to be directed to the intestinal lumen by the pIgR-mediated excretory pathway in association with dIgA (a "piggyback" fashion) [Kaetzel *et al.*, 1991b].

At the intestinal mucosa, the humoral immune response is prevailed by preference of IgA production and the trans-epithelial transport mechanisms facilitating IgA secretion. This holds important implications for the development of vaccines that can induce significant production of specific IgA against infection.

1.3.4   Cellular immune responses

1.3.4.a   T-cell mediated immune responses

After completing differentiation, antigen-specific effector T cells emigrate from DLNs through the thoracic duct and enter the bloodstream. Under the guidance of cell-adhesion molecules on local blood vessels supplying the mucosa, circulating effector T cells migrate into the mucosal tissues, particularly to the site of infection where a gradient of local chemoattractants is formed as a result of infection [Kunkel and Butcher, 2002; Kunkel and Butcher, 2003]. Specific binding of the antigen to effector cells occurs when an antigen on target cells is recognized by effector T cells. This allows effector T cells to

specifically focus on their target. Whereas $CD4^+$ T cells can function to stimulate B cells for further differentiation or activate macrophages, $CD8^+$ cytotoxic T cells are triggered to release cytotoxic factors and cytokines that can kill, for example, virally-infected (target) cells [Kagi et al., 1996; Russell and Ley, 2002] (Figure 1.1b). These functions of specific T cells provide important host defense mechanisms by which effector $CD8^+$ T cells efficiently clear intracellular infection that is occurring and $CD4^+$ T cells stimulate B cells to produce antibodies to prevent pathogens from attacking other cells. Hence, T-cell mediated immune responses are critical in the control and prevention of infection.

1.3.4.b   Cytotoxic effector T cells

Cytotoxic T cells (or cytotoxic T lymphocytes, CTLs referred to $CD8^+$ effector T cells) play an essential role in the control of infection. CTLs can be generated in the presence of $CD4^+$ T cells which provide critical help by either stimulating APCs to express high levels of co-stimulatory molecules to interact with $CD8^+$ T cells or secrete IL-2 to activate neighboring $CD8^+$ T cells [Miceli and Parnes, 1991] (Figure 1.1a). $CD8^+$ effector T cells are able to identify and attack target cells, e.g., virally-infected cells expressing antigen via the MHC class I molecules [Harty et al., 2000]. Recognition of MHC/peptide complexes on target cells by the TCR triggers $CD8^+$ cytotoxic T cells to rapidly release various cytotoxic molecules, exerting cytolytic activity in an antigen-specific way [Zinkernagel and Doherty, 1974; Lukacher et al., 1984]. Perforin is required to interact with the membrane of target cells by forming a transmembrane pore, causing

osmotic damage of the cell membrane which allows granzyme B to penetrate the cell

membrane and program the target cell to death [Kagi *et al.*, 1994a; Wilson, 1996;

Topham *et al.*, 1997]. Also, $CD8^+$ cytotoxic T cells can kill target cells by providing

FasL, which interacts with Fas expressed on target cells and induces apoptosis [Topham

et al., 1997; Russell and Ley, 2002]. Therefore, upon recognition of antigens, CTLs can

kill their targets to control infection by utilizing performed cytotoxic molecules or

expressing death ligands.

1.3.4.c   Th1 T-cell response to intracellular pathogens

On the basis of their bioactivity pathway, cytokines are classified as types 1, 2 and

3. In type 1 cytokine pathway, IL-12 is the initiating molecule [Trinchieri, 2003], and the

"master" cytokine that induces tumor necrosis factors (TNFs) and IFN-γ which both are

the effector cytokines in cell-mediated immune responses [Guidotti and Chisari, 2001] as

well as in Th1 type gut inflammation [Strober *et al.*, 2002]. Type 2 cytokines, including

IL-4, IL-5 and IL-6, play an important part in B-cell activation, isotype switching and

production of IgA and IgE. Transforming growth factor (TGF)-β, constitutively

expressed in the intestine as a typical type 3 cytokine, down-regulates IFN-γ response and

plays a critical role in IgA isotype switching [Letterio and Roberts, 1998]. Therefore,

binding of cytokines and their receptors leads to activation, enhancement or even

alteration of gene expression in the responding cells, thereby shaping the immune

response that follows.

Other than through a cell-cell interaction with targets, T cell-mediated mucosal immune responses against viral infection are achieved also by focal regulation of various cytokines produced upon activation. The involvement of cytokines from either DCs or Th cells is critical in determining the type of immune responses. Effector $CD4^+$ Th cells are classified as Th1, Th2 and Th3 according to the type of skewing cytokines they produce [Murphy and Reiner, 2002]. Depending on the circumstances when they first encounter antigens, activated $CD4^+$ T cells staging at Th0 are able to differentiate into either Th1 cells or Th2 cells [Kelso et al., 1991]. The Th1/Th2 division appears to be driven by different DC subsets [Maldonado-Lopez et al., 1999; Pulendran et al., 1999], which interact specifically with T cell subsets [den Haan et al., 2000; Kronin et al., 2001; Pooley et al., 2001] and induce T cells to proliferate or undergo apoptosis [Suss and Shortman, 1996; Hochrein et al., 2001]. Therefore, DCs can push Th0 toward either Th1 or Th2, while biased Th cells will influence ensuing responses by further releasing skewing cytokines [Moser and Murphy, 2000].

Host resistance to infections critically depends on the Th response polarization. Given a appropriate situation, such as pathogenic products, stimulatory molecules, T cell-derived impact and preexisting cytokine microenvironment, DCs can be induced to secrete IL-12 and by which DCs mediate a Th1 cell-based cytokine polarization [Langenkamp et al., 2000; Schulz et al., 2000; Maldonado-Lopez et al., 2001; Straw et al., 2003; Trinchieri, 2003]. In general, Th1 cells lead the immune response toward cell-mediated immunity, whereas Th2 cells favor humoral immunity [Constant and Bottomly,

1997]. Attenuation of Th1 T-cell responses can disable the immune system to undertake

virus clearance, while enhanced Th1 polarization promotes resistance to viral infections

[Sher *et al.*, 1992; Clerici and Shearer, 1993; Romagnani *et al.*, 1994; Suhrbier and Linn,

2003]. Thus, successfully induced and maintained Th1 cell responses are typically

involved in controlling the infection caused by intracellular pathogens.

IFN-γ displays its critical roles in the augmentation and maintenance of the Th1

cell response, including IL-12 production, cytotoxic effector T-cell development, and

suppresses IL-4-mediated Th2 responses [Parronchi *et al.*, 1992; Mosmann and Sad,

1996]. IFN-γ stimulates IgG2a subclass switching but suppresses IL-4 and antagonizes

IL-4-induced IgG1 and IgE differentiation [Snapper and Paul, 1987; Stevens *et al.*, 1988].

IFN-γ is secreted by activated $CD4^+$ Th1 and $CD8^+$ cytotoxic T cells as well as NK cells

and exhibits an important non-cytolytic way to clear viruses from infected cells without

causing massive tissue destruction [Guidotti and Chisari, 2001]. It is thus defined as the

key direct antiviral cytokine in addition to other immunobiological functions such as

bactericidal activity, inducing cell proliferation, apoptosis and stimulating expression of

MHC class I and II and other genes [Boehm *et al.*, 1997; Katze *et al.*, 2002]. It has been

shown that IFN-γ can reverse the downregulated MHC class I expression on the cells

infected with herpes simplex virus (HSV) type 1 (HSV-1) [Mikloska *et al.*, 1996]. This

thus suggested that IFN-γ also provided help to CD8-mediated cytotoxic T-cell

responses.

1.3.5   Mucosally-induced tolerance

Tolerance involves either deficient T-cell responses due to insufficient activation

[Ohashi *et al.*, 1991], anergy or apoptosis [Jenkins and Schwartz, 1987; Shevach, 2000],

or downregulated T-cell activities due to active suppression by T regulatory (Tr1) cells

[Shevach, 2000; Roncarolo *et al.*, 2001]. Induction of mucosal tolerance to an antigen

depends on several parameters including the nature of the antigen, its association with

adjuvants, the locale where antigen is taken up, and local cytokine milieu [Mowat and

Viney, 1997]. Mucosally-induced tolerance is usually induced by oral administration of

soluble antigens in the absence of adjuvant [Weiner, 1997; Strober *et al.*, 1998].

DCs might play a critical role in the induction and maintenance of tolerance

[Steinman *et al.*, 2000; Dhodapkar *et al.*, 2001; Albert *et al.*, 2001]. IECs might also play

a role as they normally lack expression of co-stimulatory molecules when infection or

inflammation is absent [Sanderson *et al.*, 1993; Zimmer *et al.*, 2000]. Ingestion of soluble

antigen repeatedly at low doses leads to the induction of tolerance mediated by $CD4^+$ T

cells, which produce TGF-β and IL-10 to suppress both Th1 and Th2 cell responses in an

antigen-specific manner [Chen *et al.*, 1995; Khoo *et al.*, 1997; Gutgemann *et al.*, 1998]. It

has been postulated that antigen presentation in the PP leads to the development of TGF-

β-producing cells, possibly the Tr1 cells, which self-sustain themselves and are

independent of, but can be influenced by, the presence of Th1 or Th2 cell responses

[Kelsall and Strober, 1999; Fujihashi *et al.*, 2001]. Although PPs might be an important

organ in the induction of tolerance in addition to the activation of immune responses, recent studies suggest that PPs are not indispensable due to the role of MLNs as the absence of PPs does not abolish the induction of tolerance in mice [Spahn *et al.*, 2001; Spahn *et al.*, 2002]. The involvement of isolated lymphoid follicles (ILF) in this regard is unknown. Peripheral immune response might also contribute significantly to oral tolerance induction due to the systemic presentation of orally administered antigen [Gutgemann *et al.*, 1998]. Overall, mucosal tolerance is generated via different mechanisms in order to avoid unwanted immune responses and potentially damage in the mucosal tissues.

1.3.6   Mucosal immune response to virus infection

It has long been observed that high levels of antibodies, such as IgA and IgG, in sera and at mucosal surfaces are associated with reduced viral titers following infection with viruses such as respiratory syncytial virus, simian immunodeficiency virus (SIV) and HSV [Kuklin *et al.*, 1998; Wang *et al.*, 2000; Kato *et al.*, 2001; Haynes *et al.*, 2002]. The protective role for IgA in mucosal defense against viral infection has been found primarily in the gut (Figure 1.1b), while IgG antibodies predominate in the lung and vaginal tract [Milligan and Bernstein, 1995b; Yuan *et al.*, 1996; Parr *et al.*, 1998]. Antibodies can be the response against both free virus particles in the intestinal lumen or LP and infected cells. Both antigen-specific IgA and IgG can recognize virus-derived antigenic epitopes such as glycoproteins and, through physical binding, the potential

interactions of the virus with host cells are prohibited, i.e. virus neutralization [Heyman, 2000; Parren and Burton, 2001]. IgA-bound virus complexes can be transcytosed by epithelial cells into the lumen and expelled to the exterior, or from the apical surface to the basolateral membrane and then taken up by LP APCs for antigen presentation [Kaetzel *et al.*, 1991; Sixbey and Yao, 1992; Kaetzel *et al.*, 1994; Gan *et al.*, 1997]. Antibodies also bind viral proteins expressed by virus-infected cells to either direct cell lysis or prevent cell-to-cell transmission by inhibiting virus release [Burton, 2002]. Enhanced antibody responses result from increased numbers of viral-specific, antibody-producing plasma cells. It has been shown that induction of higher numbers of local IgA-producing cells correlates with better protection against rotavirus challenge (through the oral route) [Yuan *et al.*, 1996]. IgG-producing cells have been shown to be increased dramatically by 80-fold in the vaginal tissues after vaginal immunization with attenuated herpes simplex virus type 2 (HSV-2) [Parr and Parr, 1997a]. Therefore, antibodies act through various mechanisms to prevent, reduce and control viral infection.

Although antibodies are important in host defense of mucosal surfaces against virus infection, antibody-mediated immune responses might not suffice to eradicate virus infection. This is probably due to the low binding activity to the virus protein on infected cells [Burton, 2002]. Once hidden inside LP cells (negative for pIgR), viruses might not be accessible to antibodies [Lamm, 1997]. In this case, viruses can be eliminated primarily through either destruction of infected cells or cessation of virus replication inside cells [Doherty and Christensen, 2000; Guidotti and Chisari, 2001]. Under such

circumstances, cell-mediated cytotoxic anti-virus immune responses are necessary.

NK cells are one type of cytotoxic cells that might be involved in early host response to viral infection. This idea is suggested by the observation that NK-deficient patients did not completely lose the ability to control the infection as compared to immunocompetent populations but experienced more severe infection, for example, with HSV [Biron et al., 1989]. However, animal studies showed that NK cells produced IFN-γ during the early phase of infection, and depletion of NK cells did not delay virus clearance [Milligan and Bernstein, 1997]. It was demonstrated that NK cells failed to clear the influenza virus in T- and B-deficient mice [Bot et al., 1996]. These observations suggested that NK cells might be synergetic in anti-virus responses but not essential.

CD4$^+$ T cells also demonstrated activity to resist viral infections, such as influenza [McDermott et al., 1987; Taylor et al., 1990; Eichelberger et al., 1991] and HSV [Manickan et al., 1995; Sin et al., 1999; Sin et al., 2000]. Due to the limitation in access to MHC class I expressing virus infected cells, CD4$^+$ T cells were found to be more important in helping CD8$^+$ T cells to be primed through DCs, exert cytotoxic functions and establish immune memory [McMichael and Rowland-Jones, 2001]. Therefore, reduced CD8$^+$ T-cell activity due to the impaired response of CD4$^+$ T cells has been a major problem for patients to combat infection of human immunodeficiency virus (HIV) [McMichael and Rowland-Jones, 2001].

Viral antigens are typically associated with MHC class I molecules or a pathway that leads to cross-presentation by DCs. Various lines of evidence have shown that cytotoxic $CD8^+$ T effector cells (or CTLs) are able to fulfill the task of virus clearance [Doherty and Christensen, 2000; Berzofsky *et al.*, 2001] (Figure 1.1b). These cells recognize viral peptides complexed with MHC molecules on target cells via their T-cell receptor (TCR). Thus, the killing of targets is mediated by the αβ TCR expressed on virus-specific $CD8^+$ effector T cells which are restricted to MHC class I presented viral peptides [London and Rubin, 1999]. The perspective on the critical role of $CD8^+$ T effectors in anti-viral infection is strengthened by recent studies on acquired immunodeficiency syndrome (AIDS) demonstrating that depletion of $CD8^+$ T cells led to a pronounced increase in plasma virus load [Jin *et al.*, 1999; Schmitz *et al.*, 1999], while reconstitution of these population re-suppressed viremia [Schmitz *et al.*, 1999].

Virus-specific cytotoxic T cells in mucosal tissues were first demonstrated in the lung and the bronchoalveolar washings of mice after intravenous (IV) or intranasal (IN) infection with influenza virus [Yap *et al.*, 1978]. The protective activity against viral infection was conferred by these mucosal T cells rather than by systemic counterparts [Yap and Ada, 1978]. Such effect was confirmed subsequently by the adoptive transfer of cytotoxic $CD8^+$ T cells [Lukacher *et al.*, 1984; Taylor and Askonas, 1986]. In the gut, it was shown that immunization with reovirus induced the antigen-specific precursor CTLs (pCTLs, before gaining cytolytic effector function), and upon *in vitro* re-stimulation, there was a higher frequency of these cells in MLNs and PPs as compared to in the spleen

46

[London *et al.*, 1987; London *et al.*, 1990]. Adoptively transferred immune CTLs from PPs have the ability to repopulate the gut epithelium of severe combined immunodeficient (SCID) mice and respond to reovirus infection [George *et al.*, 1990; Guy-Grand *et al.*, 1991]. Finally, clearance of viruses (e.g., rotavirus) by CTLs was closely associated with the expression of $\alpha 4\beta 7$ on these cells [Rose *et al.*, 1998], thus suggesting the central role of CMIS in the homing of effector cells to the site of infection. Therefore, local cytotoxic $CD8^+$ T cells can be primed and exert killing activities to control viral infections.

To prevent viral infection or reduce virus loading, induction of antibody responses is important. However, when infection occurs, it is critical to obtain adaptive cellular mucosal immunity characterized by specific $CD8^+$ T-cell mediated killing and professional help from $CD4^+$ T cells. This makes it possible to effectively control the existent infection

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

1.4.1   HSV-2 epidemiology and transmission

Sexually-transmitted diseases (STDs) are one of the most prevalent infections in

the world. STDs cause severe and frequent health problems as most of the time the infections are initially asymptomatic and are often ignored until serious manifestations have occurred. HSV is one of the most common STDs that is incurable and affect millions of people. HSV is transmitted by close skin contact, and manifested or blisters and sores almost anywhere on the skin but mostly either on the face or on the genitals. The HSV viruses are members of the family *Herpesviridae* and comprise two serotypes, HSV-1 and HSV-2. HSV-1 usually affects the body above the waist, mostly the mouth, while HSV-2 primarily infects the genitourinary tract and surrounding areas [O'Farrell, 1999]. However, both serotypes can infect the rest of the body. HSV-2 is usually transmitted by sexual contact with infected persons who are shedding the virus even in the absence of symptoms. Anal and rectal tissues can become infected also as a result of sexual contact, especially homosexuals behaviors or other sexual practices [Owen, Jr., 1980; Wexner, 1990]. HSV-2 can also be vertically transmitted during birth and cause encephalitis in children and young adults [Schleiss, 2003].

The prevalence of HSV-2 is in increasing worldwide and tends to be higher in younger generations particularly in the developing world [Fleming *et al.*, 1997; Halioua and Malkin, 1999; O'Farrell, 1999]. In many countries of sub-Saharan Africa and the Caribbean, the prevalence in adults is approximately 50% [Armstrong *et al.*, 2001]. A high prevalence is seen also in the USA (above 22% in adults) [Armstrong *et al.*, 2001; Krone *et al.*, 2000]. A very recent study on the regional distribution of seroprevalence demonstrated that HSV-2 infection in Ontario, Canada (9.1%) was not as common as in

USA but high prevalences were identified in women under prenatal care (23.1%) [Howard *et al.*, 2003].

The major public health concern about HSV-2 lies in its potential role as co-factor for the transmission of HIV [Holmberg *et al.*, 1988; Chen *et al.*, 2000; Renzi *et al.*, 2003]. Genital ulceration increases the risk for HIV infection. It has been found that HSV induces HIV replication and, thus, leads to HIV reactivation [Fennema *et al.*, 1995; Moriuchi *et al.*, 2000; Moriuchi and Moriuchi, 2002].

1.4.2   HSV-2 pathogenesis

HSV-2 is an icosahedral virion containing 152 kbp linear double-stranded deoxyribonucleic acid (DNA) [Haarr and Skulstad, 1994]. Although infection of a host cell is initiated by the association with glycoprotein C and glycosaminoglycans (GAG) on the cell surface, stable attachment and virus entry is mediated by other glycoproteins including glycoprotein B (gB) [Spear *et al.*, 1992; Rajcani and Vojvodova, 1998]. Although the overall homology between HSV-1 and HSV-2 is approximately only 50%, the sequence of gB is well conserved. HSV can infect various host cells and has a relatively short reproductive cycle and a high efficiency in destroying infected cells. Virus assembly begins in the nucleus and early gene products include enzymes, such as thymidine kinase (TK) and DNA polymerase [Tenser, 1991]. The incubation period of the virus varies, ranging from 1 day to 4 weeks with a median of 6-8 days. The inability

to control virus replication at mucosal surfaces results in herpes viremia, through which the virus is disseminated widely to other organs, such as the liver, the lung, the pancreas, the adrenal glands, the bone marrow, and the small and large intestines. By infecting the nervous system, HSV has the capacity to establish latent infections in sensory neurons [Baringer, 1975]. Reactivation is an indication of the occurrence of latency [Tenser, 1991] and the viral TK is required for the reactivation of HSV from its latent state since HSV lacking TK does not reactivate from the latency [Tenser *et al.*, 1996; Chen *et al.*, 1998].

The production of infectious progeny is accompanied by cell killing. In cultures of cells infected with HSV-2, rapid spreading of the virus is observed. After entering the body, virus shedding starts around 1 week and lasts the duration of about 1-2 weeks. HSV-2 can even be isolated in the vaginal secretions from infected individuals who display no symptoms [Krone *et al.*, 2000]. As compared to asymptomatic individuals, the virus titers from individuals with vaginal lesions are 100- to1000-fold higher and the efficiency of transmission during such active phases is significant increased.

On the basis of the understanding of the natural properties of HSV-2 infection, vaccine-based therapies should aim at establishing a great capacity of immunological barrier at the mucosal surfaces to prevent viral transmission and the development of latency.

1.4.3   HSV-2 infectious mouse model

Although the infectious disease caused by HSV-2 is naturally seen only in humans, HSV-2 infection can be established in mice [Morahan *et al.*, 1977; McDermott *et al.*, 1984; Parr *et al.*, 1994] and guinea pigs [Stanberry *et al.*, 1982]. The mouse model that has now been extensively used for studies on host resistance to lethal vaginal infection of HSV-2 was first developed in 1984 by McDermott *et al.* [1984] using BALB/c mice. The mouse model on some certain mouse strains, such as C57BL/6 which are naturally resistant to HSV-2, have been modified by Parr *et al.* [1994], who pretreated these mice with Depo-Provera® (a derivative of progesterone made by Pharmacia & Upjohn) five days before vaginal inoculation of the virus. By suppressing and antagonizing estrogen and prolonging diestrus, this treatment thins the lining of vaginal wall and hence increases the susceptibility of the genital mucosa to virus infection.

The host resistance to HSV infection directly reflects successfully induced mucosal immunity. Due to the temperature effects on viral replication, HSV pathogenesis occurs locally at the mucosal level rather than the systemic [Letchworth and Carmichael, 1984]. Local gross inflammation and ulcerative lesions in the skin close to the infected mucosal site can be generated in non-immunized mice and, when infection overwhelms the mucosal defense, HSV enters the dorsal root ganglia and leads to paralysis of hindlimbs [Morahan *et al.*, 1977; McDermott *et al.*, 1984, Parr *et al.*, 1994; Parr and Parr, 1997a]. These signs are reliable criteria that allow the severity of herpes infection to be

measured and used to determine anti-viral immunity at the mucosal surfaces [Gallichan and Rosenthal, 1996a]. Severe inflammations and the development of paralysis imply an inefficient immunological barrier, while improvements of the clinical pathology and the absence of paralysis are indicative of effectively induced mucosal immunity.

Only a number of studies have been carried out to infect mice with HSV-2 at the rectal mucosa. Phillips et al. [1998] observed that BALB/c mice became susceptible to rectal challenge of HSV-2 (~5×10^3 plaque-forming units, PFU) only after topical application of nonoxynol-9 (a commonly used vaginal spermicide), which was found to cause epithelium destruction. Zeitlin et al. [2001] were able to infect C57BL/6 mice with HSV-2 (~1×10^7 PFU) by introducing an aluminum oxide-coated cylinder to abrade the rectal mucosa, and investigated the protective effect of microbicide contained in BufferGel™ (ReProduct, a spermicidal and microbicidal gel). These studies suggested that intact mice were resistant to HSV-2 infection and pretreatment of the rectal mucosal surface was required to establish HSV-2 infection and develop the disease as seen in humans.

Since HSV-2-infected mice progress and develop the disease that somewhat resembles infection in humans, the HSV-2 infection in mice substantially provides valuable insights of the mucosal immune system in increasing immunological resistance to viral infection and, thus, has direct implications for the development of mucosal vaccines.

1.5   Mucosal vaccination

1.5.1   Antigen delivery system

As the mucosal inductive site displays effectiveness in the induction of mucosally-induced tolerance, the mucosal immune system can be rendered irresponsive to the antigen upon subsequent encounters. Vaccination with soluble proteins have been found to be less effective in the induction of protective immunity and failed in clinical trials [Connor *et al.*, 1998; Eriksson and Holmgren, 2002]. Thus, the introduction of proteins is suggested to be with adjuvants to prevent the tendency toward mucosally-induced tolerance.

The enterotoxins cholera toxin (CT) and heat-labile toxin (LT) produced by *Vibrio cholera* and *Escherichia coli* (*E. coli*), respectively, have been studied in experimental systems and found to be effective as adjuvants for co-administered unrelated proteins in the induction of both cellular and humoral immune response by mounting either Th1 or Th2 cell responses [Elson and Ealding, 1984; Lycke and Holmgren, 1986; Clements *et al.*, 1988; Xu-Amano *et al.*, 1993; Marinaro *et al.*, 1995; Ogra *et al.*, 2001]. Rectally administered peptide vaccines with CT were shown to be effective in stimulating host resistance to rectal challenge of vaccinia viruses (VV) in mice [Belyakov *et al.*, 1998b] and in the induction of cell-mediated mucosal immune responses in primates [Belyakov *et al.*, 2001]. However, CT and LT holotoxins are

potentially limited for human use because of their toxicity (inducing epithelial cells to secrete water and ions into the intestinal lumen and, thus, causes diarrhea) [Clements and Finkelstein, 1979; Levine *et al.*, 1983; Spangler, 1992]. Additional problem is the requirement of multiple administrations to induce primary immune responses, which might be an obstacle in clinical applications. Although mucosally administration of the non-toxic B subunit of CT (CTB) can induce local antibody responses [Holmgren *et al.*, 1993] and safely used in humans [Kozlowski *et al.*, 1997; Kozlowski *et al.*, 1999; Jertborn *et al.*, 2001], CTB probably acts more as a trans-mucosal antigen carrier since it also facilitates tolerance induction [Sun *et al.*, 1994; Aspord and Thivolet, 2002; Bregenholt *et al.*, 2003]. Moreover, the adjuvant effect of detoxified mutant CT and LT is currently still questionable [Fujihashi *et al.*, 2002].

Recent studies showed that oligodeoxynucleotides containing unmethylated CpG motifs serves as a safe adjuvant in mucosal vaccine to stimulate Th1 and CTL responses [Gallichan *et al.*, 2001; Ashkar and Rosenthal, 2002; Dumais *et al.*, 2002], suggesting a more acceptable alternative adjuvant therapy. Since stimulation of the production of critical cytokines appears to be the underlying mechanism for adjuvants, administration of cytokine activity, which are less toxic than bacterial-derived toxins, might be more effective in providing Th skewing effect [Ogra *et al.*, 2001; Eriksson and Holmgren, 2002].

Other than coadministration with adjuvants derived from microbial constituents,

antigens also become more immunogenic when incorporated into genetically engineered organisms, for example, when their genes are carried by live viral or bacterial vectors. Live recombinant vaccine vectors are capable of specifically targeting the cells of interest and producing antigens at the expected site to induce immune responses [Bouvet *et al.*, 2002]. One of the goals of vaccines is to prevent infectious diseases such as STDs by introducing genetic material into selected cells to induce immune responses against pathogens. However, these vectors have not been fully appreciated for vaccination at rectal mucosa. DNA and VV vaccines were only examined for mucosal immune responses but not for protection [Belyakov *et al.*, 1998c; Hamajima *et al.*, 2002]. Due to their specific targeting on M cells [Jones *et al.*, 1994], *Salmonella* vectors were studied for rectal administration in humans [Nardelli-Haefliger *et al.*, 1996; Kantele *et al.*, 1998; Kutteh *et al.*, 2001], but only local IgA production rather than cellular immune responses was detected. Multiple administrations of some vectors seemed to indicate that a single immunization with the vectors was not sufficient to induce detectable primary immune responses. Three-time rectal administrations of Sindbis virus-based replicon particles induced vaginal immune responses against VV challenge [Vajdy *et al.*, 2001]. Four rectal administrations of *E. coli* vectors expressing OVA (ECOVA) for 4 weeks induced IgG and IgA antibodies in serum and proliferation of specific splenic $CD4^+$ T cells [Yoshida *et al.*, 2001]. The replication deficient adenoviral vector (Adv), one of the most important gene transfer vehicles, remains to be studied in this regard. Taken together, a more comprehensive approach to gene-based vaccines is needed to improve our understandings to the lower GI mucosa as a route of mucosal vaccination.

1.5.2   Adenoviral vector as an antigen delivery vehicle

Adenovirus, a frequent cause of upper respiratory tract infections, was initially

isolated from cell lines of adenoidal tissue obtained by tonsillectomy. Adenovirus is often

associated with enteritis in children (usually self-limited) or in immunocomprised

patients [Munoz et al., 1997; Ruuskanen, 1997]. Adenovirus is a non-enveloped,

icosahedral particle containing hexon and penton capsomeres and a double stranded DNA

genome sized at 30-38K bp. To enable replication, the virus penetrates the plasma

membrane before entering the cell nucleus [Whittaker et al., 2000; Cullen, 2001]. Penton

fibres projecting from the particle are associated with the attachment to the host cell by

binding to the coxsackievirus and adenovirus receptor (CAR) on target cells [Nemerow,

2000]. The subsequent interactions of the penton base with integrin molecules on the cell

surface mediate endocytosis of the virus, which is then trapped into an endosome

[Wickham et al., 1993]. With help from the penton base, the virus is able to escape the

endosome and localize to the nucleus for DNA replication [Greber et al., 1993]. The gene

transcription of adenovirus involves the early phase and late phase. Viral DNA

replication is initiated by the early region approximately 7 hours post-infection, after

which late proteins are expressed under control of the late region, and viral assembly

begins at ~24 hours and progeny viruses are released from the infected host by 2-3 days.

Viral replication involves the E1 region. Deletion of E1 results in inability of the virus to

replicate and lyse the infected cell, as well as abolishes the potential oncogenicity of the

virus. E3 region plays a regulatory role in the immune response of the host cell. Deletion

of E3 does not affect virus replication and, thus, is used for transgene insertion [Graham

and Prevec, 1991]. Adv with double deleted E1 and E3 regions has increased space for

insertion of heterologous genes and are an ideal system for gene transduction, gene

therapeutics and gene-based vaccines.

As compared to its wild type, Adv retains its capacity in infection of and gene

transfer to a variety of host cells and thus are currently widely used in gene-based vaccine

studies [Graham, 1990; Bramson *et al.*, 1995; Wilson, 1996, Babiuk and Tikoo, 2000;

Hitt and Graham, 2000; Young and Mautner, 2001]. Adv-mediated *in vivo* gene transfer

has proved to be one of the most successful ways in vaccination by expressing

heterologous gene products that trigger or modulate immune responses. Although early

anti-adenoviral inflammatory responses are induced following administration [Worgall *et*

al., 1997; Cartmell *et al.*, 1999], efficient infection of the virus in appropriate cells can

lead to later immune responses specific for the product of transferred immunogen [Yang

et al., 1996; Michou *et al.*, 1997] as well as for the viral antigens [Yang *et al.*, 1994b;

Yang *et al.*, 1994c]. Adv can specifically make use of MHC class I molecules to direct

prime CTLs or activate these cells through cross-priming by professional APCs [Letvin *et*

al., 2002; Robinson, 2002]. Secreted proteins or antigens released from the infected cells

after infection can be further processed and presented by APCs through MHC class II

pathway for either antibody response or activation of $CD4^+$ Th cells to stimulate B cells

or CTL response [Benihoud *et al.*, 1999]. As viruses target the same specific arms of the

immune system, vaccination with the viral vector Adv takes full advantages of immune

system in the induction of antiviral immune responses.

Adv is a good candidate as a vaccine also owing to its adjuvant function. The antiviral immune response inevitably induced following Adv-based vaccination renders the host resistant to revaccination with the same vector [Yang *et al.*, 1995a], but might provide effective adjuvantive mechanism by which Adv acts on the specific immune response to transgene-encoded antigens. Administration of Adv induced early anti-adenoviral inflammatory responses [Worgall *et al.*, 1997; Cartmell *et al.*, 1999], in which the hexon protein appears to be the major contribution [Molinier-Frenkel *et al.*, 2002]. Empty Adv (lacking heterologous genes) induced both antibody responses [Van Ginkel *et al.*, 1995] as well as viral antigen specific splenic CTLs that can lyse Adv-infected targets *in vitro* [Kafri *et al.*, 1998]. Adv deprived of one or more early regions (E1, E2A, E3 or E4) still demonstrated the ability to activate DCs *in vitro* in synergy with CD40L and enhance the role of IL-12 in simulating T cells to produce IFN-γ [Rea *et al.*, 1999]. Adv on its own also induced nuclear transcription factor NF-κB-mediated production of chemokine interferon-inducible protein-10 [Borgland *et al.*, 2000] which has been found to be of an important part in T-cell generation and effector T-cell trafficking [Dufour *et al.*, 2002]. Therefore, deletion of early genes (E1-E4) in Adv does not reduce such anti-vector immune responses as an adjuvant mechanism [Lusky *et al.*, 1998].

1.5.3   Adv-based vaccine at the mucosa

Immunization through mucosal routes with Adv to induce mucosal immunity to virus infections has been studied in various animal models [Rosenthal *et al.*, 1996]. Intranasal (IN) administration of Adv encoding HSV glycoproteins induced specific cellular and humoral mucosal immunity [Johnson *et al.*, 1988; Witmer *et al.*, 1990; Hanke *et al.*, 1991; Zheng *et al.*, 1993] and protection against lethal challenge of HSV-2 [McDermott *et al.*, 1989b; Gallichan *et al.*, 1993; Gallichan *et al.*, 1995; Gallichan and Rosenthal, 1995; Gallichan and Rosenthal, 1996b; Gallichan and Rosenthal, 1998] and HSV-1 [Endresz *et al.*, 1995]. Adv administered alone as well as a booster has also been shown to be promising in the induction of anti-HIV immune responses [Buge *et al.*, 1997; Edghill-Smith *et al.*, 2002; Shiver *et al.*, 2002; Casimiro *et al.*, 2003]. These suggested that Adv might be a good candidate for mucosal vaccination.

In the small and large intestines, Adv-mediated gene transfer into the epithelial cells was quantitatively shown in both humans [Croyle *et al.*, 1998; Jobin *et al.*, 1998] and rodents [Huard *et al.*, 1995; Cheng *et al.*, 1997; Croyle *et al.*, 1998; Foreman *et al.*, 1998; Wirtz *et al.*, 1999]. Transfer of therapeutic levels of genes to the colon to treat colitis in animal models was achieved. Intrarectal (IR) administrations of Adv encoding IL-18 antisense messenger ribonucleic acid (mRNA) (at a dose of 1×10^{10} PFU for three times with 2 days intervals after onset of colitis) significantly reduced local IL-18 levels and suppressed the development of colitis in the $CD4^+$-transfer mouse model [Wirtz *et*

al., 2002]. A single IR administration of Adv expressing IL-10 (AdIL-10, 5×10^8 PFU)

was competent to subvert spontaneously developed colitis in IL-10$^{-/-}$ mice [Lindsay *et*

al., 2003]. In terms of immune induction, few studies have carried out to particularly

examine mucosal immune responses induced after antigen gene transfer to the rectal

mucosa by Adv.

1.5.4   Mucosal vaccine design

How to induce robust mucosal immune responses remains an elusive goal in

mucosal immunology. Since systemic immunity can only deal with pathogens which

have entered the body through the bloodstream, it is essential to establish mucosal

immunological barriers to effectively prevent and control infection at mucosal sites

[McGhee *et al.*, 1992]. Earlier studies by McDermott *et al.* [1990] revealed that vaginal

immunization of mice with TK$^-$ HSV-2 stimulated significant levels of local IgG

production and led to protection against subsequent vaginal challenge of wild type HSV-

2, while parenteral immunization did not. Rosenthal and his colleagues [Gallichan *et al.*,

1993; Gallichan and Rosenthal, 1995; Gallichan and Rosenthal, 1996b; Gallichan and

Rosenthal, 1998] also demonstrated that immunization via the nasal cavity with Adv

expressing gB elicited immune responses in the genital tract and conferred immune

protection against vaginal challenge of HSV-2. Other studies using different models in

other species provided support for the concept of mucosal immunization as a more

effective vaccination strategy against infection at mucosal surfaces [Belyakov *et al.*,

2001; Vajdy *et al.*, 2001].

Within the mucosal immune system, the route of immunization is an important factor to determine immune protection at a specific mucosal site. Gallichan *et al.* [1998; 2001] showed that intranasal immunization with Adv expressing glycoprotein B (AdgB) provided genital protection against HSV-2 challenge at a relatively low challenge dose $(2 \times 10^4$ PFU), and protection was overwhelmed by a 10-fold higher challenge dose. A growing body of literature suggested that local mucosal immunization was more effective than distant mucosal immunization. After nasal, rectal or vaginal immunization of mice with group B streptococci-derived capsular polysaccharides as an antigen conjugated to CT, levels of IgA were found to be highest induced by each local rather than a distant mucosal immunization [Shen *et al.*, 2000]. Similar results were also found in monkeys after rectal or vaginal immunization with CT [Eriksson *et al.*, 1998]. Studies also specifically looked at induction of colonic mucosal immune responses and protection. Rectal immunization with CT induced significant levels of local immune responses such as IgA [Haneberg *et al.*, 1994]. Peptide-based immunization via rectal mucosa was more effective than via nasal or upper gastric mucosa in the induction of rectal protection against VV challenge [Belyakov *et al.*, 1998b]. Thus, rectal immune responses are better induced by local mucosal immunization as compared to distant mucosal immunization.

Whether the rectum can be an alternative route to induce genital protection has become a topic in anti-STD vaccinations. This is because the male population may not be

vaccinated practically through the genitourinary tract. In addition, vaginal immunization showed to be less effective to induce local immune responses and protection than immunization at a distant site such as nasal cavity [Wu *et al.*, 2000b; Vajdy *et al.*, 2001]. It was suggested that that induction of immune responses in DLNs might be the key for local protection. Immunization at the ILNs (the DLNs that drain lymph from rectal and genital tracts) by injection of proteins in the proximity of the nodes induced both rectal and genital CTL responses and protection against challenge of simian immunodeficient virus (SIV) at both sites [Klavinskis *et al.*, 1996; Lehner *et al.*, 1996]. In contrast, immune protection was not achieved by immunization at non-DLNs [Mitchell *et al.*, 1998]. Only a few studies showed that rectal immunization conferred genital immunity although ILN responses were not investigated. Rectal immunization with group B streptococci conjugated to CT significantly induced specific IgA not only in the feces but also vaginal secretions [Haneberg *et al.*, 1995]. In a guinea pig HSV-2 genital infection model, rectal immunization with VV vectors expressing HSV glycoprotein D appeared to result in lower virus shedding from the genital tract than intranasal immunization although the data were not statistically analyzed (virus titers in log: 2.9 vs. 4.1 at day 3, and 1.8 vs. 2.8 at day 4 post-infection) [Bernstein, 2000]. In a more recent study, genital protection against VV challenge was achieved following rectal immunization using replicons as a vaccine vector [Vajdy *et al.*, 2001]. Emerging results suggested the likelihood of vaccination through the lower GI tract for the induction of immune protection in the GU tract.

## 1.6	Hypotheses and objectives of the study

It was hypothesized in the present study that the rectal mucosa might be an ideal route for vaccination against STD infection at the rectal and genital mucosae. This was established based on previous observations that mucosal immunization was more advantageous and efficient via the local route than via a distant mucosal or a systemic, non-mucosal route [Lehner *et al.*, 1999; Cripps *et al.*, 2001; Ogra *et al.*, 2001]. First, mucosal vaccines induced a certain degree of immune responses at another mucosal site to which a vaccine might be inaccessible. Second, mucosal vaccines elicited not only mucosal but also systemic immunity and both of these were protective. Third, local mucosal immunization induced stronger local immune responses as compared to distant mucosal immunization. Moreover, induction of ILN immune responses conferred genital protection. However, further converging evidence is required to support the view on local mucosal immunization. This is because that most previous studies showed differences between local and distant mucosal immunizations only in generating antibody immune responses; Few studies looked specifically at local cellular immune responses and used pathogenic infectious agents to challenge animals. Therefore, the present study was to investigate the rectal mucosa as a route of local mucosal immunization in mice and addressed the question about the effectiveness of local mucosal versus distant mucosal versus systemic, non-mucosal immunization.

It was also hypothesized in the present study that Adv might be a useful antigen

delivery system for rectal immunization to elicit cellular immune responses in the colonic

tissue and the ILNs and confer local protection against viral infection. Adv was shown to

be competent in gene transfer to colonic epithelial cells *in vivo* [Wirtz *et al.*, 1999] and

achieve local therapeutic effects in cytokine therapy to treat colitis [Wirtz *et al.*, 2002;

Lindsay *et al.*, 2003]. Adv proved to be effective in the induction of mucosal immunity

via some other mucosal routes [Rosenthal *et al.*, 1996]. Thus, Adv was chosen as a

vaccine for rectal immunization.

The first objective of the present study was to develop a new method for IR

delivery of Adv and investigate colonic gene transfer both quantitatively and

qualitatively. The second objective was to detect antigen-specific cellular and humoral

immune responses after IR immunization with Adv encoding a model antigen gene. Both

local as well as systemic compartments were examined. Immune responses were also

tested by challenge of an infectious virus carrying the antigen previously used for

immunization. The third objective was to establish a rectal challenge model of HSV-2

and use this model to validate the intrarectal immunization regimen as compared to other

routes of immunization, i.e. intranasal (distant mucosal) and subcutaneous (systemic,

non-mucosal).

CHAPTER 2   MATERIALS AND METHODS

2.1   Animals

Female C57BL/6 (H-2^b) mice age 6-8 weeks were purchased from Harlan (Indianapolis, IN). IFN-$\gamma^{-/-}$ (B6.129S7-$Ifng^{tm1Ts}$) mice [Dalton et al., 1993] were purchased from the Jackson Laboratory (Bar Harbor, ME). IL-12 p40$^{-/-}$ [Magram et al., 1996] and β2m$^{-/-}$/CD8$^{-/-}$ [Freland and Ljunggren, 2000] mice were kindly provided by Dr. J. Magram (Hoffmann-La Roche, Nutley, NJ) and Dr. H.G. Ljunggren (Karolinska Institute, Sweden), respectively. All mice were housed in pathogen-free conditions at Central Animal Facility at McMaster University. All animal experiments were approved by the Animal Ethics Research Board of McMaster University and conducted according to regulations of the Canadian Council on Animal Care. Animal handling was carried out in biological safety cabinets.

2.2   Cell lines

EL4 (H-2^b) cell lines were obtained from the American Type Culture Collection (Manassas, VA). E.G7-OVA was an EL4 transfectant cell line that stably expressed

chicken ovalbumin (OVA) protein [Moore *et al.*, 1988]. Both cell lines were cultured in RPMI-1640 supplemented with 10% w/v fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. In the culture medium of E.G7-OVA, geneticin (G418, Invitrogen Corp, Carlsbad, CA) was added at a concentration of 0.4 µg/ml. African green monkey kidney Vero and CV-1 cells were cultured as monolayers in α-MEM medium supplemented with 5% w/v FBS and 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.01 M HEPES [McDermott *et al.*, 1984]. E.G7-OVA was used for subcutaneous challenge after immunization with Adv expressing OVA, or as target cells in a chromium release assay. EL4 was used as control targets also in the chromium release assay. Vero cells were used for propagation and titration of HSV-2. CV-1 cells were used for titration of recombinant vaccinia viruses.

2.3   Viruses

AdLuc (Ad5E1mCMVDK1) and Adβ-gal (Ad5E1mCMVCA35LacZ) are the replication-deficient adenoviral vectors (Adv) that express firefly *Photinus pyralis* luciferase and *Escherichia coli* (*E. coli*) β-galactosidase, respectively [Addison *et al.*, 1997]. AdOVA (Ad5delE1MCMV-OVA.1) expresses chicken ovalbumin (OVA) [Thomson, 2001], and AdgB (Ad5E3SV40gB) encodes gB of HSV-1 [Hutchinson *et al.*, 1993]. VV-OVA is a recombinant vaccinia virus (VV) expressing full length OVA. All replication-deficient Adv were propagated in 293 cells and purified by cesium chloride

gradient centrifugation [Hitt *et al.*, 1998]. All virus stocks were aliquoted and stored at $-70°C$ before use. AdLuc was used in gene transfer study for quantification analysis of transgene expression, and Adβ-gal was used for visualization of gene expression. AdOVA and AdgB were used for mucosal and parenteral immunization. VV-OVA was used for rectal or vaginal challenge after immunization with AdOVA.

HSV-2 strain 333 was propagated on Vero cells as previously described [McDermott *et al.*, 1984]. Briefly, Vero cells were infected with HSV-2 at a multiplicity of infection (MOI) dose of 0.1 in a minimal volume of Vero culture medium without FBS at $37°C$ for 1-2 hrs. Vero culture medium was added to the culture. Infected cells were harvested at 24-48 hrs. To release the virus, cells were centrifuged and disrupted by three cycles of freezing/thawing followed by sonication. After centrifugation, the virus was recovered from supernatant, aliquoted and stored at $-70°C$. HSV-2 was diluted in phosphate buffered saline (PBS) and used for rectal and vaginal inoculation.

2.4   Delivery of Adv

2.4.1   Intrarectal (IR) delivery of Adv ·

Mice were anesthetized by isoflurane (Abbott Laboratories, Montreal, PQ,

Canada). 150 µl of 50% v/v ethanol was pipetted into the colon via the anal canal through

a fire-polished 1000-µl pipette tip. After 40-min incubation, the colon was rinsed with

PBS using a stainless steel animal feeding needle placed in the anal canal (Poppe & Sons

Inc., New Hyde park, NY). Mice were then inoculated IR with 100 µl Adv by pipetting.

To prevent the leakage of delivered Adv, the anal opening was temporally sealed with

one drop of Dermabond® topical skin adhesive (Ethicon, Somerville, NJ). Anesthetized

mice were allowed to lie flat in the anesthetic machine chamber for 1 hr during which

their positions were changed by turning the body in order to presumably insure a better

contact of the delivered agents with the intestinal wall. Mice were then allowed to move

freely. To increase contact time, the Dermabond® seal was maintained to keep virus

inside the lumen for additional 4-5 hrs before being removed. This pipetting and

Dermabond® (P&D) method was also used for rectal inoculation of other viruses

2.4.2   Intravaginal (IVAG) administration

Prior to IVAG inoculation, mice were subcutaneously injected with 2 mg of

Depo-Provera® (medroxyprogesterone acetate, Pharmacia & Upjohn Co., Kalamazoo,

MI) as previously described [Gallichan and Rosenthal, 1996a]. Five days later, at which

time the lining of vaginal wall becomes thinner, the mice were anesthetized and the

vaginal wall was rinsed with PBS and swabbed with a Dacron Swab-pak® (Medical

Packing Corp, Camarillo, CA) to remove secretions. Adv in 10 µl PBS were given by

pipetting through a fire-polished 100-µl micropipette tip inserted into the vaginal lumen,

followed by Dermabond® application on the vaginal opening. After 1-hr incubation under

anesthesia, mice were allowed to move freely. Dermabond® was removed 4-5 hrs later.

The procedures were also used for vaginal inoculation of other viruses.

2.4.3   Intranasal (IN), intragastric (IG), and subcutaneous (SC)

administration

For IN administration, mice were anesthetized and held on the back with head

tilted back and the nose pointed up. A total volume of 30 μl Adv was introduced by two

injections into each nostril for complete inhalation [Gallichan and Rosenthal, 1996a]. For

IG administration, mice were restrained manually and 50 μl Adv were delivered by

introducing a feeding needle into the left diastema rami and further down to esophagus.

Mice were SC injected with 200 μl Adv in PBS by sliding a needle under the skin in the

left flank of mice. The SC injection procedures were also used for tumor inoculation.

2.5   Challenge of viruses and tumors

2.5.1   Rectal challenge of HSV-2 or VV

The procedures for virus challenge were as same as described earlier for IR

delivery of Adv but different in given volume. In short, a 50% v/v ethanol was given

rectally and the colonic lumen was rinsed with PBS before inoculation. Thirty microliters

of HSV-2 (at different doses) or VV-OVA (5×10^7 plaque-forming units, PFU) were

introduced into the colon by the P&D method. Mice were maintained under anesthesia

for 1 hr incubation and Dermabond® was removed 4-5 hrs after the procedure. HSV-2

infected animals were monitored daily for morbidity and experimental endpoint as

described below (see Section 2.6). To evaluate virus replication, rectal secretions were

sampled by rectal swabbing daily from day 1 to day 5 and days 7 and 10. VV-infected

mice were sacrificed at day 6 to evaluate virus replication in ovaries and rectal tract.

2.5.2   Vaginal challenge of HSV-2 or VV-OVA

The procedures for vaginal challenge were adapted from previous studies

[Gallichan and Rosenthal, 1996a; Dumais *et al.*, 2002]. C57BL/6 mice were

subcutaneously injected with 2 mg of Depo-Provera®. Five days later, mice were

anesthetized and the vaginal tract was rinsed with PBS and swabbed followed by vaginal

inoculation of HSV-2 (at different doses) or VV-OVA (2×10^7 PFU) in 10 μl PBS.

Dermabond® was applied as described above. HSV-2 infected animals were monitored

daily for morbidity and experimental endpoint as described below (see Section 2.6). To

evaluate virus replication, vaginal secretions were sampled by rectal swabbing daily from

day 1 to day 5 and days 7 and 10. VV-infected mice were sacrificed at day 6 to evaluate

virus replication in ovaries and vaginal tract.

2.5.3   Subcutaneous challenge of E.G7-OVA

E.G7-OVA tumor cells at doses of 2×10^5 or 5×10^5 were suspended in 200 μl of PBS and injected subcutaneously in the right flank of mice. Tumor appearance and growth was monitored every 3-4 days and determined by performing three measurements of tumor diameter (length, width and height that are perpendicular to each other and span the largest portion of the tumor in each direction). Any palpable tumors were considered as positive. Tumor-bearing mice were euthanized when became moribund or the sum of three dimensional measurements reached 30 mm or ulcerative lesions on the tumor were found.

2.6   Clinical   scores   of   pathology   of   HSV-induced   perineal inflammation

A 5-point pathology score (Table 2.1) was used to measure and indicate the progress of disease based on definitions in previous studies [Morahan *et al.*, 1977; Stanberry *et al.*, 1982; Goodell *et al.*, 1983; Gallichan and Rosenthal, 1996a; Parr and

Table 2.1 A clinical score developed to assess the severity of rectal HSV-2 infection.

Point	Manifestations	Location	
		Rectal challenge	Vaginal challenge
0	No sign of disease	Anal opening	External vagina
1	Redness	Anal/perianal area	External vaginal area
2	Swelling	Perianal	External vaginal area
3	Hair loss, severe redness	Perineum	Perineum
4	Ulcerative lesion	Perianal/perineum	External vaginal area/perineum
5[§]	Paralysis (or moribund)	Hindlimbs	Hindlimbs

[§] Experimental endpoint.

Parr, 1997a; Phillips and Zacharopoulos, 1998; Zeitlin *et al.*, 2001].

2.7 Sample collection and preparation

2.7.1 Collection of serum

Blood was sampled from orbital plexus using a microhematocrit tube and placed in a microcentrifuge tube. Blood samples were kept at room temperature for 30 min to allow clot formation and then centrifuged. The sera were transferred to a new tube and stored at $-20°C$ until use. Sera were used to determine antigen-specific antibody concentrations.

2.7.2 Collection of vaginal wash and rectal swabbing

Vaginal secretions were collected by inserting a fire-polished micropipette tip into the vaginal lumen to aspirate 30 µl PBS in and out 8-10 times. Vaginal wash was repeated once. To collect rectal secretions, the anorectal canal was swabbed with a premoistened Dacron Swab-pak® (Medical Packing Corp, Camarillo, CA). The swab tip was then cut and put into a microcentrifuge tube containing α-MEM medium at 0°C. The tube was vortexed and the fecal contents were pelleted. The supernatant was transferred

to a new tube and stored at −70°C until use. Rectal swabbing and vaginal washes were used to determine HSV-2 shedding from each site.

2.7.3   Collection of tissues

Mice were euthanized and their abdominal cavities were opened. Lungs and spleens were first removed. Iliac lymph nodes (ILNs) were identified on both sides of the abdominal aorta above its bifurcation into the iliac arteries, and mesenteric lymph nodes (MLNs) were found at the corner of terminal ileum by lifting the cecum up. Lymph nodes were teased free from surrounding tissues or the mesenteries with forceps. The colon (in between the anal canal and cecum) and the cecum together with 2-3 cm distal ileum were also removed. All tissues were aseptically removed and placed on ice for further process.

2.7.4   Tissue homogenation

Lungs, colons, genital tissues and paired ovaries were removed, pre-weighed, cut into small pieces and homogenized using a homogenizer (POLYTRON®, Kinematica, Inc., Cincinnati, OH) and kept on ice for further process. Colon homogenates were used for antibody concentration determination. Colon, genital and lung homogenates were used for luciferase quantification. Ovary, colon, genital homogenates were used for virus titration after HSV-2 or VV infection.

2.7.5   Lymphocyte isolation

The procedures for isolation of colonic lamina propria lymphocytes (LPLs) have

been described elsewhere [Coligan *et al.*, 2002]. Colons were cut longitudinally and then

cut into small pieces. Tissues were washed by 3-4 changes of Ca^{2+}- and Mg^{2+}-free Hanks'

balanced salt solution (HBSS, Mediatech, Herndon, VA) to remove colonic contents.

Tissue pieces were then transferred into a 75-ml culture flask with 10 ml of Ca^{2+}- and

Mg^{2+}-free HBSS/ethylene diaminetetraacetic acid (EDTA) (10% w/v FBS, 15 mM

HEPES, 5 mM disodium EDTA, 0.014% w/v dithiothreitol, and 100 μg/ml gentamycin).

To disrupt the epithelial cells from the mucosa, the flask was incubated at 37°C with

stirring (using a magnetic stirring bar sized at 20×6 mm) at a speed of 80-100 rpm for 30

min. Released intraepithelial cells were passed through 50-μ nylon screens and were

discarded. Intestinal pieces were transferred into a new flask with fresh 37°C RPMI-1640

medium containing 10% w/v FBS, 15 mM HEPES, 100 μg/ml gentamycin, and 250 U/ml

collagenase VIII (Clostridiopeptidase A, Sigma Chemical Co., Somerville, NJ). The flask

contents were stirred at 37°C at the same speed for 1 hr or until small pieces were

invisible. The cells liberated from digested tissues were recovered by filtering the

medium through 50-μ nylon screens. Filtered cells were purified in a 30%/75% v/v

gradient of Percoll™ (Sigma Chemical Co., Somerville, NJ). The colonic LPLs were

retrieved from the interface and washed twice with PBS free of Ca^{2+} and Mg^{2+}. Purified

colonic LPLs were placed in culture medium to rest 1 hr before use. This procedures was

also used for isolating LPLs from the ileocecum.

Lungs were rinsed in PBS and placed into a Petri dish and then cut into small

pieces for digestion with collagenase as described above until single-cell suspension in

RPMI-1640 culture medium was achieved. Spleens were cut in several places, and

splenocytes were squeezed out with the syringe plunger, passed through nylon screen and

centrifuged followed by the removal of red blood cells using ACK (abbreviator for

ammonium chloride and potassium chloride) cell lysing buffer (0.15 M NH_4Cl, 10 mM

$KHCO_3$ 0.1 mM disodium EDTA). Lymph node (LN) cells were squeezed out by

applying gentle pressure on cut-open lymph nodes with the blunt end of a syringe plunger

and then filtered through 50-μ nylon mesh and re-suspended in culture medium after

centrifuged.

Freshly isolated splenocytes, LN cells, and LPLs were used for IFN-γ ELISPOT

assays. Splenocytes and LN cells were also used as effectors in the chromium release

assay.

2.8   Gene expression detection

2.8.1   Luciferase gene expression

Luciferase activity was measured using Luciferase Assay System Kit (Promega,

Madison, WI) according to the manufacture's instructions. Briefly, tissue homogenates were placed in Cell Culture Lysis Reagent for cell lysis. After centrifugation of the tissue homogenates, 20 μl of supernatant were plated in a LumiNunc™ MicroWell® Plate (Nalge Nunc, Rochester, NY) and assayed in a Tropix® TR-717 microplate luminometer (Applied Biosystem, Bedford, MA) which automatically repeated the inject-then-read process by adding 100 μl of the Luciferase Assay Reagent (*Photinus pyralis* luciferin) to a single well and immediately detecting emitted light.

2.8.2   β-galactosidase (β-gal) gene expression

β-gal staining was performed as previously described [Sanes *et al.*, 1986; Login *et al.*, 1987]. Fresh colonic tissues were placed in tissue fixative (2% w/v formaldehyde, 0.05% w/v glutaradehyde, 1.6 g sodium cacodylate and 0.025% w/v calcium chloride) on ice for 30 min exposed to microwaves (50% power of a 800-watt microwave oven (Easy Touch®, Panasonic, Japan) for 5-8 sec at ~45°C and immediately returned ice. The sample was then overlaid with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, Invitrogen Corp, Carlsbad, CA) at ~37°C overnight in staining buffer (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide and 2 mM MgCl$_2$ in PBS containing 0.02% w/v NP40 and 0.01% w/v sodium deoxycholate). After staining, colon samples were washed thoroughly with PBS, fixed in 10% v/v formalin for 48 hrs, paraffin-embedded, and sectioned (4-5 μ) and counterstained with eosin and nuclear fast red. Cell-based blue clusters representing positive staining of β-gal were enumerated

under the microscope.

2.9   Mucus staining

Alcian blue and periodic acid-Schiff's (AB/PAS) techniques were used to stain glycoproteins on the apical surface of intestinal epithelial cells. Paraffin-embedded tissue section on slides were rinsed in 3% w/v acetic acid solution and stained with 1% w/v Alcian blue, pH 2.5 for 30 min. The slides were rinsed again in 3% acetic acid solution and washed in tap water. The sections were then treated with 1% v/v periodic acid for 15 min followed by staining with Schiff's reagent (40% w/v basic fusin and 40% w/v sodium metabisulfite) for 30 min. A counterstain was performed with Mayer's haematoxylin.

2.10   Virus titration

For titration of HSV-2, rectal swabbing and vaginal washes were collected daily from day 1 to day 5 and days 7 and 10 after rectal and vaginal challenge of HSV-2. To determine virus replication within the tissue, colons were taken at day 5 post-infection. For titration of VV titers, mice were sacrificed at day 6 after VV-OVA challenge.

Ovaries, colons and vaginal tissues were harvested, homogenized and sonicated. Virus titers were determined by the plaque-forming assay as previously described [McDermott *et al.*, 1984]. Briefly, serial 10-fold dilutions of virus were plated in a 24-well plate containing a monolayer of Vero cells and incubated at 37°C for 48 hrs. The medium was removed and the plaques were counterstained with 5% w/v crystal violet and counted at each dilution using dissecting scope. Virus presence was expressed as PFU/sample or PFU/tissue weight (tw).

2.11 Antibody ELISA

Enzyme-linked immunosorbant assay (ELISA) was used to quantify IgA and IgG titers as previously described [Gallichan and Rosenthal, 1996a; Braciak *et al.*, 2000]. Ninety-six-well MaxiSorp™ flat-bottomed MicroWell® plates (Nalge Nunc, Rochester, NY) were coated with OVA protein (Grade V, Sigma-Aldrich Corp. St. Louis, MO) in borate-buffered saline (BBS, pH 8.5) at a concentration of 10 µg/ml for 2 hrs at 37°C in a sealed, humidified chamber. After coating, each well was washed with PBS containing 0.05% v/v Tween-20 (PBST) and blocked with blocking buffer (PBST containing 1% w/v bovine serum albumin, BSA) for 1 hr at room temperature. Blocking buffer was decanted and wells were rinsed with PBST. Intestinal homogenates and sera serially diluted previously in blocking buffer were incubated in designated wells in duplicate for 2 hrs at room temperature. Each well was then rinsed with PBST and incubated with

alkaline phosphatase-conjugated goat anti-mouse IgA, IgG1 or IgG2a antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL) at a 1:3000 dilution in blocking buffer for 2 hr at room temperature. The wells were then rinsed and freshly dissolved p-nitrophenyl phosphate (pNPP, Southern Biotechnology Associates, Inc., Birmingham, AL) was added at a final concentration of 1 mg/ml and incubated in darkness for 30 min at room temperature. The optical density was determined at 405 nm using an absorbance and fluorescence microplate reader (TECAN, Research Triangle Park, NC). Antibody titers were derived from the inverse dilution at which the sample yielded an optical density that was twice that of the background of control specimens obtained from non-immunized mice.

2.12 IFN-γ ELISPOT assay

The frequency of interferon (IFN)-γ-secreting cells was determined by enzyme-linked immunospot (ELISPOT) assay according to the manufacture's directions using Multiscreen-IP plates (Millipore, Bedford, MA) and the Mouse IFN-γ ELISpot Development Module with Strep-AP/BCIP-NBT ELISpot Blue Color Module (R&D Systems Inc., Minneapolis, MN). Multiscreen-IP plates were coated with mouse IFN-γ capture antibodies and incubated overnight at 4°C. The following day, excess antibodies were removed by washing wells using PBST, and well membranes were blocked with ELISPOT blocking buffer (1% w/v BSA and 5% w/v sucrose in PBS) for 2 hrs. The

blocking buffer was then removed and the plate was filled with culture medium RPMI-1640, incubated at room temperature for 20 min, and the medium was decanted. Isolated lymphocytes were immediately added to the wells in duplicate in numbers ranging from 1×10^5 to 1×10^6 cells/100 µl/well and cultured in the presence of the $OVA_{257-264}$ peptide SIINFEKL (Sigma Genosys, Woodlands, TX) at a final concentration of 1 µg/ml for 24 hrs at 37°C. To detect captured IFN-γ protein, each well was washed with PBST and incubated with biotinylated IFN-γ detection antibodies at 2–8°C overnight. Excess detection antibodies were removed and streptavidin-conjugated alkaline phosphatase was added to the wells. These were incubated for 2 hrs at room temperature. Each well was then washed. The chromogenic substrate 5-bromo-4-chloro-3' indolylphosphate p-toluidine salt (BCIP) together with nitro blue tetrazolium chloride (NBT) was added into wells for enzymatic conversion into a colored black-purple precipitate by alkaline phosphatase. After a 30-min incubation in the darkness at room temperature, the BCIP/NBT solution was discarded and wells were rinsed with deionized water. The well membranes were air dried completely at 37°C and inspected for colored spots under a dissecting microscope. Background spots varied from tissue to tissue in the absence of peptide stimulation. The spots in 10^6 cells of IFN-γ-secreting LN and intestinal lymphocytes of non-immunized mice were 0-1, and those of splenocytes ~5. Spot numbers 2-fold higher than the background were considered as significant. Frequencies of IFN-γ-secreting cells from immunized mice were derived by subtracting the background spots and expressed as spot-forming cells per 10^6 cells.

2.13 Intracellular IFN-γ staining and flow cytometry

Intracellular cytokine staining (ICCS) was performed using Cytofix/Cytoperm Plus™ Kit containing GolgiPlug™ and Perm/Wash™ solutions (BD Biosciences Pharmingen, San Diego, CA) according to the manufacture's instructions. The antibodies used for both surface and intracellular staining were purchased from BD Biosciences Pharmingen (San Diego, CA). Splenocytes were stimulated for 4-6 hrs at 37°C with 2 μg/ml OVA peptide SIINFEKL in the presence of a protein transport inhibitor, GolgiPlug™ containing brefeldin A at a concentration of 1 μl/10^6 cells/ml. The cells were harvested, distributed into polypropylene tubes, and placed on ice. Non-antigen-specific binding of immunoglobulins to the Fcγ receptors on cell surfaces was blocked with antibodies (clone 2.4G2) specific for anti-mouse anti-FcγIII/II receptor (15 min incubation). The cells were incubated with fluorescein isothiocyanate (FITC)-labeled anti-CD8 mAbs (clone 53-6.7) for 30 min at 4°C and then washed with staining buffer (0.2% w/v BSA in PBS) followed by centrifugation. The cell pellet was re-suspended in 250 μl of Cytofix/Cytoperm™ solution and incubated for 15 min at 4°C to permeabilize cell membrane. The cells were then washed with Perm/Wash™ to maintain a permeabilized state and incubated for 30 min at 4°C with phycoerythrin (PE)-conjugated anti-IFN-γ mAbs (clone XMG1.2) in Perm/Wash™ solution. The cells were washed with Perm/Wash™ and re-suspended in staining buffer. Samples were analyzed with a FACScan (Becton Dickinson, Sunnyvale, CA) and WinMDI 2.8 software (The Scripps Research Institute, La Jolla, CA).

2.14 Chromium release assay

Cytolytic assays were performed as previously described [Wan *et al.*, 1999]. To prepare re-stimulator cells, EL4 cells were incubated with $OVA_{257-264}$ peptide SIINFELK and irradiated (5,000 rad). Isolated splenocytes were re-stimulated for 3 days with re-stimulator cells at a ratio of 50:1 in EL4 cell culture medium. LN cells were cultured without re-stimulator cells prior to the cytolytic assay. On the day of assay, fresh EL4 target cells were incubated with 1 µg/ml SIINFEKL (EL4/SIINFEKL) for 2 hrs and labeled with sodium chromate (^{51}Cr, PerkinElmer Life Science Inc., Boston, MA) at a dose of 100 µCi (1 µCi = 37 kBq) per 10^6 cells for 70 min (EL4 cells were also radioactively labeled as controls). Target cells were then washed to remove excess peptides and sodium chromate. Lymphocytes were cultured as effectors with targets in a V-bottom microtiter plate in 3-fold dilution duplicates (10:1, 30:1, and 90:1 based on 5,000 target cells/well) for 4 hrs at 37°C. In some wells, 10 µl of ascites fluid containing anti-CD8 mAbs (clone 53-8.7, American Type Culture Collection, Manassas, VA) were added into 20 min before the addition of target cells to block CD8-mediated cytolytic activities. Supernatants containing sodium chromate released from lysed target cells were harvested and measured for radioactivity on a TopCount® NXT™ Microplate Scintillation and Luminescence Counter (Packard Bioscience Company, Meriden, CT). The experimental release was a value relative to the maximum release generated by addition of 1N HCl into wells containing target cells only. The minimum value measured in the supernatant of untreated target cells referred to spontaneous release. The specific

lysis was calculated as percentage of (experimental release − spontaneous release)/(maximal release − spontaneous release).

2.15 Statistical Analyses

Results are expressed as mean ± standard deviations (SD) or range where applicable. Comparisons between groups were analyzed by Student's t-test. Comparisons among means of more than two groups were determined by one-way ANOVA *post hoc* with *Bonferroni* adjustment. Analyses were performed with SPSS 11.5 for Windows (SPSS, Chicago, Illinois). *P* values less than 0.05 were considered statistically significant.

CHAPTER 3   ADV GENE TRANSFER AND RECTAL CHALLENGE MODEL OF HSV-2

3.1   The pipetting and Dermabond® method for IR administration

To elicit a potent mucosal immune response, an antigen must be introduced across the epithelium to the mucosal system and be present at appropriate levels. Previous studies demonstrated that after IR delivery of Adv colonic epithelial cells served as a locale for gene transduction and enabled antigen access to the colonic mucosa on the basis of two observations [Thomson, 2001]. First, Adv-encoded β-gal was mainly expressed within epithelial cells along the entire length of colonic epithelium. This was achieved by removing mucus with 50% v/v ethanol enema in a 3-hr process. Second, IR delivery of Adv-encoded OVA resulted in marked colonic inflammatory responses (lymphocytic infiltration and enlarged lymphoid follicles) in the mice that had been sensitized with OVA absorbed to aluminum hydroxide by two intraperitoneal injections. However, this IR model might not be adequately developed. When Adv-encoded OVA was given IR to naïve mice, OVA-specific cytotoxic T lymphocyte (CTL) responses were not detected in the iliac lymph nodes (ILNs) as an important measure of cell-mediated mucosal immunity [Thomson, 2001]. Gene transfer in these animals to a lesser degree than in sensitized mice was unlikely to be the reason because there was no statistical

difference in gene expression level between naïve and sensitized mice (data not shown). It was speculated that the amount of antigen was sufficient to stimulate primed lymphocytes to respond but might not be great enough to prime sufficient naïve lymphocytes. Although giving an increasing dose might be necessary, the 3-hr incubation of ethanol might be detrimental to cells because an *in vitro* 3-hr ethanol enema resulted in death of most epithelial cells (T84 cells, human colonic crypt-like epithelial cells). It was observed that mucus was expelled from the rectum and could be rinsed out 40 min after ethanol instillation, thus this procedure time could be lessened. Another concern about insufficient gene transfer was severe leakage of administered material from the colon during and immediately after IR infusion due to the occupancy of catheters inserted in the intestinal lumen and the bowel movement, respectively.

In the present study, three solutions were proposed to improve gene transfer. The first solution was to increase the Adv dose to 2×10^9 plaque-forming units (PFU), i.e., twice of that used previously [Thomson, 2001]. The second solution was to reduce the duration of ethanol enema to 40 min (from 3 hrs of the standard protocol). The third solution was to prevent leakage by developing a new injection method without catheterization. The main drawback of catheterization is the risk of colon perforation and epithelial damage because rectal bleeding is seen from time to time [Coligan *et al.*, 2002]. Thus, a pipetting and Dermabond® (P&D) method was developed as a non-invasive delivery of material by pipetting material into the colon through a fire-polished 1000 µl micropipette tip placed in the anal canal. The P&D avoided during-infusion leakage and

physical contact of the delivery device with the epithelium and, thus, completely prevented colonic perforation and epithelial damage. To prevent leakage after administration, one drop of Dermabond® (a skin adhesive) was applied topically at the anal opening. Dermabond® was removed with ease after 4-5 hours and no anal blockade was observed after Dermabond® removal. It was found that both pipetting and Dermabond® effectively prevented loss of administered material. Under such circumstances, there was a need to re-examine the mucus status and re-visit gene expression.

3.2   Ethanol treatment of colon

The present study first examined whether 40-min incubation with 50% v/v ethanol administered IR by pipetting was sufficient to remove mucus. At 20 min (the rest time before IR administration of viruses) after the 40-min ethanol incubation, the presence of mucus was examined by staining with Alcian blue and periodic acid-Schiff's (AB/PAS, reacting with glycoproteins of mucus on the apical surface of epithelial cells). There were no significant abnormalities in histological morphology. As compared to control tissues (PBS treatment) that were positive for AB/PAS staining (**Figure 3.1a-c**), ethanol-treated intestinal wall along the length of 4-cm colon from the anal portion was AB/PAS negative (**Figure 3.1d,e**). The very proximal colon (near the cecum, **Figure 3.1f**), the entire cecum and distal ileum (data not shown) were AB/PAS positive

regardless of ethanol treatment. This indicated that pipetting plus 40-min ethanol incubation was efficient to remove mucus from the majority of the colon.

Figure 3.1 Histological assessment of mucus on mouse colon. 50% ethanol or PBS was

pipetted intrarectally (IR) and incubated for 40 min. Colons were then rinsed with PBS.

Twenty minutes later, colons were removed, fixed in 10% formalin, paraffin embedded,

sectioned and stained with Alcian blue/periodic acid-Schiff's (AB/PAS) reagents. (a–c)

Mucus and goblet cells were positively stained deep blue on the apical surface of the

colon in PBS treated mice (black arrows). (d,e) After ethanol treatment, the majority

colonic epithelial surface was AB/PAS negative (white arrows) although goblet cells

were still positive. (f) Mucus was not removed from the colon near cecum (black arrow).

There were no significant abnormalities in histological morphology. One of two

experiments is shown. A magnification of ×400 diameters.

Distal colon (PBS treated)	a	Distal colon (ethanol treated)	d
Proximal colon (PBS treated)	b	Proximal colon (ethanol treated)	e
Colon near cecum (PBS treated)	c	Colon near cecum (ethanol treated)	f
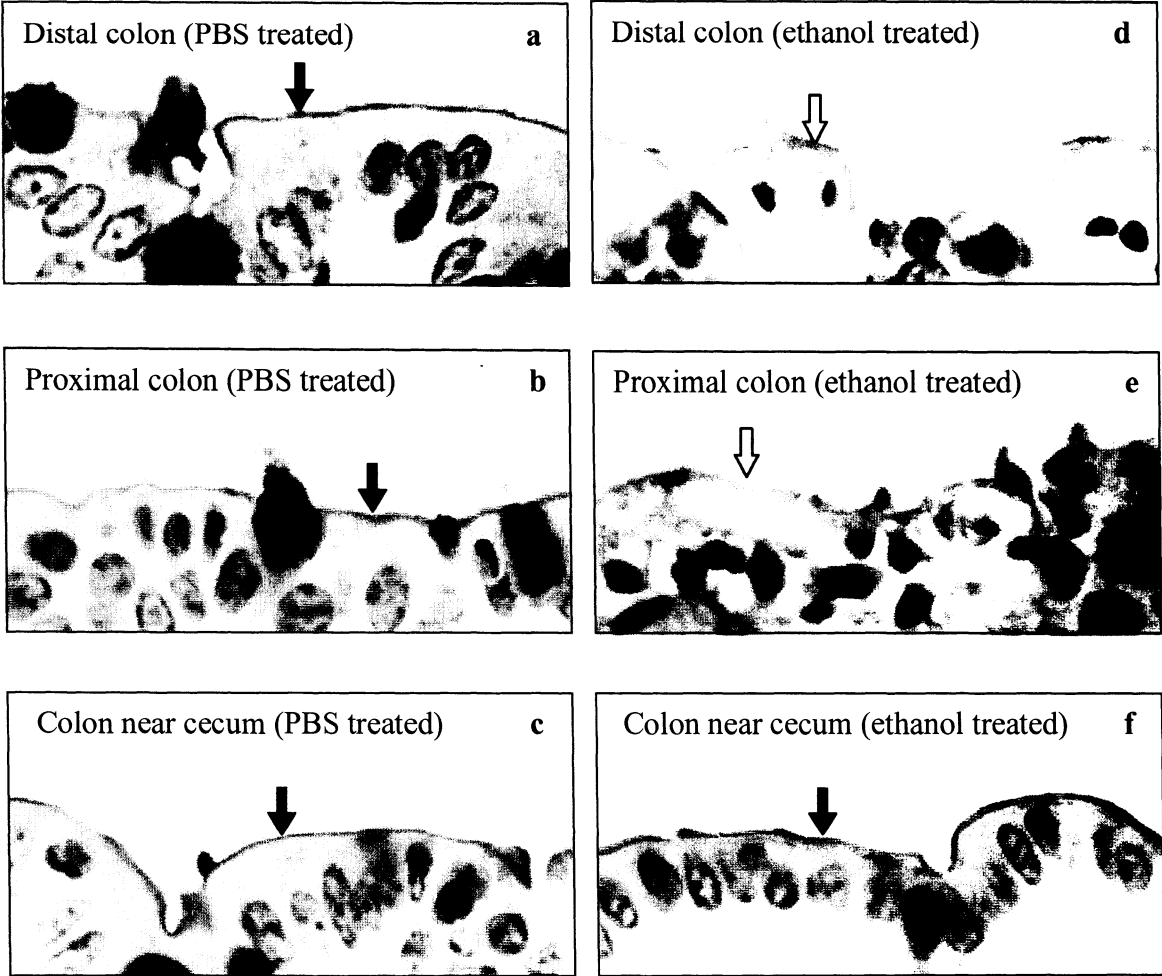
(Figure 3.1)

It was questioned whether the presence of mucus in the cecum and ileum was due to the inaccessibility to instilled ethanol. To examine the possibility, India ink (a colloidal carbon solution) was given using the pipetting method and its distribution was inspected by gross examination. The ink was mainly present in the colon with some in the cecum but rarely in the small intestine. The ileocecal valve probably prevented instilled material from entering the ileum. Although found in the cecum, the ink was largely trapped in the cecal feces, which likely reduced the contact of ethanol with the mucosal surface and, thus, prevented mucus from being removed.

3.3 Quantitative characterization of Adv-mediated gene transfer in the colon

Previous studies showed that β-gal expression in the colon could not be visualized unless ethanol pretreatment was performed, thus highlighting the use of ethanol to allow gene transfer by Adv [Thomson, 2001]. The present study was to confirm this by performing luciferase quantification assays at day 2 (the time that β-gal transgene was detectable in previous studies) after IR administration of AdLuc. The luciferase activity was detected in the colon without ethanol pretreatment, suggesting that gene could be transferred to the colon in the presence of mucus. However, ethanol pretreatment dramatically enhanced gene transfer efficiency by approximately 100-fold (**Figure 3.2a**). It was probable that colonic epithelial cells were completely exposed to Adv in the

Figure 3.2 Quantification of transgene expression in the mouse colon. Adenoviral vectors (Adv) encoding luciferase (AdLuc) were IR administrated by the pipetting and Dermabond® (A&D) method. The intestinal tissues were removed at day 1 2, 3, 4, 6 and 8, and the luciferase activities were determined in tissue homogenates. (a) Gene expression at different intestinal segments was detected. Comparisons of luciferase activity were performed amongst different intestinal segments and corresponding segments between ethanol- and PBS-treatment groups. Ethanol treatment significantly increased gene expression in colon ($P < 0.01$) but not in the cecum. Transgenes were mainly found in the colon, minimal in the cecum (colon vs. cecum $P < 0.001$) and at baseline levels in the distal ileum. (b) The capacity of the colon for gene transfer was evaluated at day 2. There was a dose-dependent increase in luciferase expression. (c) Time course of transgene expression in the colon was examined. The luciferase activities were high at day 1-3, decayed from day 3 but significantly at day 4. The enzyme activity is expressed as mean ±SD from 3 mice in each group and determined by an increase in relative light unit (RLU) per tissue weight (tw) compared to background measured in untreated mice. Unless indicated, mice were pretreated with ethanol in the colon prior to IR administration of Adv. *: $P < 0.05$. **: $P < 0.01$. ***: $P < 0.001$.
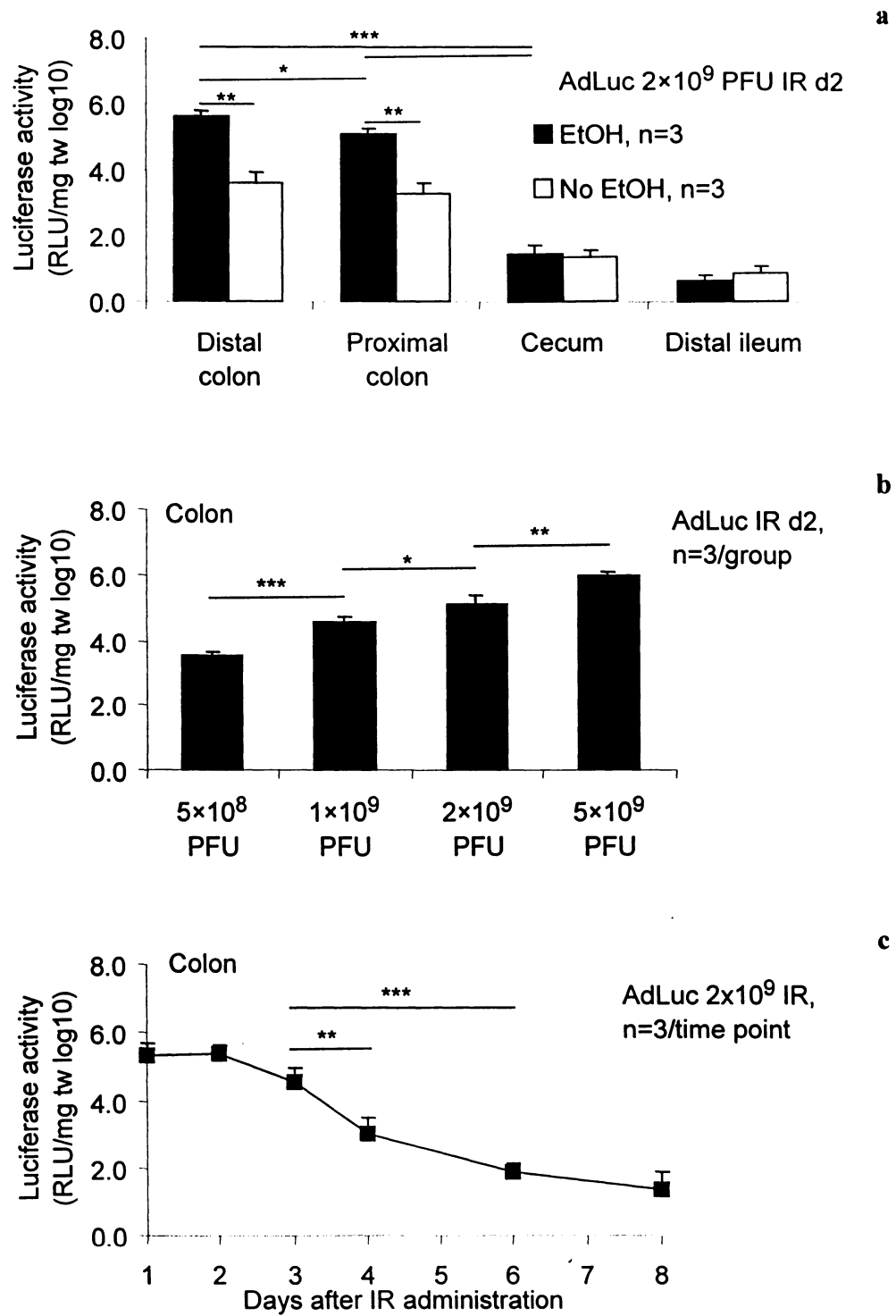
(Figure 3.2)

absence of mucus and viral infection was greatly enhanced, resulting in a larger amount of gene transduction by infected cells. This clearly indicated that removing mucus was essential for effective gene transfer to the colon.

The luciferase quantification assay was used to examine gene distribution along the lower intestines. Regardless of ethanol pretreatment, luciferase activities were mainly detected in the colon (**Figure 3.2a**). Gene expression in the distal half of colon was 2-3-fold higher than that in the proximal half irrelevant to ethanol pretreatment (**Figure 3.2a**). Cecal luciferase expression occurred at very low levels and did not increase after ethanol treatment (**Figure 3.2a**), which might indicate the presence of mucus prohibiting Adv infection and gene transfer. In the ileum, gene expression was almost undetectable (**Figure 3.2a**). This was probably because Adv did not reach the small intestine as described earlier (see Section 3.2). Therefore, these data indicated that colon was the major site for gene transfer and adjacent segments such as cecum played a minor role.

The capacity of colon for gene transfer was further examined using the luciferase reporter system at day 2. There was a dose-dependent increase in luciferase expression; administration of the highest dose available, 5×10^9 PFU AdLuc in 100 μl, gave rise to the highest gene expression over other doses (**Figure 3.2b**). These results were consistent with previous observations on β-gal gene expression showing a dose-dependent increase in positively stained β-gal spots [Thomson, 2001].

The time course of gene transfer to the colon was analyzed by the quantitative measurement of luciferase activity after IR administration of AdLuc. It was found that luciferase was highly expressed from day 1 to day 3 (**Figure 3.2c**). The expression tended to decline at day 3 but significantly at day 4 and remained at low levels until approximately one week after administration.

3.4   Qualitative characterization of Adv-mediated gene transfer

Previous studies using β-gal showed that Adv-mediated gene transfer occurred almost exclusively within epithelial cells [Thomson, 2001]. Here, the locale of gene expression was reassessed. Other than within the epithelium, β-gal expression was also detected beneath the epithelium, either diffuse in the LP (**Figure 3.3a**) or associated with lymphoid follicles (**Figure 3.3b**). The frequency of LP cells expressing Adv-transduced gene was however significantly lower than that in epithelial cells ($P < 0.05$, **Figure 3.3c**). This suggested that Adv-mediated gene transfer also took place beneath the epithelium.

3.5   Determination of Adv dose at each site based on gene expression

One of the objectives of the present study was to compare effectiveness of the IR

Figure 3.3 Gene expression immediately beneath the epithelium of mice. β-galactosidase

encoded Adv (Adβ-gal) was administrated IR. Two days later, colonic tissues were

removed and immediately stained with X-gal at 37°C for overnight. Tissues were then

formalin fixed, paraffin-embedded, sectioned (4-5 μ) and counterstained with eosin and

nuclear fast red stain. (a) β-gal staining was found to be cell-associated and located in

lamina propria (LP) interstitium or (b) lymphoid follicles. (c) The frequency of β-gal

expressing cells immediately beneath the epithelium was significantly lower than within

the epithelial layer ($P < 0.05$, n = 3). Image: a magnification ×200 diameters.
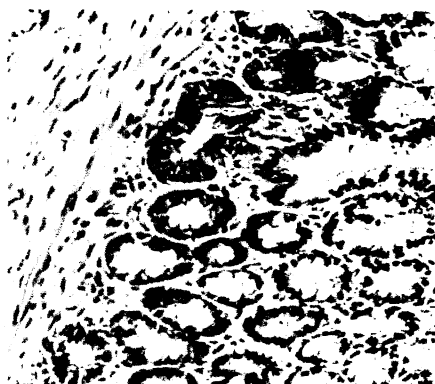
a
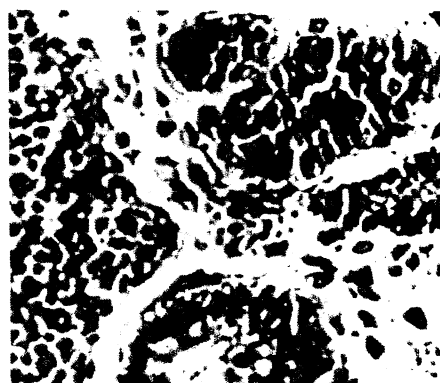
b
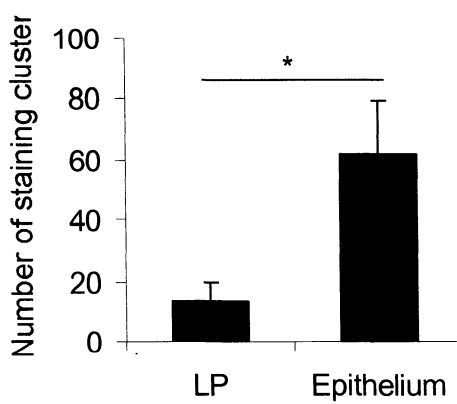
c

(Figure 3.3)

route with others such as intranasal (IN), intravaginal (IVAG) and subcutaneous (SC) in providing rectal and genital protection. Due to the differences in organ size, immune structure and cell compositions, there is no defined way to direct compare immunological functions in different organs or tissues. Also, there is no corresponding property of gene uptake and gene transfer at each site. "One dose" for all different routes appears to be inappropriate. The dose of 5×10^8 PFU Adv, which induced immune responses via the IN route, proved to be insufficient to induce rectal immune responses, while the dose of 2×10^9 PFU that was able to prime colonic lymphocytes was found to be toxic to mice when given IN (in a pilot experiment, 3 out of 6 mice were moribund). Thus, a comparison was performed based on a relatively constant gene expression level of either density (amount per unit tissue weight) or total antigen mass in the entire tissue. The former one appears to be a more appropriate to use because antigen concentration is an important parameter to determine immune induction [Healy and Goodnow, 1998].

3.6   Comparison of gene transfer by different routes

Depo-Provera® contains medroxyprogesterone acetate (a derivative of progesterone), which, in mice [Parr et al., 1994; Khanna et al., 2002] and monkeys [Marx et al., 1996], acts similarly to progesterone, markedly thinning the epithelial layer of vaginal wall, thus reducing the barriers to virus transmission. It was found that the

luciferase density in the female genital tract was 10-fold lower after IVAG administration

of AdLuc as compared to IR and IN routes unless the mice were pretreated with Depo-

Provera®. After Depo-Provera® treatment, luciferase density increased to a level

comparable to those at other sites. It was found that administration of 2×10^9, 5×10^8 and

1×10^9 PFU by the IR, IN and IVAG (with Depo-Provera®) route, respectively, gave rise

to similar levels of antigen density (IR vs. IN $P = 0.698$, IR vs. IVAG $P = 0.054$, **Figure**

3.4a) and antigen mass (IR vs. IN $P = 0.616$, IR vs. IVAG $P = 0.172$, **Figure 3.4a**). Since

there was no similar way to measure antigen deposit for SC gene delivery, the SC

injection dose was set at 5×10^8 PFU which ensured no underdose compared to that for IN

administration. When 1×10^9 PFU was applied in the upper GI tract, gene expression was

only at baseline levels as compared to controls (**Figure 3.4b**). It showed that when Adv

was delivered directly to the duodenum, gene transfer could be detected in the colon but

still very low as compared to the small intestine [Cheng *et al.*, 1997]. Thus, IG

immunization was not further studied at this point.

3.7   Establishment of a rectal challenge mouse model with HSV-2

The critical step to be taken toward the objectives of this study was to verify the

Adv IR immunization strategy by applying it in an infectious model. The mouse model of

genital HSV-2 infection is attractive because of its relevance to human disease. Rectal

Figure 3.4 Comparison of gene transfer by different mucosal routes in mice. At two days

after AdLuc administration via IR, intranasal (IN), intravaginal (IVAG), or intragastric

(IG) route, luciferase activities were determined by measuring luminescence in each

corresponding tissue as RLU per tw or organ. At specified doses used for immune

function studies, there was no statistical difference in the density or total mass of gene

expression at each site (a, $P > 0.05$) except the upper GI tract (b), where gene expression

was substantially low. Averaged density of gene expression in the colon did not exceed

that in the lung or vaginal tract. The values are the mean $\pm$SD of 3 mice in each group.
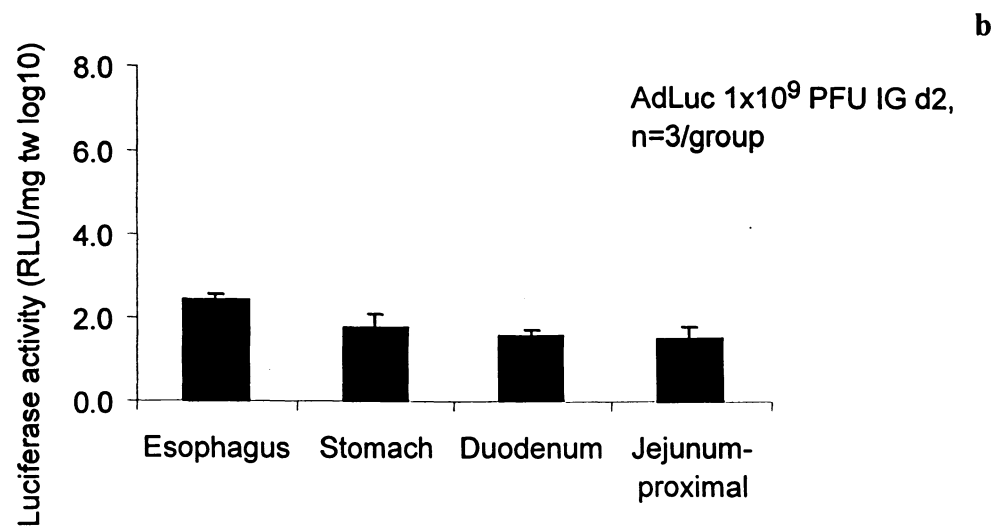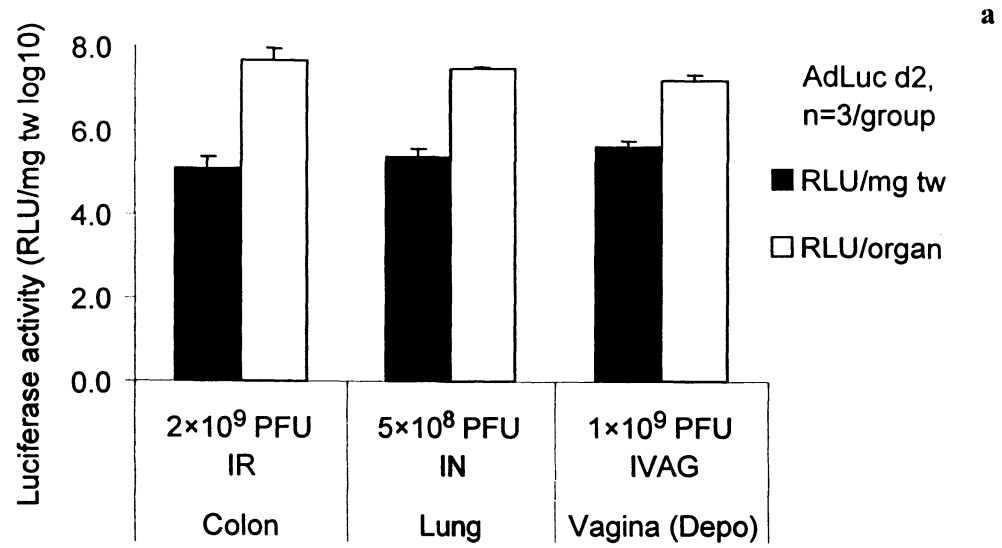
(Figure 3.4)

infection of HSV-2 is seen in humans as a result of sexual behavior. Therefore, a mouse challenge model was developed to suit the need to investigate rectal protection.

The initial attempts to rectally-inoculate C57BL/6 mice with of HSV-2 strain 333 being used in genital model did not succeed in the establishment of overt infection. Among all experimentally determined doses, inoculation of the highest dose, 2×10^7 PFU, resulted in only a mild infection in 50% of mice (n = 4) and only 25% of them developed paralysis. Previous studies achieved HSV-2 infection by pretreating mice rectally with contraceptive jelly nonoxynol-9, which was shown to disrupt the epithelium [Phillips and Zacharopoulos, 1998; Phillips et al., 2000], or mechanical abrasion with metal cylinders on the mucosal surface [Zeitlin et al., 2001]. As clinically observed rectal infections of HSV-2 are often associated with mucosal abrasion, it is reasonable to speculate that the presence of the integrated mucus layer might confer resistance to virus infection. In the present study, ethanol was thus used again to remove the mucus layer and it was found that mice became infected. A pilot experiment showed that perianal inflammation became increasingly severe and developed perianal ulcerative lesions, paralysis of hindlimbs, and death within 7-10 days after rectal inoculation. Removing the mucus rendered mice susceptible to HSV-2 infection via the rectal route and, thus, was used as a standard protocol for rectal challenge of viruses including vaccinia viruses (VV).

To determine the absolute lethal dose (LD_{100}) of rectal infection, HSV-2 was given at various doses ranging from 2×10^1 to 2×10^6 PFU. The mortality of infected mice

was based on the development of hindlimb paralysis, which was considered as the endpoint proceeding lethality. No mouse developed disease at doses ranging from 2×10^1 to 2×10^2 PFU although some of these mice were apparently infected (**Figure 3.5a**). At the dose range from 2×10^3 to 2×10^4 PFU, the majority, if not all, of mice were infected and a proportion of them reached the end point. When doses were elevated to 1×10^5, 2×10^5 and 2×10^6 PFU, all mice succumbed to infection by day 10 and no mice survived the disease (**Figure 3.5a**). As 1×10^5 PFU was the minimum dose that caused 100% death, it was determined to be the LD_{100}. Both 2×10^5 PFU (2-fold LD_{100}) and 2×10^6 PFU (20-fold LD_{100}) were used for subsequent challenge experiments.

The mice receiving HSV-2 rectally at the doses of 2×10^5 and 2×10^6 PFU developed the disease and worsened progressively. The overall features of clinical pathology did not substantially differ from those found in vaginally-infected mice. On the basis of pathology scales for genital infection and previous observations on rectal infection in mice, a 5-point clinical score was developed to describe the severity and progression of the disease (see Chapter 2.6 and Table 2.1). Previous studies reported that rectally-infected mice began with perianal inflammation [Phillips and Zacharopoulos, 1998] and developed ulcerative lesions in the late phase of the disease [Zeitlin *et al.*, 2001]. Similar manifestations were observed in the present study. Redness was the sign of infection first seen 2-4 days after inoculation, followed by mild swelling and severe swelling with hair loss. Lesions usually started around anal opening and expanded to the

Figure 3.5 Rectal challenge model of HSV-2 in mice. Animals were inoculated rectally with HSV-2 at various doses for determination of LD_{100}. (a) Mortality and (b) clinical pathology were monitored daily. For details about measurements, see Section 2.6 and Table 2.1. Mice 100% developed paralysis after IR inoculation of HSV-2 at doses of 2×10^5 and 2×10^6 PFU. (c) To localize HSV-2 replication in the rectal tissue, colons were removed 5 days after rectal inoculation and equally divided into 3 segments: distal, middle, and proximal. Each intestinal segment was homogenized and determined for virus replication by plaque-forming assays on Vero cell monolayers. Virus replication occurred mainly in distal colon. (d) To determine virus shedding, rectal swabbing was performed daily from day 1-5 and day 7. Swab collections were transferred to culture media and titers (PFU/swabbing sample) determined by plaque-forming assays on Vero cell monolayers. Viruses were shed at high levels throughout entire course of the disease. The clinical scores and virus titers are expressed as mean $\pm$SD derived from mice numbered as indicated in each group.
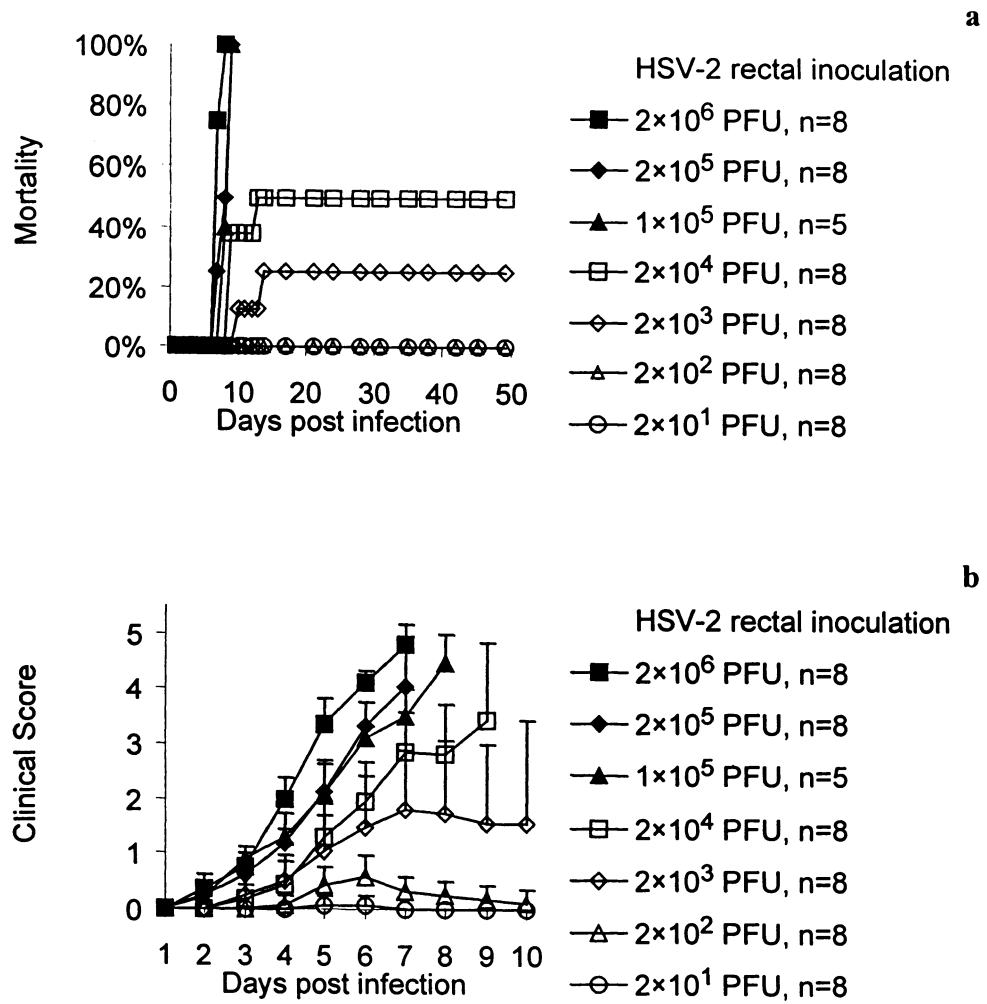
(Figure 3.5 a,b)
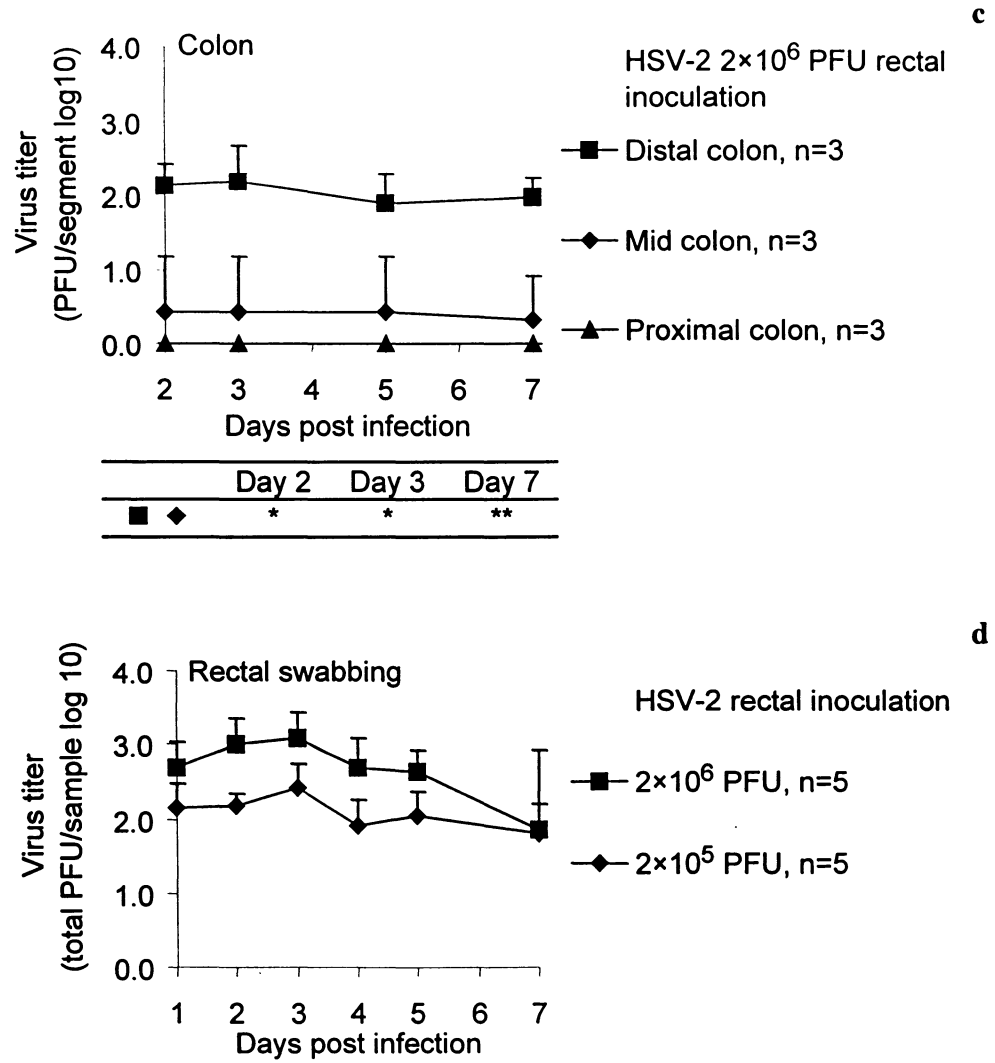
(Figure 3.5 c,d)

skin of perianal area around day 6-7. Mice were 100% paralyzed within 10 days. When

were infected with less than the LD_{100}, some mice were able to resolve the disease and

the mice that did not display morbidity in the initial 10 days never developed disease.

The rectal swabbing method has been clinically used to retrieve shed viruses from

the rectal tract of HSV-2-infected patients. To examine the validity of this method in

mice, the location of viral replication in the colon was examined. Colons were resected

from HSV-2 infected mice at 2, 3, 5 and 7 days post-infection and infectious HSV-2 was

determined in homogenates using the viral plaque-forming assay. Along the colon, the

highest virus titers were found in the distal segment and considerably less in the middle,

while no viral particles were detected in the proximal end (**Figure 3.5c**). This indicated

that distal colon was the major site for HSV-2 replication and rectal swabbing should be

able to recover the majority of viruses shed into the lumen. Because swabbing samples

were contaminated by fecal contents, it was difficult to estimate recovered volume for the

calculation of virus concentration. Alternatively, total virus PFU of each sample was used

to represent the degree of virus shedding. It was found that, although tended to be higher

around day 2-3, HSV-2 was shed at relatively constant levels until day 7, at which time

most mice developed paralysis (**Figure 3.5d**). Overall, rectal swabbing could be used to

measure shed viruses in the rectal lumen, and viral titers reflected the levels of virus

replication in the rectal tissue.

It was found that the rectal challenge model of HSV-2 was reproducible when

mucus was removed with ethanol prior to inoculation and appeared to be an *in vivo* means of examining the significance of mucosal immunological barriers. Mortality, clinical pathology, and virus shedding were useful measures in this regard.

3.8   Summary

The P&D IR delivery method was non-invasive and effectively prevented leakage of delivered material. The ethanol treatment removed mucus from the majority of the colon and remarkably enhanced Adv-mediated gene transfer. Adv-encoded heterologous genes were transferred mainly to the colon and minimal to upper intestinal segments. Colonic transgenes were highly expressed during the first 1-3 days and maintained at relatively low levels for approximately one week after administration. Gene transfer not only occurred within the epithelium but also, to a lesser extent, immediately beneath the epithelium.

Mice become susceptible to rectal infection of HSV-2 after ethanol treatment in the rectum. The disease developed in rectally-infected mice resembled mouse genital infection with respect to local inflammation, ulcerative lesions and hindlimb paralysis. Virus shedding occurred throughout the entire course of the disease. This rectal HSV-2 challenge model might be useful to examine mucosal immunity against viral infection via the lower GI tract.

CHAPTER 4   INDUCTION OF MUCOSAL IMMUNE RESPONSES

4.1   T-cell mediated interferon (IFN)-γ responses

When a foreign antigen enters the mucosal immune system, it is captured and processed by antigen-presenting cells (APCs). It is then presented to stimulate lymphocytes accessing there to proliferate and differentiate into effectors that can respond to the antigen at the next encounter. It was shown that a significant amount of antigen was transferred to the colonic mucosa with the use of Adv via the rectal route (see Chapter 3). Whether this gene-based antigen delivery could stimulate the colonic mucosal immune system to mount an antigen-specific mucosal immune responses was a main question to be addressed. To explore the possibility of immune induction via the colon, ovalbumin (OVA) was chosen as a foreign antigen to be given by IR with Adv. OVA is the model antigen that has been widely used in murine experimental models to evaluate host immune responses against foreign antigens. The significance of the OVA model system lies in increasingly wide availability of techniques and reagents for characterization of antigen-specific immune responses. Previously cloned the full-length chicken OVA gene was cloned into an adenovirus vector (AdOVA) and its functions were confirmed to induce specific immune responses [Thomson, 2001]. Infection of target cells with this vector led to expression of gene product and secretion of authentic

OVA. SC immunization with AdOVA induced both antigen-specific CTLs and antibody responses, and immunized mice had the ability to inhibit the growth of OVA-expressing tumor cells inoculated subcutaneously.

The IFN-γ secretion from T cells in response to antigens is indicative of the generation of antigen-specific effectors and is also a hallmark of the induction of T-cell mediated adaptive immune responses [Boehm *et al.*, 1997; Murphy and Reiner, 2002]. It was investigated whether antigen-specific IFN-γ-secreting cells could be induced by immunization with Adv via the rectal mucosa. After intrarectal (IR) administration of 2×10^9 PFU AdOVA in C57BL/6 mice, colonic lamina propria lymphocytes (LPLs), ILN cells and splenocytes were recovered at days 4, 6 and 14. Following a 24-hr incubation with $OVA_{257-264}$ peptide SIIENFEKL (an immunodominant MHC class I specific epitope of C57BL/6 mice), OVA-specific IFN-γ-secreting spots, each of which represents an individual IFN-γ-secreting cell, were inspected in an ELISPOT plate pre-coated with anti-IFN-γ mAbs (**Figure 4.1**). As early as day 4, IFN-γ secretions were detected from isolated lymphocytes from immunized mice (**Figure 4.1**). The number of IFN-γ-secreting cells from both colonic LPLs and splenocytes increased over time (**Figure 4.1a,b**). The results suggested that IR immunization with Adv induced not only local but also systemic T-cell-mediated immune responses. It was noteworthy that ILN lymphocytes displayed responses with a distinct pattern. The frequency of OVA-specific ILN cells was high at days 4 and 6 but became substantially lower at day 14 (**Figure 4.1c**). As compared to their counterparts in the spleen and colon, antigen-specific ILN T cells seemed to be

Figure 4.1 Frequency of antigen-specific IFN-γ-secreting cells in mice. At days 4, 6 and

14 after IR immunization with 2×10^9 PFU Adv encoding ovalbumin (AdOVA), (a)

colonic lamina propria lymphocytes (LPLs), (b) splenocytes and (c) ILN cells were

isolated and cultured at 37°C in the presence of OVA SIINFEKL peptides in a plate pre-

coated with capture mAbs against IFN-γ. After 24-hr incubation, the cells were removed

and IFN-γ-secreting spots were measured by adding detection antibodies specific for the

cytokine followed by color development. OVA-specific, IFN-γ-secreting colonic LPLs

and splenocytes increased over time, while large quantity of specific ILN cells were

present at the beginning and disappeared by day 14. The results are shown as range of

ELISPOT from two independent experiments.
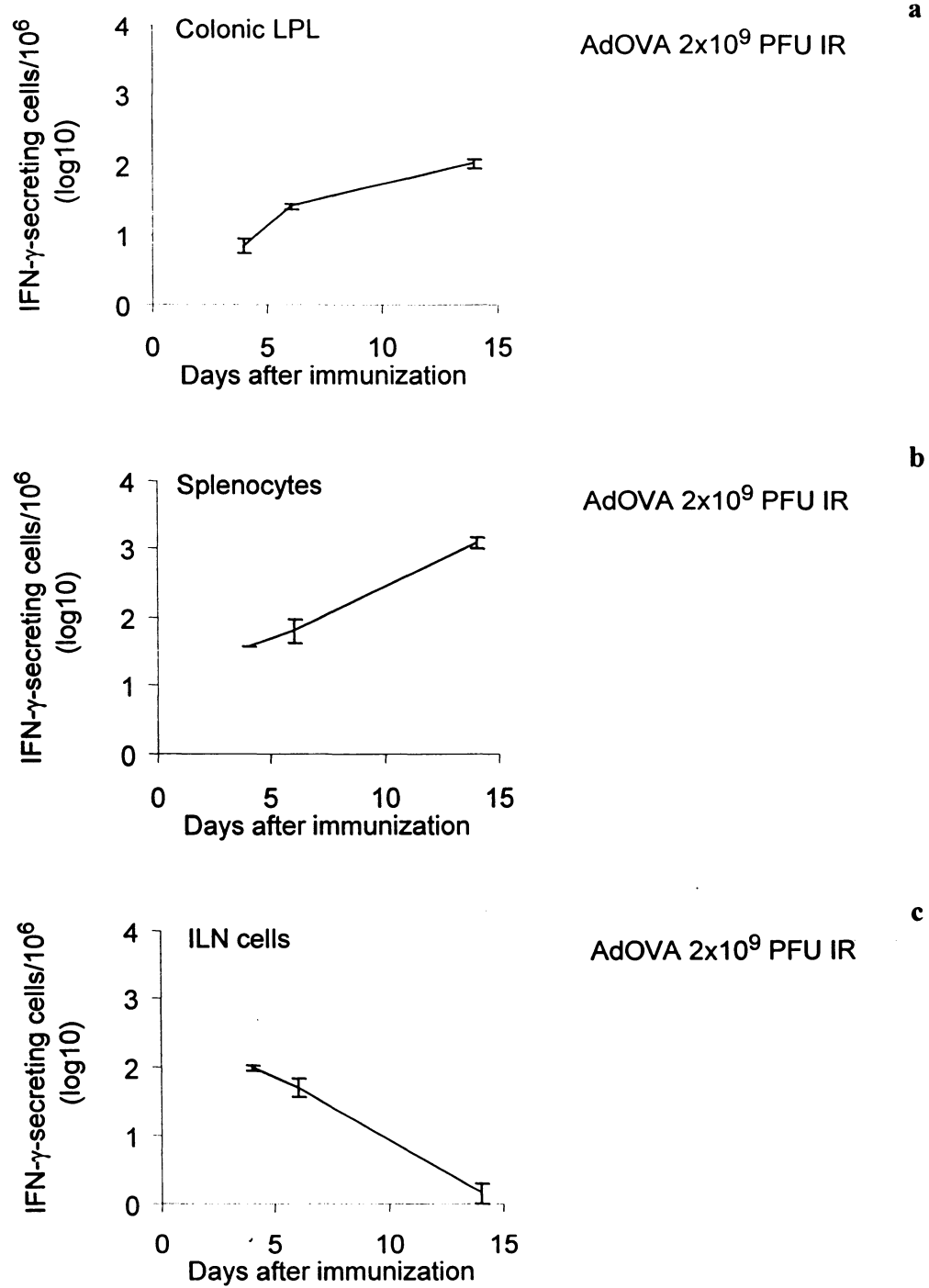
(Figure 4.1)

highly generated earlier but persisted transiently; while ILN numbers decreased, both

specific LPL and splenocyte populations were still growing. These results suggested that

Adv given by the rectal route induced T-cell mediated immune responses and also

implied that ILNs might play an early inductive role in the colonic mucosal immune

system.

CD8$^+$ effector T cells are essential in responding to viral antigens by secreting

IFN-γ following interactions of their TCRs with MHC-I/peptide complex on infected

cells. In the present study, this was examined by staining intracellular IFN-γ in CD8$^+$

spleen cell populations after immunization with AdOVA. It was found that CD8$^+$

splenocytes were able to produce IFN-γ upon exposure to MHC class I restricted

SIINFEKL peptides with a frequency of ~1% (**Figure 4.2**). On the contrary, cells isolated

from mice receiving a control vector that did not express transgene were unable to

produce IFN-γ in the presence of SIINFEKL. Thus, the data suggested that IR

immunization with an Adv-carried foreign antigen stimulated CD8$^+$ T cells to be able to

specifically respond by producing IFN-γ when encountered the antigen.

4.2   Induction of lymphocyte cytotoxic activity

CD8$^+$ T effectors (CTLs) employ a number of cytotoxic mechanisms to kill

Figure 4.2 Intracellular IFN-γ staining of mouse CD8$^+$ T cells. Three-four weeks after IR

immunization with 2×10^9 PFU AdOVA, splenocytes were isolated and stimulated with

SIINFEKL peptides in the presence of brefeldin A for 4-6 hrs. Cells were then incubated

with anti-CD8 mAbs followed by anti-IFN-γ mAbs staining in a permeabilization

condition of the cell membrane. The percentage of CD8$^+$ cells producing IFN-γ was

analyzed with FACScan and indicated in the plot. Approximately 1% of splenocytes from

AdOVA IR immunized mice produced IFN-γ (left panel), while cells derived from mice

treated with control Adv without transgene expression did not (right panel). One of two

experiments is shown.
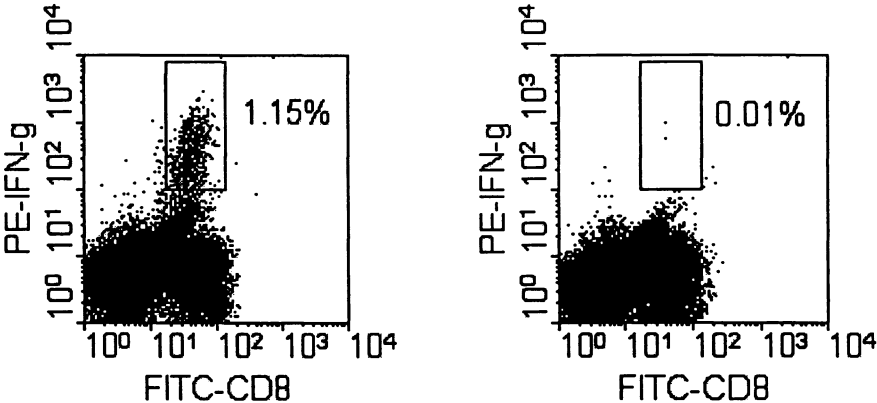
(Figure 4.2)

virally-infected cells and, therefore, are essential in the control of virus infection [Kagi *et al.*, 1996; Russell and Ley, 2002]. On the basis of the above observations that IR immunization with Adv induced T-cell mediated IFN-γ production, it was next examined whether antigen-specific CD8$^+$ T cells were cytotoxic. This was determined with chromium release assays, in which lymphocytes, as effectors, were isolated from lymphoid tissues of immunized mice and measured for their capacity to kill ^{51}Cr-labelled antigen-expressing target cells by quantifying the pre-ingested ^{51}Cr released from the targets upon lysis. The target cells were created by exposing EL4 thymoma cells with the SIINFEKL peptides (EL4/SIINFEKL) prior to use, and the lysis of such targets indicates MHC class I-restricted killing. Following AdOVA IR immunization at a dose of 2×10^9 PFU, ILN cells and splenocytes were isolated at various time intervals (days 4, 6, and 14). Before assay setup, fresh splenocytes were re-stimulated with EL4/SIINFEKL for 3-4 days. This allows the assay, which has low sensitivity, to measure a recall cellular response after *in vitro* proliferation of specific lymphocytes. ILN effectors were cultured for 3 days in the absence of re-stimulator cells. Assays on these cells measure the cytolytic ability of lymphocytes that have been primed without *in vitro* proliferation, hence reflecting a primary immune response in the draining lymph nodes (DLNs). It was found that ILN lymphocytes displayed cytolytic activities at days 4 and 6 (**Figure 4.3a-b**) but undetectable activity at day 14 (**Figure 4.3c**). The character of ILN cytolytic activities was a reminiscence of IFN-γ-secreting cells (see Chapter 4), suggesting that lymphocytes were primed in ILNs at the early induction phase. CTL responses were also detected in the spleen. In contrast to ILN cells, the splenocytes exhibited lower cytolytic

Figure 4.3 Cytolytic activities of ILN and spleen cells in mice. At various time after IR

immunization with 2×10^9 PFU AdOVA, ILN and spleen cells were isolated. ILN cells

were recovered at days (a) 4, (b) 6 and (c) 14, and spleen: days (d) 10 and (e) 14. For

primary cytolytic assay, ILN cells were cultured for 3 days without re-stimulation. For

recall cytolytic assay, splenocytes were re-stimulated for 3 days with EL4 cells pulsed

with SIINFEKL peptides. The chromium release assay was performed with EL4 pulsed

with or without SIINFEKL at indicated ratios of effector to target (E:T). Cytolytic

activities of ILN cells were detected at days 4 and 6 but barely at day 14. Splenocyte

cytolytic activities increased over time. The results represent the range of values from

two independent experiments.

(Figure 4.3a-c)

d

e
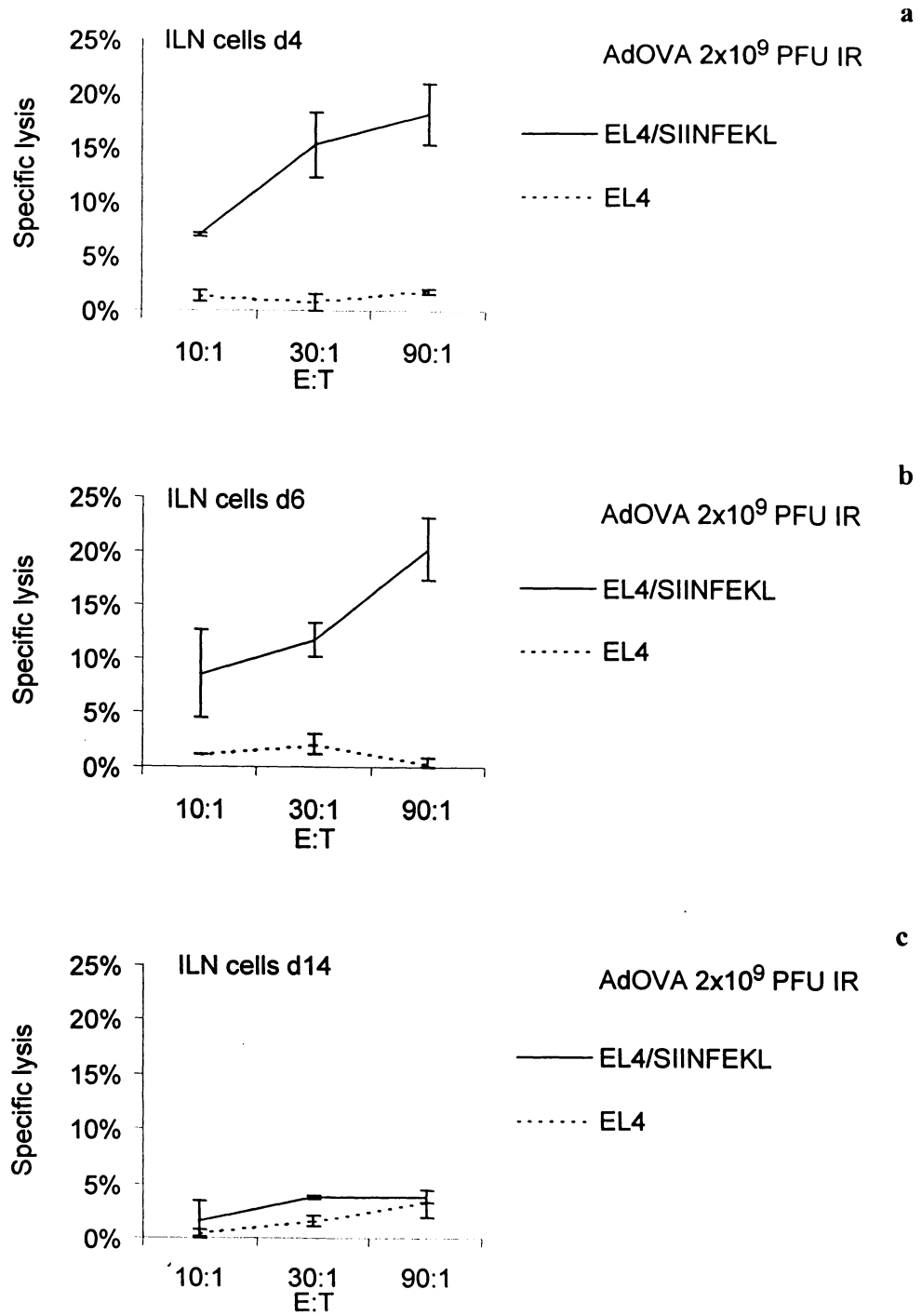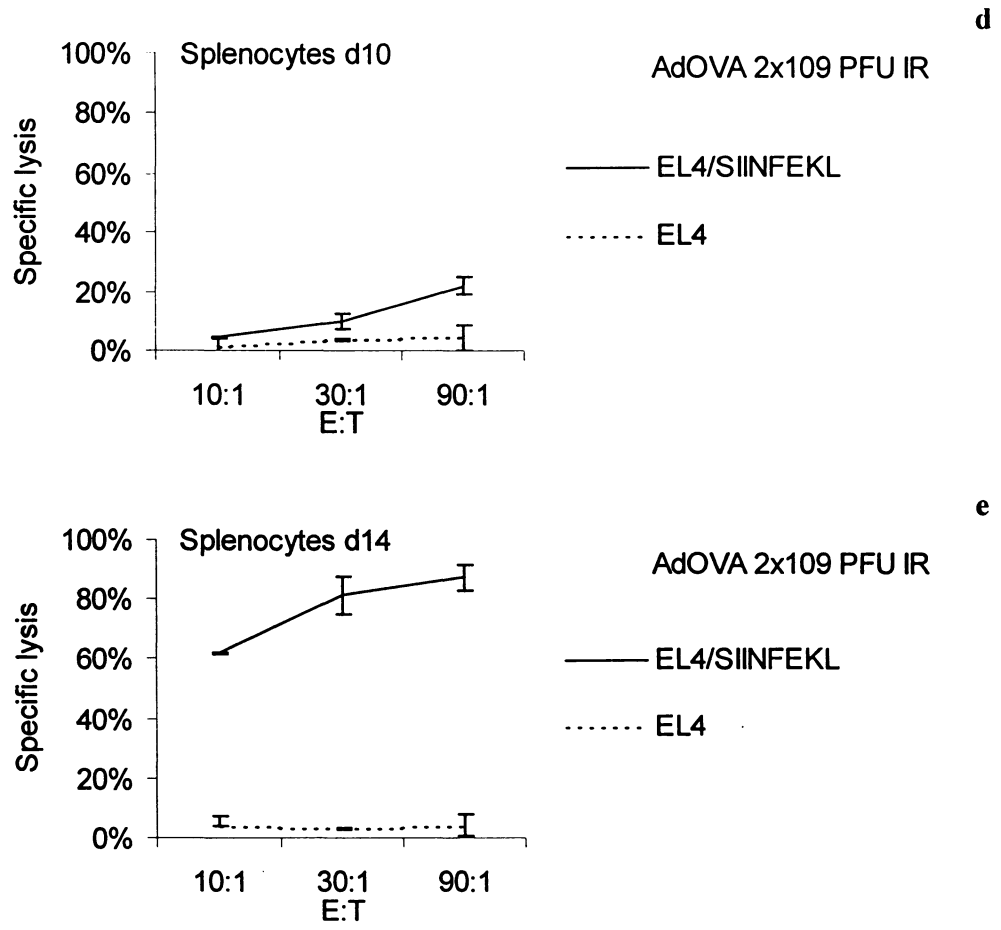
(Figure 4.3 d,e)

activities at early time but higher later on as noted at day 14 (**Figure 4.3e,f**). To determine whether the cytotoxicity was mediated by $CD8^+$ T cells, anti-CD8 mAbs were added into the incubation before the cytolytic assay. It was clearly shown that anti-CD8 antibody abolished the cytolytic response (**Figure 4.4**), thus indicating the induction of a CD8-mediated cytotoxic T-cell response. Therefore, IR immunization with Adv via the rectal mucosa was effective in priming $CD8^+$ T cells to become cytotoxic to antigen-bearing cells in an antigen-specific manner.

4.3   Colonic mucosa as an inductive site

The colonic mucosal immune system was the mucosal site of interest in immune induction after IR immunization. To clarify that colonic mucosa was the major site through which immune responses were induced, early OVA-specific immune responses (IFN-γ production and CTLs) at adjacent intestinal segments (i.e., ileoceca, the cecum and 3-cm distal ileum) and mesenteric lymph nodes (MLNs) were analyzed after IR immunization with AdOVA. The frequency of OVA-specific IFN-γ-secreting ileocecal LPLs and MLN cells were 10-fold lower than that of colonic LPL and ILN cells (**Figure 4.5a,b**). Also, MLN cytolytic activities were barely detectable as compared to ILNs (**Figure 4.5c**). This indicated that the immunological involvement of other intestines and associated LNs was minimal and implied that the colonic mucosa and ILNs played a central role in the induction of mucosal immune responses after IR immunization.

Figure 4.4 Determination of CD8-mediated cytolysis in mice. The assay was conducted

as described in Figure 4.3. Anti-CD8 mAbs (clone 53-6.7) were added into the assay

prior to the addition of target cells. Cytolytic activities of ILN and spleen lymphocytes

with E:T ratio at 90:1 are shown. Anti-CD8 treatment efficiently blocked antigen-specific

cytolytic activities, indicating CD8-mediated cytolytic responses. The results represent

one of two independent experiments.
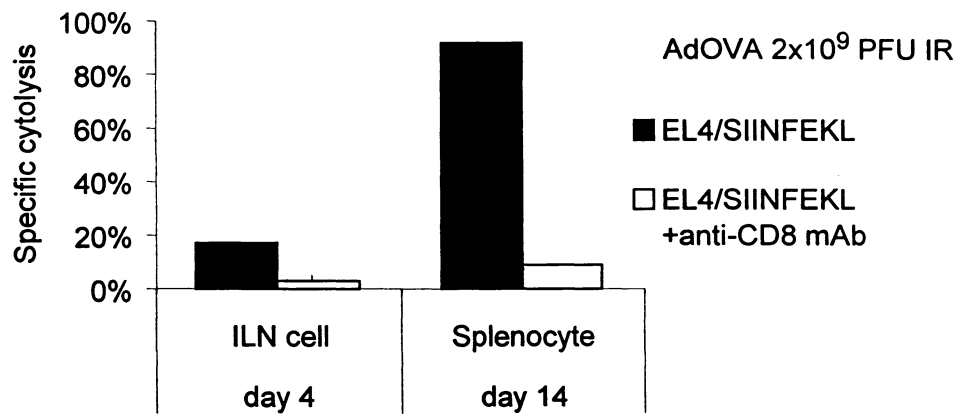
(Figure 4.4)

Figure 4.5 Determination of the colonic mucosa as the major inductive site in mice. (a,b)

The ELISPOT assay was used to measure IFN-γ-secreting cells in tissues. Following IR

immunization with 2×10^9 PFU AdOVA, colons, ileoceca (cecum and 3 cm distal ileum)

and associated LNs (ILNs and MLNs) were removed and lymphocytes were isolated at

day 4. ELISPOT was performed as described in Figure 4.1. Frequencies of IFN-γ

secreting cells were significantly higher (~10-fold) in colons and ILNs ($P < 0.01$) than in

ileoceca and MLNs, respectively. ELISPOT represents values from 3 mice each group as

mean ±SD. (c) The chromium release assay was used to measure LN CTLs. ILN and

MLN cells isolated at day 6 after AdOVA IR immunization were cultured for 3 days in

the absence of re-stimulator cells. The chromium release assay was performed as

described in Figure 4.3. Cytolytic activities were very minimally detected in MLNs as

compared to ILNs.
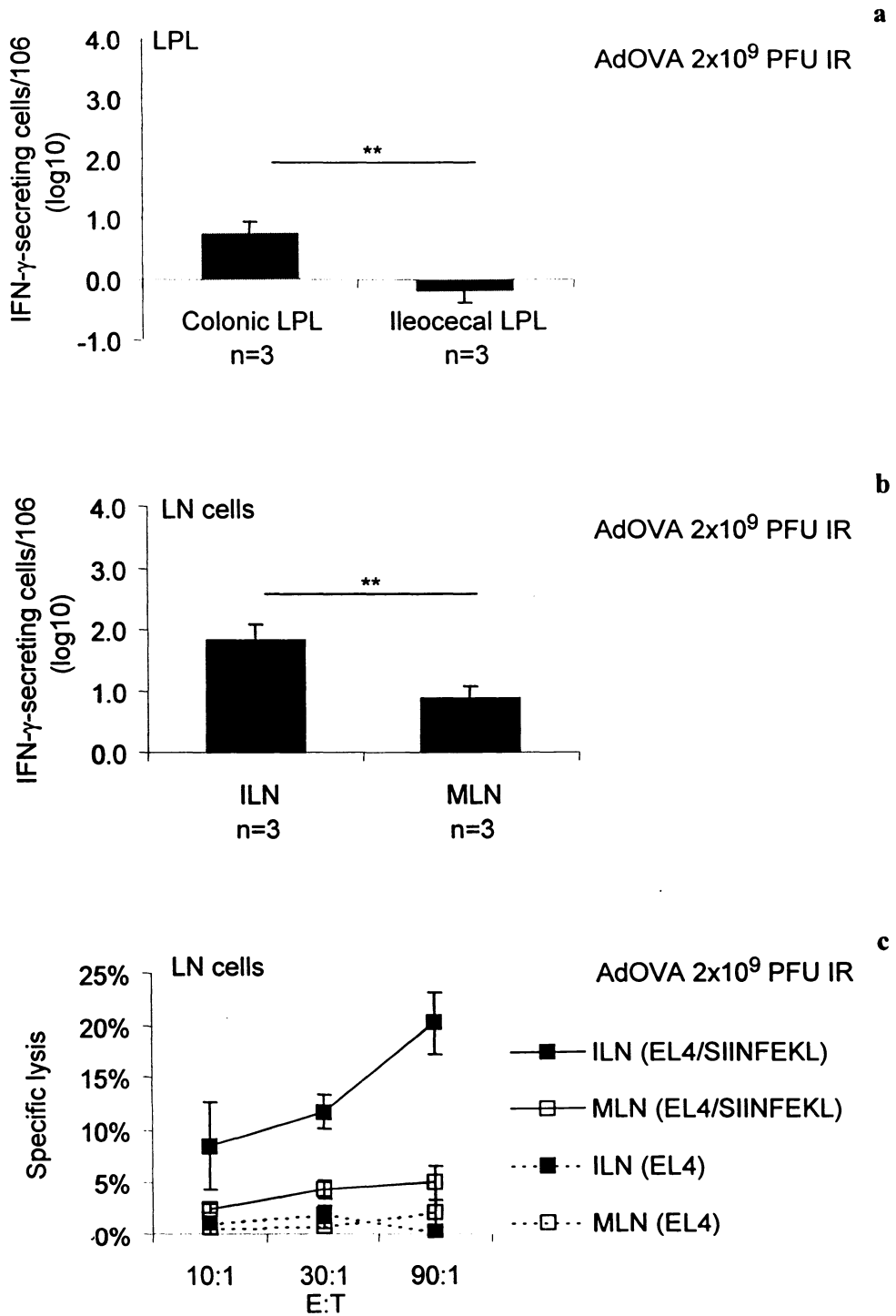
(Figure 4.5)

4.4   Determination of working doses of Adv

Since cytotoxicity of effector cells represents the ability of host to eliminate intracellular pathogens, it was used to determine the working dose of immunization with Adv. In previous studies, primary CTL responses of ILNs were not detected when 1×10^9 PFU AdOVA was administered IR [Thomson, 2001]. Here when doses were increased to 2×10^9 PFU, substantial levels of cytolytic activity were present (see Figure 4.3). This might correlate with the degree of gene expression, in which a significant difference was found between the two doses (see Figure 3.2). Since it was difficult to prepare more highly concentrated viruses, 2×10^9 PFU was chosen as the working dose for IR immunization.

4.5   Induction of Th1 dominated T-cell response

Induction of the Th1 T-cell response is important in anti-viral immunity. This can be examined by measuring subclasses of IgG2 and IgG1, which correlate with a particular type of immune response. Increased IgG2a subclass over IgG1 indicates Th1 dominant T-cell responses [Coutelier et al., 1987; DeKruyff et al., 1993]. The levels of both IgG2a and IgG1 antibodies specific for OVA were determined in the mice immunized IR with AdOVA at week 3 (**Figure 4.6**). Anti-OVA serum IgG2a antibodies

Figure 4.6 Determination of Th1 T-cell response in mice. Animals were immunized IR

with 2×10^9 PFU AdOVA, and sera were collected at week 3-4. ELISA was performed

with serial dilutions of sera in an OVA-coated plate using alkaline phosphatase-

conjugated anti-mouse IgG2a or IgG1 antibodies followed by color development with

substrate p-nitrophenyl phosphate. Serum titers of IgG2a were approximately 4-fold

higher than those of IgG1. Antibody titers are expressed as the mean ±SD of inverse

dilution of samples from 3 mice per group.
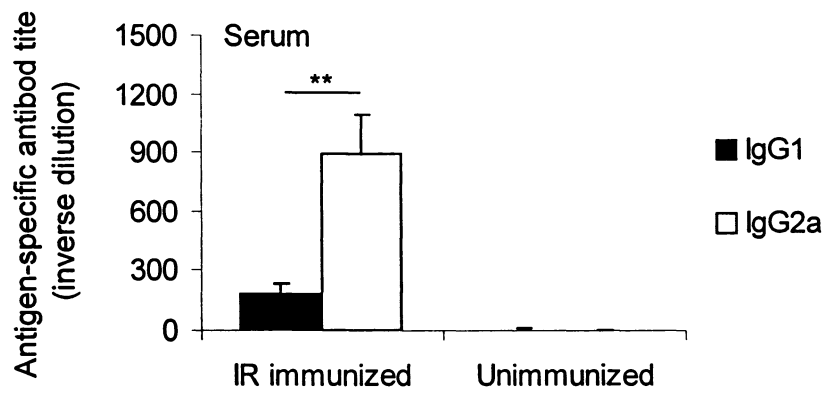
(Figure 4.6)

were more produced than IgG1 antibodies and the ratio of IgG2a to IgG1 was approximately 4:1 (**Figure 4.6**). This suggested that Adv induced a specific Th1 cell response via the rectal route.

4.6   Induction of IgA antibody response

Antibodies, particularly IgA, play a preventive role in infectious diseases at the mucosal surfaces [Kato *et al.*, 2001]. The OVA-specific IgA antibodies were determined in colon homogenates 3-4 weeks after IR immunization with AdOVA. There was a significant amount of OVA-specific IgA detected in the colon at week 2 (**Figure 4.7**). In addition, antigen-specific IgA was found also in the sera (**Figure 4.7**). This indicated that IR immunization induced antibodies both locally and, to some extent, systemically.

4.7   Induction of protective rectal immunity

To prevent mucosally-transmitted infectious disease, generation of local protective mucosal immunity is crucial. Effective mucosal immune responses can immediately control infection at the mucosal surfaces and, as a result, inhibit pathogens from propagating and disseminating to other sites of the body. In the present study, it was

Figure 4.7 Production of antigen-specific IgA in mice. Two weeks after IR immunization

with 2×10^9 PFU AdOVA, sera were collected and the colons were homogenized. ELISA

was performed with serial dilutions of sera or tissue homogenates in OVA-coated plates

using alkaline phosphatase-conjugated anti-mouse IgA antibodies followed by color

development. Significant amounts of IgA antibodies were detected in colonic tissues. The

antibody titers are expressed as the mean ±SD of inverse dilution of the samples from 3

mice per group.
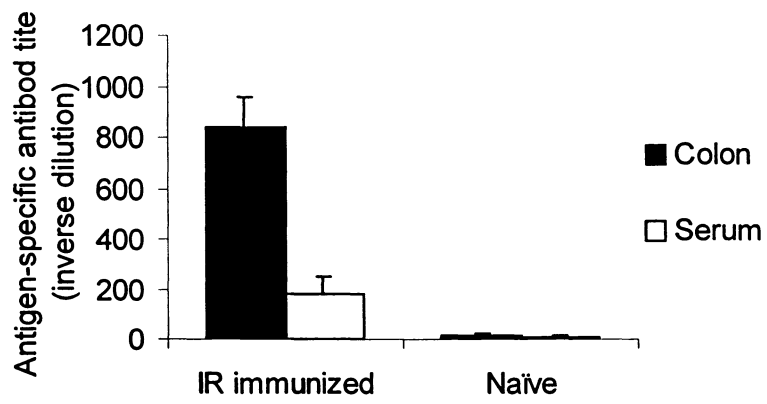
(Figure 4.7)

demonstrated that IR immunization with Adv carrying antigen gene induced antigen-specific immune responses, including IFN-γ production and cytotoxic activities. This allowed to further explore whether this immunization regimen was effective in providing protective mucosal immunity to the challenge with the antigen. This was performed in a rectal challenge model using VV expressing the same antigen used for priming. VV can potentially infect mucosal tissues and disseminate preferentially into the ovaries [Karupiah *et al.*, 1990b]. When VV were used to challenge rectal mucosa, levels of replicated VV in ovaries are indicative of the degree of rectal mucosal barrier functions [Belyakov *et al.*, 1998b; Belyakov *et al.*, 2001; Dumais *et al.*, 2002]. Mice were challenged with VV encoding OVA (VV-OVA) 2 weeks after IR administration of AdOVA, and viruses were recovered from paired ovaries as well as colons (**Figure 4.8**). High levels of VV titer were seen in both ovaries and colon tissues of unimmunized mice, while virus was not detected in either tissue of IR immunized mice (**Figure 4.8a**). This indicated that IR immunization with Adv induced a sterile immunity against antigen challenge via the rectal mucosa, thereby preventing virus spreading to the mucosal site.

4.8   Induction of protective genital immunity

It has been suggested that induction of ILN responses is the key to provide mucosal protection at not only rectal but also at genital mucosa [Lehner *et al.*, 1996, Lehner, 2003]. Since the present study showed that IR immunization with Adv induced

ILN responses and protective rectal immunity, it was important to explore whether the IR

regimen was able to also confer protective genital immunity. Two weeks following IR

immunization with 2×10^9 PFU of AdOVA, mice were challenged vaginally with VV-

OVA 5 days after treatment of Depo-Provera[®], a formulation of progesterone that causes

the lining of vagina to become thinner and increases the susceptibility to virus infection.

Six days later, virus titers were measured in both ovaries and vaginal homogenates. Virus

was not found in the ovaries and only very low levels of viral titers were detected in

genital tissues of immunized mice, while unimmunized mice had high viral titers at both

sites (**Figure 4.8b**). This result suggested that IR immunization also induced protective

genital immunity against antigen challenge and efficiently cleared antigen-bearing

viruses and, thus, prevented viral dissemination to other body sites from genital tissues.

4.9   Induction of protective systemic immunity

In terms of resistance to infectious disease, induction of systemic immune

responses is important also. Systemic immune responses play a central role in controlling

the disease that occurs due to the dissemination of pathogens through the bloodstream

[Lehner *et al.*, 1999]. The systemic immune responses were examined in a subcutaneous

challenge model with antigen-expressing tumor cell lines. Two weeks after IR

immunization with 2×10^9 PFU AdOVA, mice were challenged subcutaneously with

Figure 4.8 Protective immunity against antigen challenge in mice. At two weeks after IR immunization of 2×10^9 PFU AdOVA, animals were either (a) rectally or (b) vaginally challenged with of recombinant vaccinia virus expressing OVA (VV-OVA) dosed at 2×10^7 or 5×10^7 PFU, respectively. Six days later, ovaries and colonic or vaginal tissues were removed and homogenized for the determination of virus titers by plaque-forming assays on CV-1 monolayers. Compared to unimmunized mice, IR immunized mice had undetectable infectious virus particles in ovaries and colons and substantially low viral titers in vaginal tissues. Mean ±SD from 3 mice each group is shown.
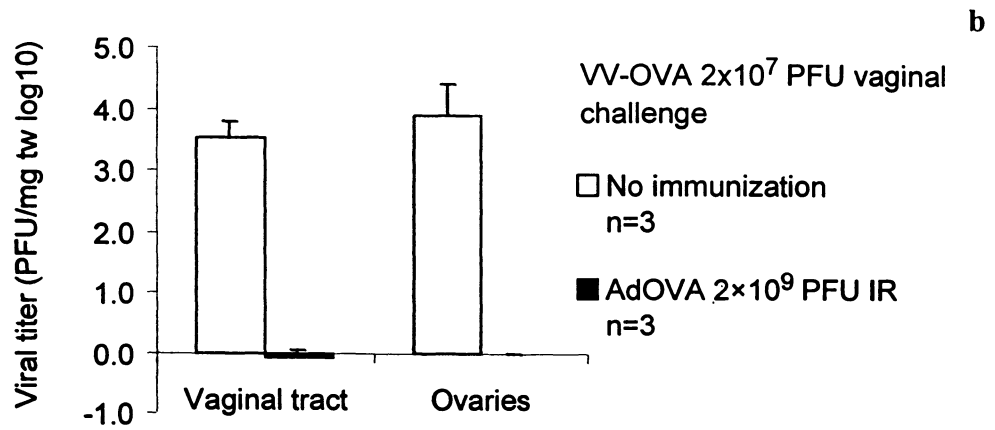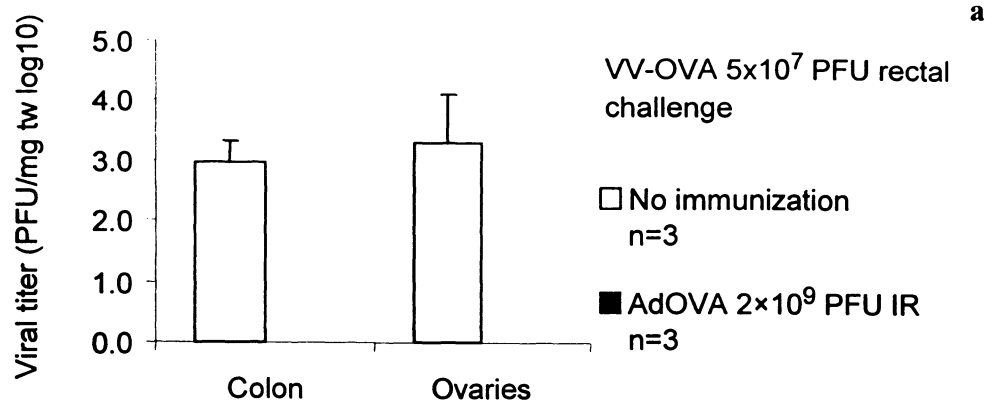
(Figure 4.8)

Figure 4.9 Protection of IR immunized mice from tumor challenge. EL4 cells expressing

OVA (E.G7-OVA) at doses of (a) 2×10^5 and (b) 5×10^5 cells were challenged

subcutaneously 14 days following IR immunization with 2×10^9 PFU AdOVA. Animals

were immunized SC with AdOVA and used as positive controls. Tumor growth was

monitored every 3-4 days. At the low challenge dose, both IR and SC immunized mice

were found to be equally protected. When the dose was increased, 80% of IR immunized

mice were protected. (c) In $CD8^{-/-}$ mice, no protection was achieved after either IR or SC

immunization. Each group consisted of 5 mice.
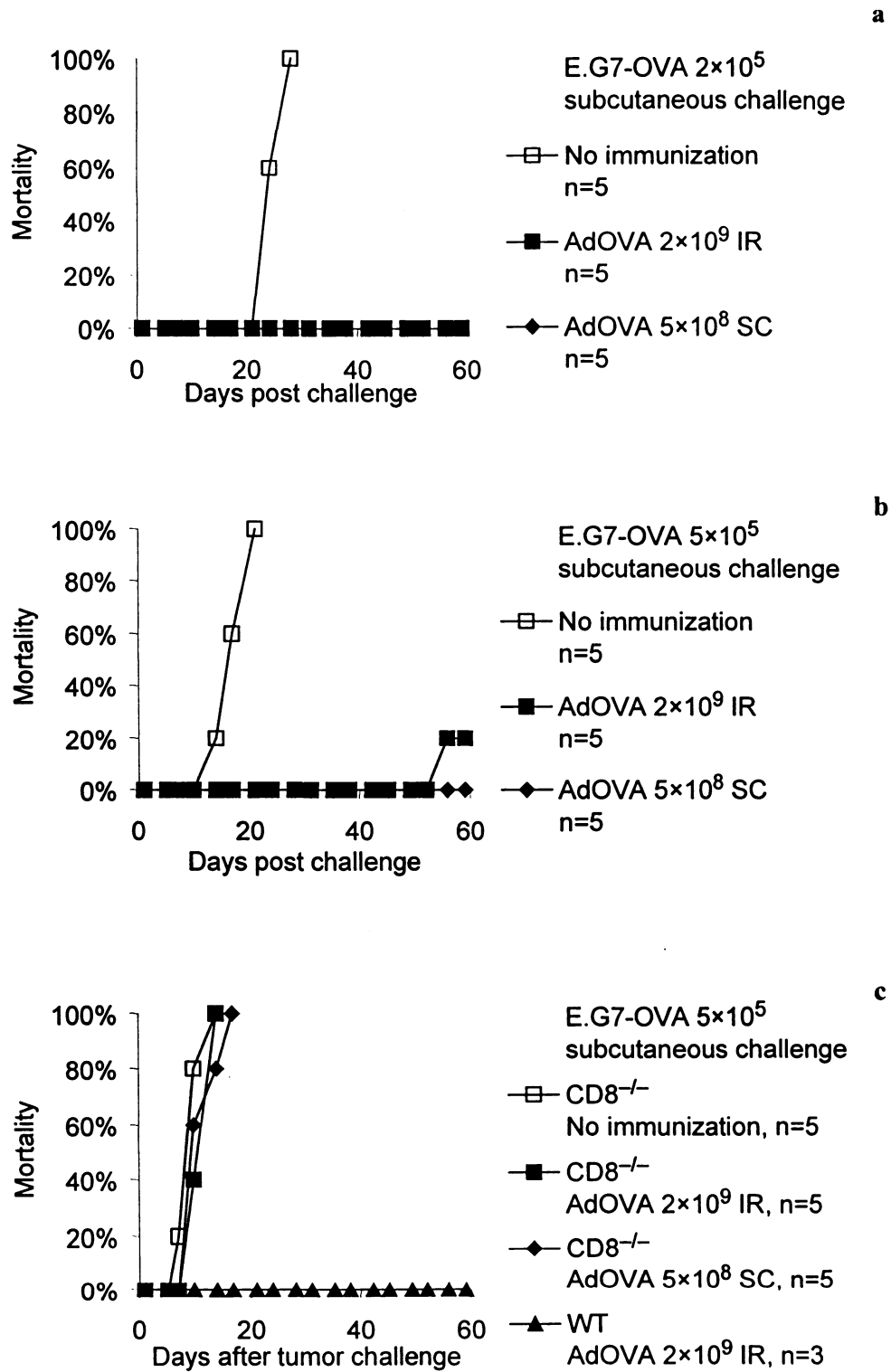
(Figure 4.9)

OVA-expressing tumor cells E.G7-OVA (**Figure 4.9**). As analyzed by mortality, IR immunization offered full protection of mice from challenge at a dose of 2×10^5 tumor cells as compared to no protection in unimmunized mice (**Figure 4.9a**). When the challenge dose was increased by 10-fold, the majority of mice were also protected (80%, **Figure 4.9b**). The protection appeared to be associated with $CD8^+$ T cells because $CD8^{-/-}$ mice (double knockout of $\beta2m$ and CD8) lacking $CD8^+$ T cells were not protected from the tumor challenge (**Figure 4.9c**). These data suggested that IR immunization with Adv induced CD8-mediated protective systemic immunity to eliminate antigen-expressing cells.

4.10 Summary

IR immunization with Adv induced antigen-specific IFN-γ secretion, cytolytic activities, and IgA production. The immune responses largely depended upon $CD8^+$ T cells and developed a Th1 T-cell immune response. The colonic mucosa was the main site for such immune induction. Both rectal and genital mucosal immunity were protective against antigen challenge with VV and efficiently control antigen-associated viruses and viral dissemination from the mucosal surface. Also, systemic immunity was induced to provide protection against antigen-expressing cells. Taken together, these results that Adv-based immunization via the rectal mucosa might be used to generate local protection against natural infection of virus.

CHAPTER 5   PROTECTION   FROM   RECTAL   AND   GENITAL

CHALLENGE WITH HSV-2

5.1   Protection from rectal challenge of HSV-2

The central question to be addressed was whether immunization at the rectal

mucosa with Adv was able to induce mucosal protection against natural infection relevant

to human infectious diseases. It was shown in the Chapters 3 and 4 that IR administration

of Adv transferred heterologous antigen genes to the colonic mucosa and induced

antigen-specific immune responses that were sufficient to protect mice from rectal

challenge with antigens encoded in a VV vector. Also, a rectal challenge model of HSV-2

(see Chapter 3) was established since HSV-2 is a cause of human STDs and latent HSV-2

infection remains incurable. Therefore, the question was first studied by examining rectal

protection against HSV-2 infection in mice immunized IR with Adv. The antigen to be

used here was HSV glycoprotein (gB), which induces anti-HSV immune responses

including CTLs [Hanke *et al.*, 1991; Bonneau *et al.*, 1993]. Adv expressing gB (AdgB)

was previously made and documented in the literature as an effective and successful

vaccine against HSV-2 infection in animal models [Johnson, 1991; Rosenthal and

Gallichan, 1997]. Nasally administered AdgB induced immune responses, including IgA

and IgG as well as CTLs, which correlated with protection from genital challenge of

HSV-2 [Gallichan *et al.*, 1993; Gallichan and Rosenthal, 1996b]. Studies showed certain functional resemblance of OVA and gB in antigen presentation and the induction of antigen-specific immune responses [Dyall *et al.*, 1996; Turner *et al.*, 1997; Kumaraguru *et al.*, 2000; Belz *et al.*, 2002]. Thus, rather than measuring rectally-induced anti-gB immune response, this part of the study was focused on *in vivo* approach of the IR immunization regimen in providing protection against HSV-2 challenges.

Unimmunized mice inoculated rectally with HSV-2 developed severe perianal inflammation, ulcerative lesions and ultimately paralysis of hindlimbs. Whether IR immunization could protect mice from developing the disease was examined. Two-three weeks after IR immunization with 2×10^9 PFU of AdgB, mice were challenged rectally with HSV-2 at the lethal dose of 2×10^5 PFU (2-fold of LD_{100}) after an ethanol enema to remove mucus (**Figure 5.1**). Compared to controls, immunized mice were fully protected from the challenge of HSV-2, with striking reductions in clinical pathology and nearly undetectable levels of viable infectious HSV-2 particles in rectal secretions (**Figure 5.1**). Immunized mice did not develop lesions or paralysis. When the challenge dose was increased to 2×10^6 PFU (20-fold of LD_{100}), 80% of immunized mice were still protected and those had significant lower clinical scores and virus shedding (**Figure 5.1**). These data suggested that IR immunization with Adv was a potent vaccination strategy against natural infection via the rectal mucosa.

Figure 5.1 Protection of IR immunized mice from rectal challenge of HSV-2. Mice were

immunized IR with 2×10^9 PFU Adv expressing gB (AdgB) and rectally challenged with

HSV-2 at doses of 2×10^5 PFU and 2×10^6 PFU at week 2-3. Mice were monitored for (a)

mortality, (b) pathology and (c) virus replication. Mice were fully protected from HSV-2

at 2×10^5 PFU and 80% at 2×10^6 PFU. The clinical scores and virus shedding in IR

immunized mice (■) were significantly reduced as compared to unimmunized mice (□)

(P < 0.001). Pathology became usually normal by day 7-10 post infection. (d)

Unimmunized mice developed ulcerative lesions and paralysis by day 7-10 (upper panel),

while immunized mice that were protected from HSV-2 infection had only minor

inflammation and usually recovered by day 7-10 (lower panel). Clinical scores and virus

titers are the mean ±SD from 5 mice each group.

(Figure 5.1a-c)

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d
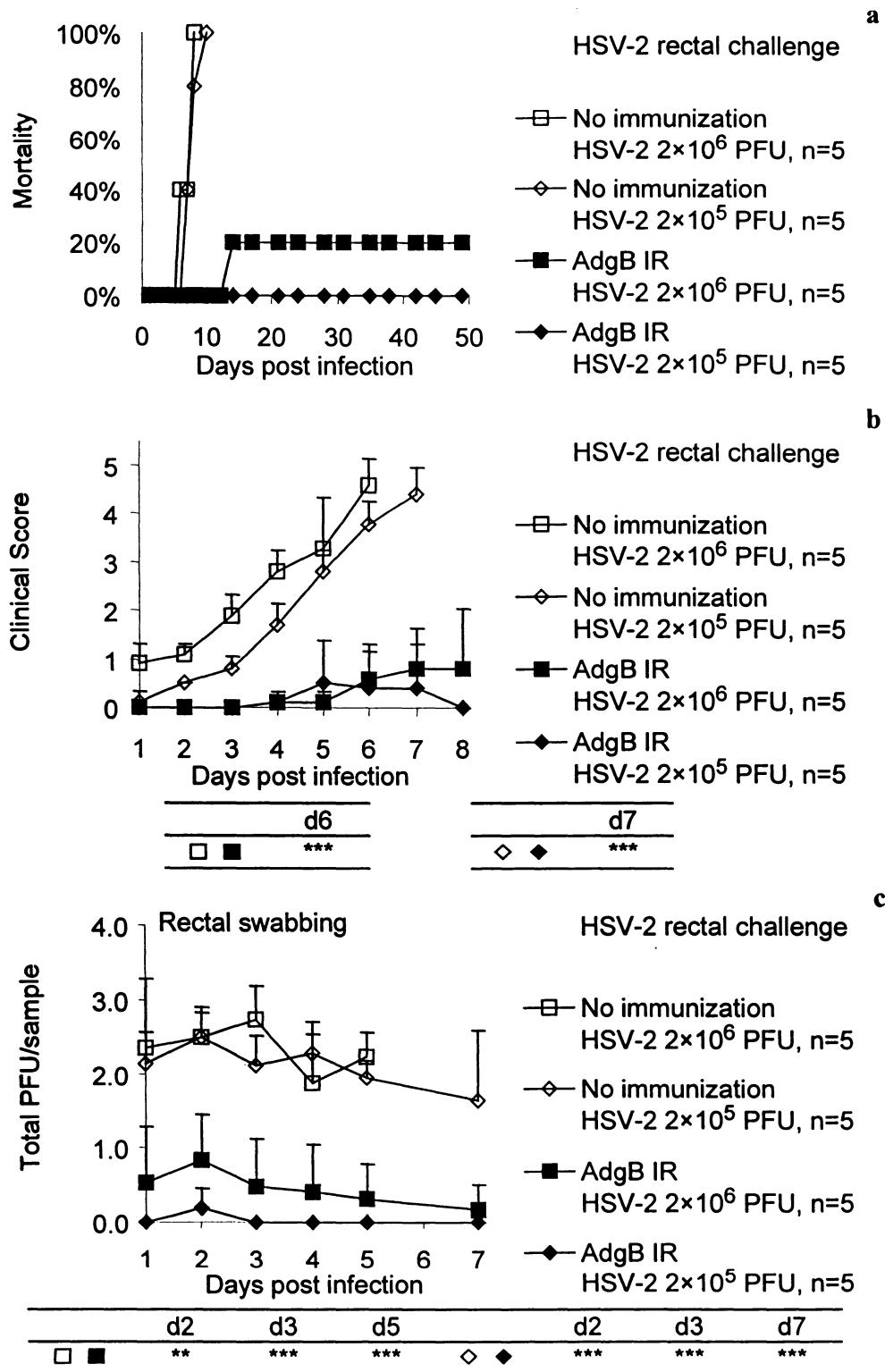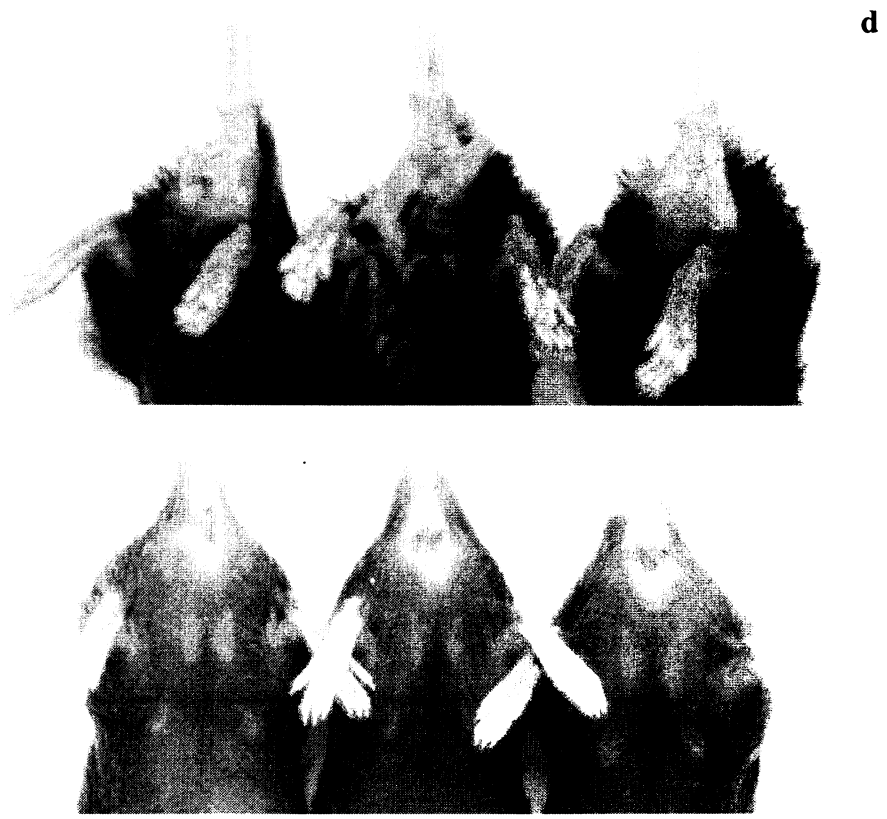
(Figure 5.1d)

5.2 Critical components that contribute to rectal immunological protection

In the present study, it was demonstrated that IR immunization with Adv resulted in $CD8^+$ T cell-mediated IFN-γ production and cytotoxic activities measured by *in vitro* means (ELISPOT and chromium release assay, see Chapter 4). The role for $CD8^+$ T cells and IFN-γ was investigated in the rectal challenge model of HSV-2. To do so, both $CD8^{-/-}$ (double knockout of $\beta2m$ and CD8) and IFN-$\gamma^{-/-}$ mice were used. The role of IL-12 was also investigated in $IL-12^{-/-}$ mice as it has been established as the inducer of IFN-γ production and the Th1 skewing molecule [Hsieh *et al.*, 1993; Manetti *et al.*, 1993; Magram *et al.*, 1996]. After IR immunization with AdgB, neither $CD8^{-/-}$ nor IFN-$\gamma^{-/-}$ mice were able to protect against rectal challenge of HSV-2 (**Figure 5.2**). $IL-12^{-/-}$ mice suffered severe infection and the majority (80%) also developed paralysis (**Figure 5.2**). These data indicated that IFN-γ, IL-12 and $CD8^+$ T cells were important immunological factors that contributed to rectal mucosal protection against HSV-2 infection.

5.3 Protection from genital challenge of HSV-2

One of the major goals of STD vaccines is to induce efficient mucosal immunity against virus transmission through the genital tract. In the present study, it was shown

Figure 5.2 Loss of protection in $CD8^{-/-}$, $IFN-\gamma^{-/-}$, and $IL-12^{-/-}$ mice. Gene-deficient mice

were immunized IR with 2×10^9 PFU AdgB and rectally challenged with HSV-2 at a dose

of 2×10^6 PFU at week 2-3. Mice were monitored for (a) mortality, (b) pathology and (c)

virus replication. In the absence of $IFN-\gamma$ or $CD8^+$ T cells, all IR immunized mice

developed disease and none of them was protected from HSV-2 infection. The majority

(80%) of IL-12-deficient mice succumbed to HSV-2 infection and developed paralysis.

Experiments performed on these gene knockout mice were run together with wild type,

which showed protection. Clinical scores and virus titers are the mean ±SD from 5 mice

each group.
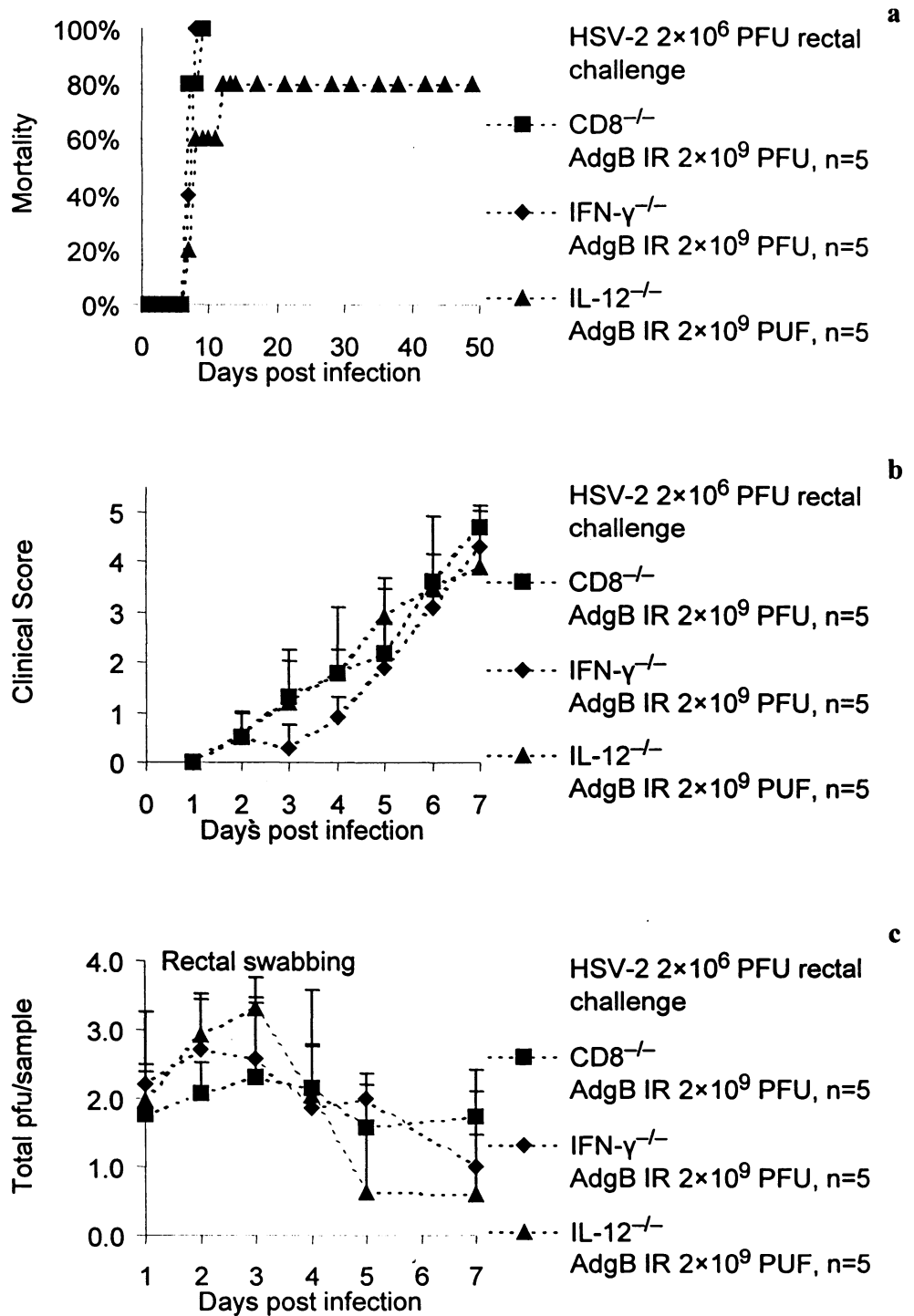
(Figure 5.2)

that IR immunization induced protective immunity against antigen challenge with VV. This suggested the possibility of IR immunization to confer genital protection against virus infection. IR immunization regimen was thus evaluated for providing genital protection against HSV-2 infection. Two-three weeks after IR immunization of AdgB, mice were challenged vaginally with HSV-2 at 2×10^5 PFU (**Figure 5.3**), 20-fold of LD_{100} as previously determined [Gallichan and Rosenthal, 1998]. All immunized mice survived the genital infection of HSV-2 (**Figure 5.3a**) with markedly reduced clinical scores (**Figure 5.3b**) and viral replication as well as shortened durations of virus shedding (**Figure 5.3c**). The results indicated that IR immunization also conferred genital protection against virus challenge via the genital route.

5.4   Ineffectiveness of genital immunization with Adv

Although the present study implied that IR immunization with Adv represented a promising vaccination strategy, there remained a question of whether the rectum was a necessary route for vaccination against viral infection at rectal tissue and, particularly, at the genital mucosa. To address this question, we studied intravaginal (IVAG) immunization as local mucosal immunization strategy in providing protection against genital HSV-2 infection. Two groups of mice were immunized IVAG with AdgB at a dose of 1×10^9 PFU. One group was given with Depo-Provera[®] 5 days prior to immunization and the other group was not treated. It was found that Depo-

Figure 5.3 Protection of IR immunized mice from vaginal challenge with HSV-2. At 2-3 weeks after IR immunization with 2×10^9 PFU AdgB, mice were challenged vaginally with 2×10^5 PFU HSV-2. Mice were monitored for (a) mortality, (b) pathology and (c) virus replication. IR immunization conferred protection against vaginal challenge of HSV-2. The clinical scores and virus shedding were significantly reduced in IR immunized mice (■) compared to unimmunized mice (□) ($P < 0.05$ and $P < 0.01$, respectively). Clinical scores and virus titers are the mean ±SD from 5 mice each group.

a

HSV-2 2×10⁵ PFU vaginal challenge

—☐— No immunization
n=5

—■— AdgB 2×10⁹ PFU IR
n=5

b

HSV-2 2×10⁵ PFU vaginal challenge

—☐— No immunization
n=5

—■— AdgB 2×10⁹ PFU IR
n=5

c

HSV-2 2×10⁵ PFU vaginal challenge

—☐— No immunization
n=5

—■— AdgB 2×10⁹ PFU IR
n=5
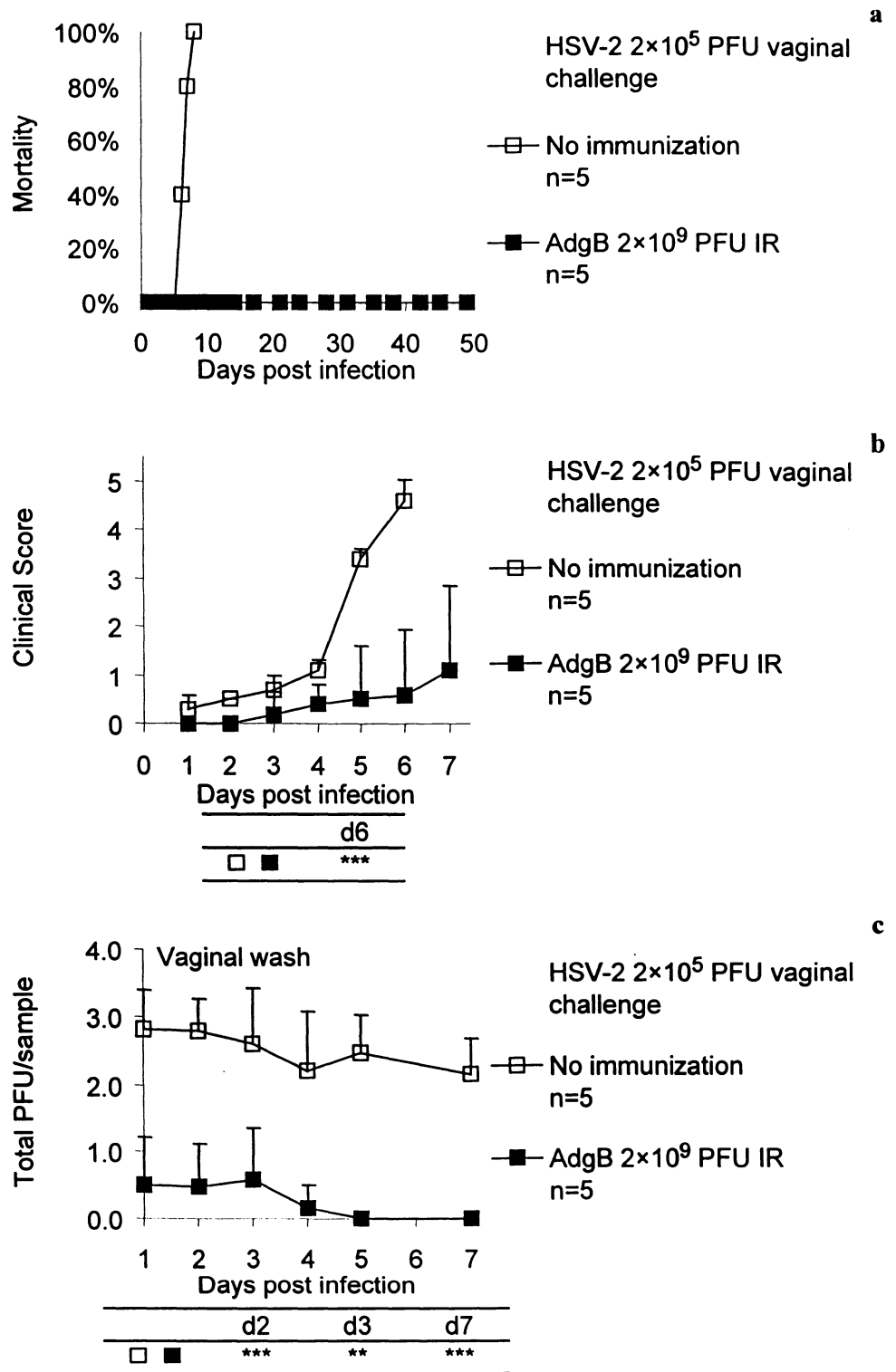
(Figure 5.3)

Provera® pretreatment gave rise to a better gene transduction at the genital mucosa by 10-fold and the levels were similar to transfer at other mucosal sites (see Chapter 3). Two-three weeks after immunization, mice were challenged intravaginally with 2×10^5 PFU HSV-2. Surprisingly, the Depo-Provera® group was not protected from the challenge (**Figure 5.4**). It has been reported that Depo-Provera® suppresses immune responses when used prior to immune induction [Kaushic et al., 2003]. However, the group without Depo-Provera® pretreatment was also unprotected from HSV-2 challenge (**Figure 5.4**). Whether immunity was generated at other mucosal sites, for example the rectum, after IVAG immunization was tested (**Figure 5.5**). It was clearly demonstrated that IVAG immunization with AdgB in either group (Depo-Provera® treated or untreated) was unable to provide protection against rectal challenge with 2×10^5 PFU HSV-2. Therefore, these data suggested that the vaginal tract might not be an ideal route for immunization, at least for Adv-based vaccines, to induce protective mucosal immunity against virus challenge.

5.5   Comparison IR with IN and SC immunization

Systemic, non-mucosal [Bouvet et al., 1994; Moldoveanu et al., 1995; Amara et al., 2001; Nilsson et al., 2001] and distant mucosal [Gallichan et al., 1993; Kantele et al., 1997; Ferko et al., 1998; Gallichan et al., 2001] immunizations are some the most studied

Figure 5.4 Inefficient genital protection after IVAG immunization of mice. At 2-3 weeks

after 1×10^9 AdgB IVAG immunization, mice were challenged vaginally with HSV-2 at a

dose of 2×10^5 PFU. Mice were monitored for (a) mortality and (b) external vaginal

pathology. No IVAG immunized mice were protected from vaginal challenge of HSV-2.

Clinical scores are expressed as the mean ±SD of 5 mice in each group.

a

HSV-2 2×10^5 PFU vaginal
challenge

—⊖— No immunization
n=5

—▲— AdgB 1×10^9 PFU IVAG(Depo)
n=5

—●— AdgB 1×10^9 PFU IVAG
n=5

b

HSV-2 2×10^5 PFU vaginal
challenge

—⊖— No immunization
n=5

—▲— AdgB 1×10^9 PFU IVAG(Depo)
n=5

—●— AdgB 1×10^9 PFU IVAG
n=5
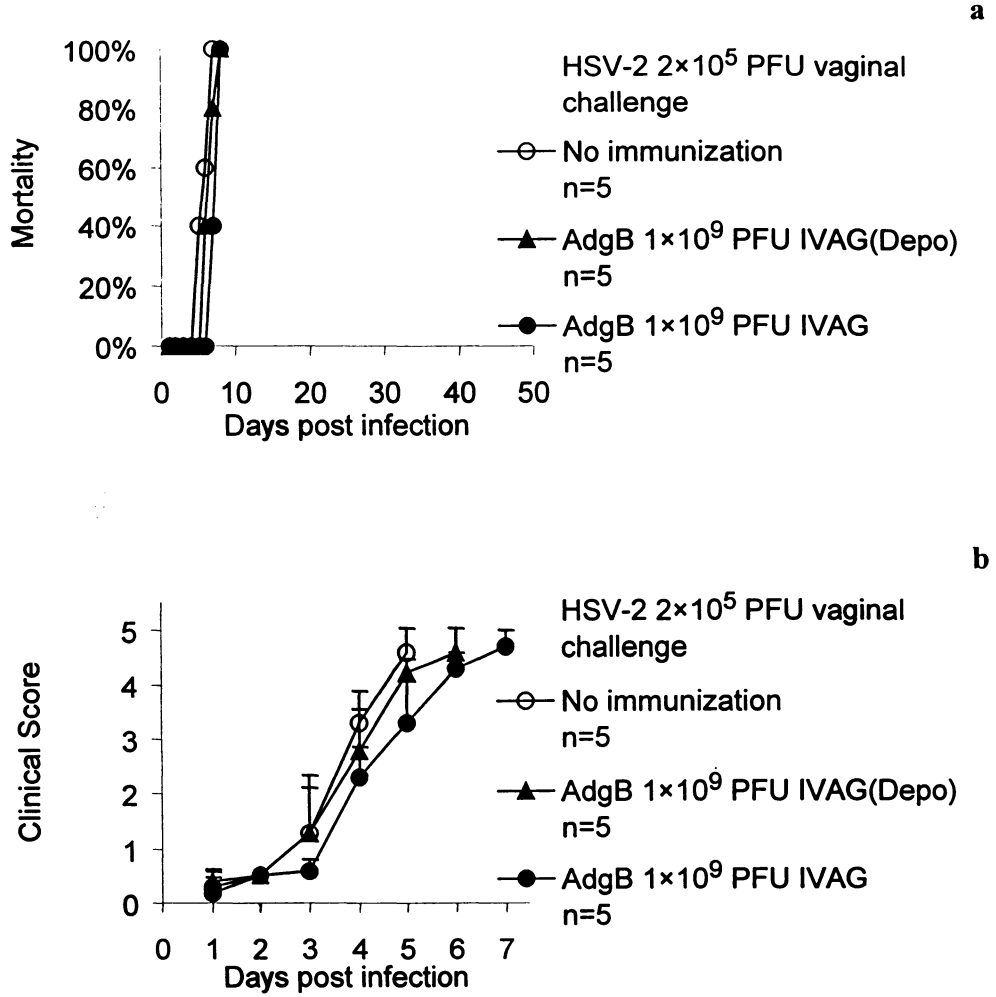
(Figure 5.4)

Figure 5.5 Inefficient rectal protection after IVAG immunization of mice. At 2-3 weeks after 1×10^9 AdgB IVAG immunization, mice were challenged rectally with HSV-2 at a dose of 2×10^5 PFU. Mice were monitored for (a) mortality and (b) perianal pathology. IVAG immunization did not confer rectal protection against HSV-2 infection. Clinical scores are expressed as the mean $\pm$SD of 5 mice in each group.
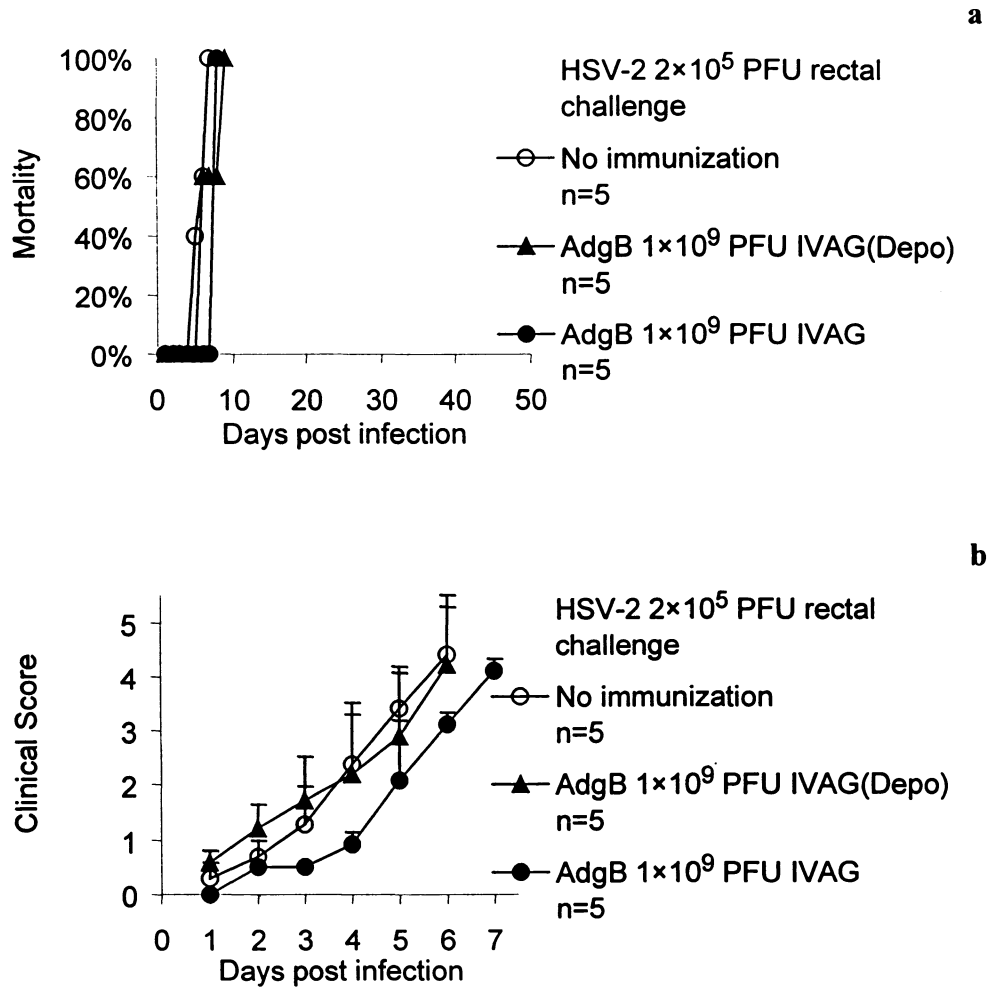
(Figure 5.5)

routes for inducing immune protection against rectal or genital viral infection. Recent

studies began to show that rectal and genital immunity was better induced by local

mucosal immunization than by systemic or distant mucosal immunization [Belyakov *et*

al., 1998b; Eriksson *et al.*, 1998; Kozlowski *et al.*, 1999; Shen *et al.*, 2000; Belyakov *et*

al., 2001; Vajdy *et al.*, 2001]. However, immunization via the rectal route requires a more

carefully elaborated procedure and is inconvenient as compared to intranasal (IN, distant

mucosal) or subcutaneous (SC, systemic non-mucosal) routes. This raised a question on

whether the rectum was a necessary immunization route for induction of local mucosal

immunity. To answer the question, a comparison was performed on three such routes (IR,

IN and SC) for the induction of rectal and genital protections. Two-three weeks after

immunization via IR, IN or SC, mice were challenged either rectally or vaginally with

2×10^5 PFU HSV-2 (**Figure 5.6**). Protection from virus challenge was judged by mortality

and local pathology. In contrast to IR immunized mice that were fully protected, both IN

and SC ammoniated mice were only partially protected (**Figure 5.6a**). Although IN and

SC immunization significantly reduced clinical scores as compared to non-immunization,

these two routes were less efficient than the rectal route (**Figure 5.6b**). Similar results

were obtained for intravaginal challenge; 100% protection was achieved by IR

immunization, while only 20% and 0% by IN and SC, respectively (**Figure 5.7a**). The

pathology was significantly less after IN and SC immunization, but IR immunization was

more effective in reducing pathology than IN and SC (**Figure 5.7b**). These data

suggested that IR immunization was more potent regimen than immunization via a distant

mucosal or a systemic route in the induction of both rectal and genital immunity.

Figure 5.6 Comparison of immunization routes for providing protection from rectal challenge of HSV-2 in mice. Animals were immunized with AdgB via SC, IN or IR route and challenged rectally with 2×10^5 PFU at week 2-3. Mice were monitored for (a) mortality and perianal (b) pathology. Both IN and SC immunization were less effective than IR in providing rectal protection. Although IN (◆) and SC (▲) immunization significantly reduced pathology compared to unimmunized mice (□) (at day 7, $P < 0.01$), these routes were less efficient than IR (■, $P < 0.001$) and were not statistically different from each other $(P = 1.00)$. Clinical scores were expressed as the mean ±SD of 5 mice in each group.
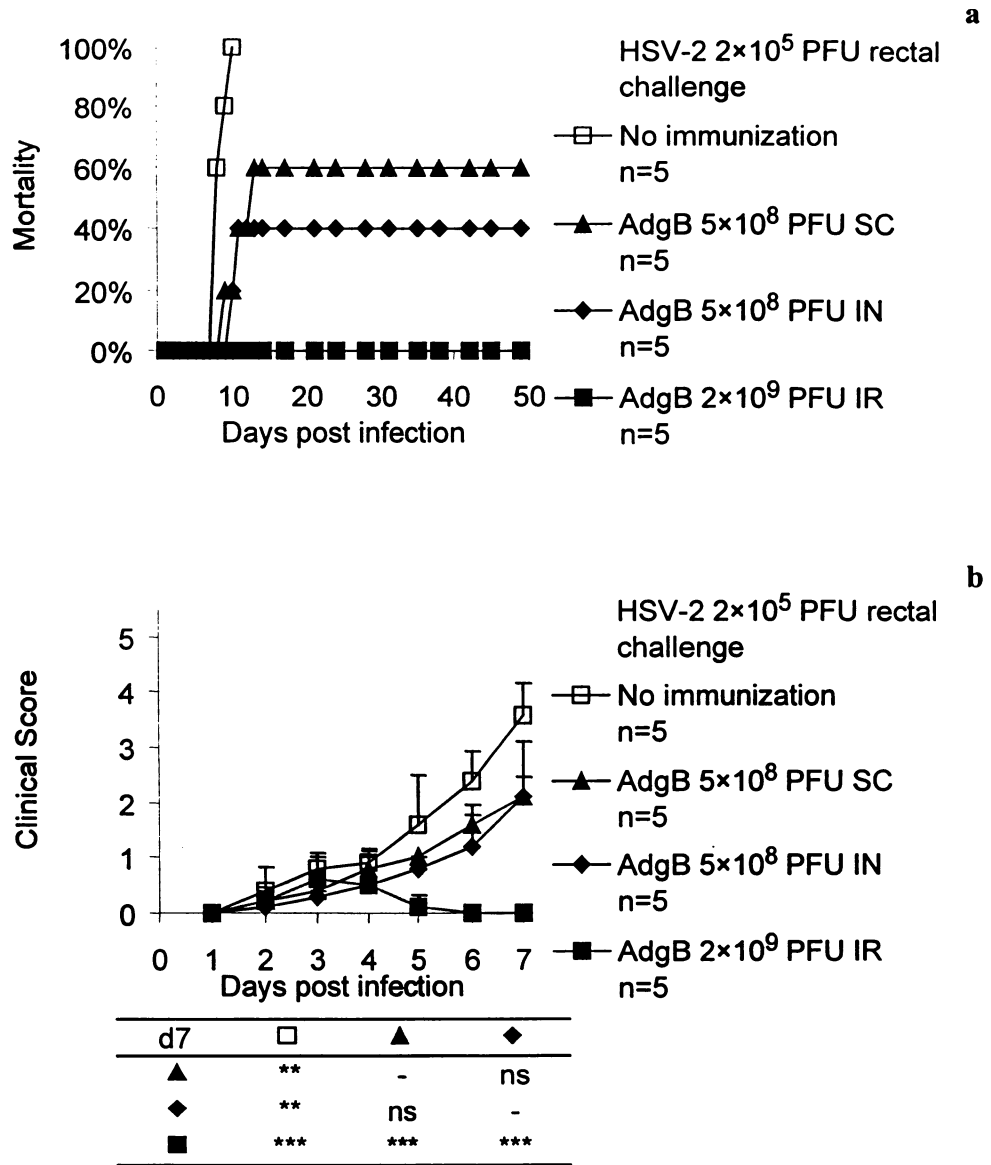
(Figure 5.6)

Figure 5.7 Comparison of immunization routes for providing protection from genital challenge of HSV-2 in mice. Animals immunized with AdgB via SC, IN or IR route were challenged intravaginally with 2×10^5 PFU HSV-2 at week 2-3. Mice were monitored for (a) mortality and (b) perianal pathology. Both SC and IN immunizations were less effective than IR in providing genital protection. Although IN (♦) and SC (▲) immunization significantly reduced pathology compared to unimmunized mice (□) (at day 6, $P < 0.05$), these routes were less efficient than IR (■, $P < 0.05$) and were not statistically different from each other $(P = 0.28)$. Clinical scores were expressed as the mean ±SD of 5 mice in each group.
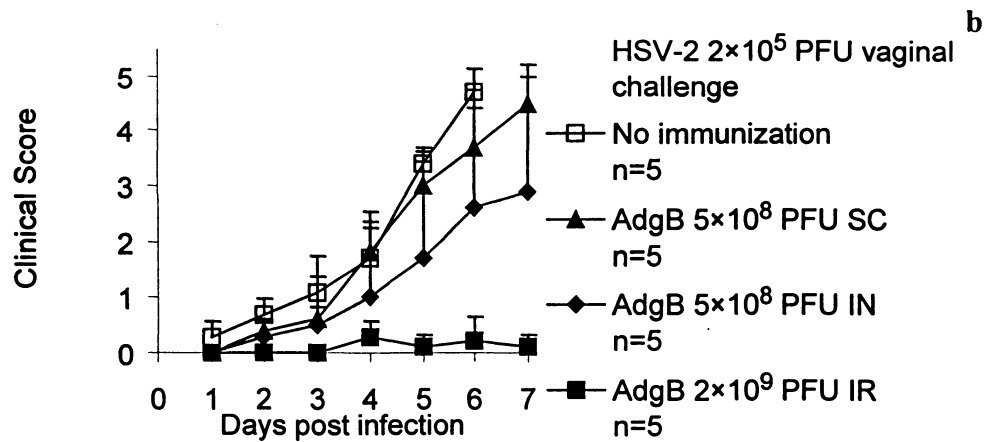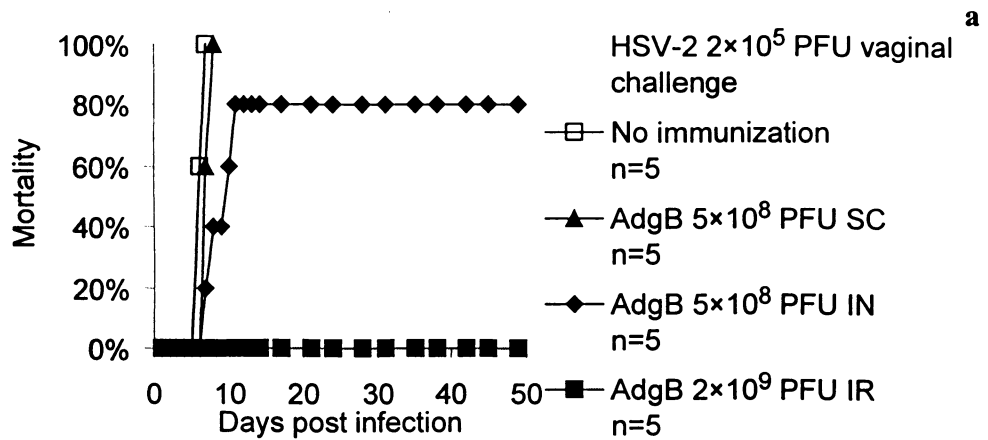
(Figure 5.7)

To strengthen the results of this work and challenge the IR immunization regimen with other models, a comparison was conducted to quantify virus replication within mucosal tissues after immunization via the three routes. Mice were immunized via different routes with AdOVA and challenged subsequently with VV-OVA. At days 6 post-infection, both the ovaries and the colonic or genital tissues were taken for the measurement of virus titers (**Figure 5.8**). IN and SC immunization did not significantly suppressed virus replication in the rectal or genital tracts as compared to non-immunization (**Figure 5.8**). As a result, neither of these two routes conferred sufficient mucosal immunity to prevent virus dissemination to the ovaries (**Figure 5.8**). In contrast, IR immunization induced sterile local immunity and cleared the virus completely in the colon and almost completely at the genital mucosa (**Figure 5.8**). No infectious virus particles were detected in the ovaries, suggesting that virus spreading was efficiently prevented (**Figure 5.8**). Therefore, IR was more efficient than IN or SC in the induction of mucosal immunity to control virus replication at both colonic and genital mucosae.

It was hypothesized that the superior protection in the rectal tissue might be associated with a higher degree of antigen-specific immune response induced by Adv via IR compared to via IN and SC. Thus, the frequency of local specific IFN-γ-secreting cells was compared after immunization with AdOVA given by the three routes. The frequency of OVA-specific, IFN-γ-secreting colonic LPLs was found to be approximately 5-fold higher after IR immunization than IN and SC immunization (**Figure 5.9**). In addition, there was no significant difference in the induction of colonic immune responses

Figure 5.8   Quantification of VV viral particles at the rectal or vaginal mucosa after immunization of mice via different routes. At two weeks after immunized with AdOVA through IN, SC or IR route, animals were challenged (a) rectally or (b) vaginally with VV-OVA at a dose of 5×10^7 PFU or 2×10^7 PFU, respectively. Six days later, ovaries and colonic or vaginal tissues were taken for the determination of virus titers by plaque-forming assays on CV-1 cells. Virus titers tended to be lower after IN or SC immunization, but there was no significant difference between IN or SC immunized mice and unimmunized mice. IN or SC immunization was less effective than IR immunization in providing rectal or genital immunity to control viral replication ($P < 0.001$). Virus titers were expressed as mean $\pm$ SD from 3 mice each group.
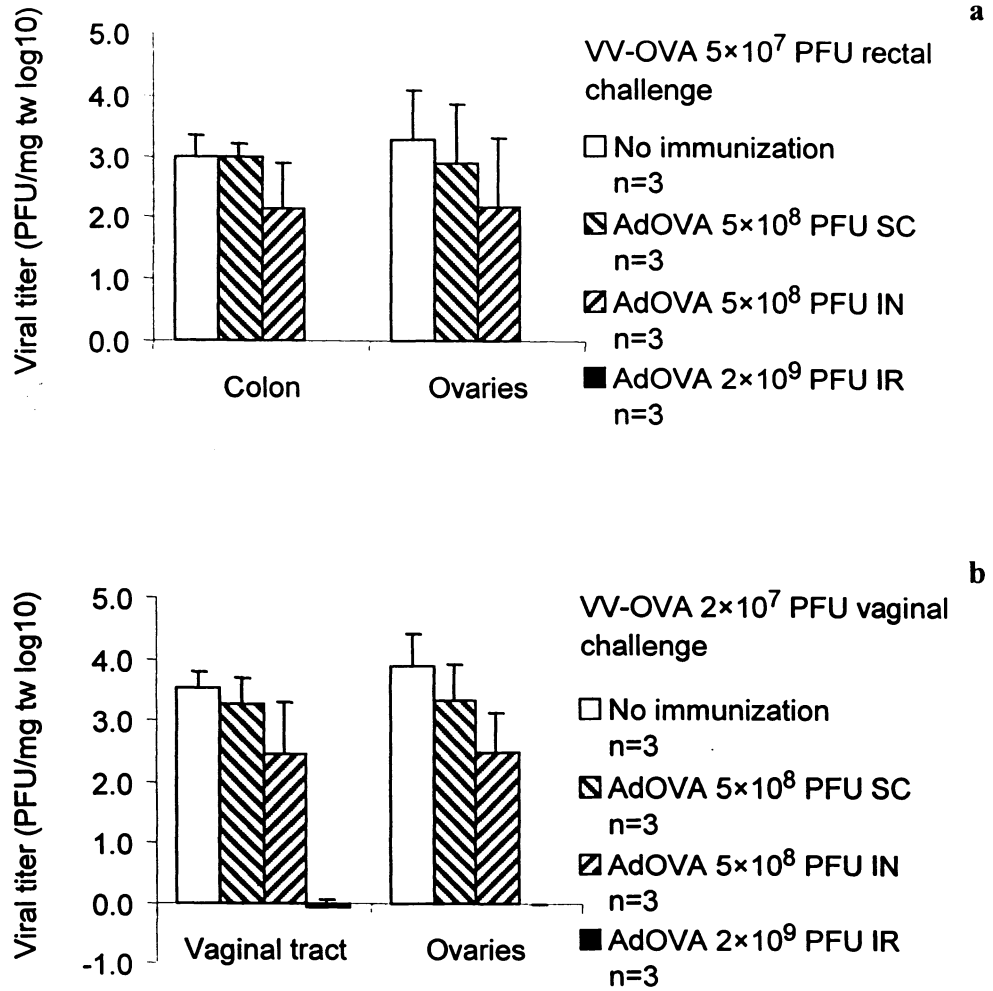
(Figure 5.8)

Figure 5.9 Comparison of colonic specific IFN-γ-secreting cells after immunization of mice by different routes. Animals were immunized with AdOVA by SC, IN or IR route. Colonic LPLs were isolated at week 2 and were used to determine OVA-specific IFN-γ-secreting cells by ELISPOT in the presence of OVA peptide SIINFEK. IR immunization induced higher frequencies of OVA-specific IFN-γ-secreting colonic LPLs than did SC or IN. Values represent the mean ±SD of 3 mice each group.
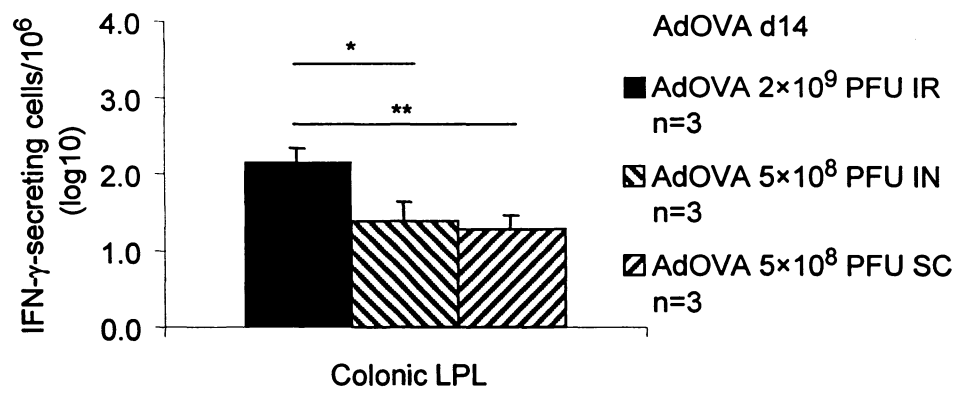
(Figure 5.9)

between IN and SC immunization. Colonic IgA production was also examined and found to be higher produced after IR immunization compared to IN and SC (**Figure 5.10**), thereby highlighting the importance of IR immunization in the induction of local antibody responses to prevent infections. Therefore, IR local mucosal immunization induced stronger colonic immune responses than distant or systemic immunization. This might provide a possible explanation for the IR immunization in conferring better immune protection.

5.6   Summary

IR immunization with Adv elicited protective immunity against rectal challenge of virulent HSV-2. IR induced local immune responses were efficient in suppressing virus replication, shortening virus-shedding period and preventing paralysis. $CD8^+$ T cells, IFN-γ and IL-12 were important factors that contribute to such protection. IR immunization also conferred genital immunity against HSV-2 infection, providing implications for the development of STD vaccines.

This study also suggested that the route of immunization was of crucial importance in vaccine design. Compared to IN and SC, IR immunization was more efficient in providing local protection from virus infection and induces stronger cellular and humoral immune responses at rectal and genital mucosal surfaces. Therefore, the

Figure 5.10 Comparison of colonic IgA production after immunization of mice via different routes. Animals were immunized with AdOVA via SC, IN or IR route. Colons were removed and homogenated at week 2 and were used to determine local IgA production by ELISA. IR immunization induced higher colonic IgA production in the colon than did SC or IN. Values represent the mean ±SD of 3 mice each group.
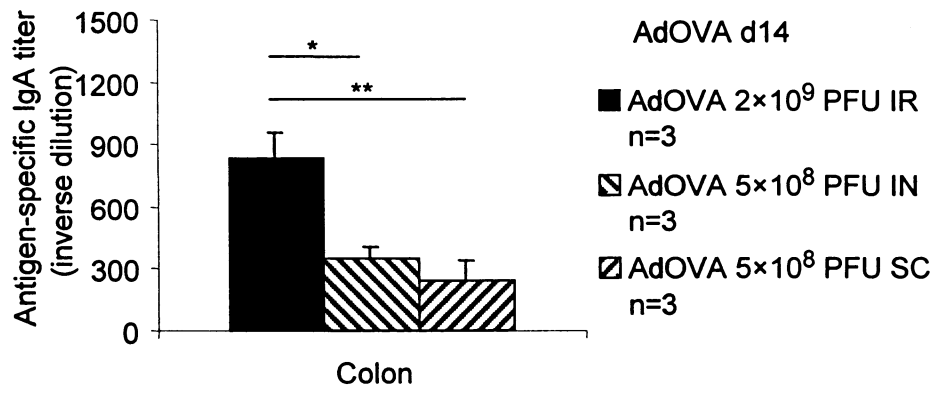
(Figure 5.10)

rectum was a more effective route for vaccination against viral infection in both rectal and genital tracts.

CHAPTER 6   DISCUSSION

6.1   Background review and summary of the study

Induction of protective immunity at the rectal and genital mucosae is critical because virus transmission often occurs at these mucosal surfaces and causes sexually-transmitted diseases (STDs) which can be life-threatening. Viruses enter the mucosa by overcoming a series of innate mucosal immunological barriers [Lehner et al., 1999]. The ability of viruses to attach and infect epithelial cells can be neutralized by antibodies, especially secretory IgA (SIgA) in the intestinal lumen and dimeric IgA (dIgA) bound to polymeric-immunoglobulin receptor (pIgR) within epithelial cells. Antibodies can also neutralize viruses that have intruded the lamina propria (LP). If viruses evade neutralization by these antibodies, they gain access to epithelial cells or other cells immediately beneath the epithelium to replicate and infect new cells, such that infection propagates. Cell-mediated immune responses eliminate cell-invading viruses by utilizing cytotoxic mechanisms and eventually control the infection in the mucosa. If infection is spread via the bloodstream to other sites of the body, systemic immune responses take place to provide further protection. As systemic immunity usually does not deal with mucosal infection, the establishment of mucosal immunity is of crucial importance to control mucosal infection and prevent viruses from disseminating [Lehner et al., 1999].

The route of immunization is a critical determinant for mucosal immune induction. There has been an increasing interest in vaccination at the rectal or genital tract to induce anti-STD mucosal immunity. Systemic, non-mucosal immunization generally does not induce efficient mucosal immunity, while distant mucosal immunization (at a mucosal site but anatomically distant from the mucosal site for protection) appears to be not very efficient. Studies have suggested that local (rectal or genital) mucosal immunization (at the mucosal site to provide protection) induces effective immune protection at the rectal or genital mucosa [Haneberg et al., 1994; Belyakov et al., 1998b; Eriksson et al., 1998; Shen et al., 2000; Wu et al., 2000b; Vajdy et al., 2001]. Therefore, local mucosal immunization against STD might be more appropriate and beneficial.

To elicit effective mucosal immunity, an antigen must be introduced into the mucosal immune system. This requires antigens to be transported across the epithelium. Since the ability of Adv infect a variety of cells and transfer antigen genes capable of inducing antigen-specific immune responses, Adv appears to be a good candidate to target the monolayer of colonic epithelial cells as delivery interface. Previous studies showed that Adv was able delivered heterologous genes into the colon [Cheng et al., 1997; Foreman et al., 1998] and, after a simple intrarectal (IR) infusion, transduced therapeutic genes to modulate immune responses in colitis models [Wirtz et al., 2002; Lindsay et al., 2003]. It was previously demonstrated that Adv was able to transfer antigens via the colonic epithelial cells and stimulate colon inflammation in the LP of antigen-sensitized mice [Thomson, 2001]. Whether IR administration of Adv can

introduce sufficiently high levels of antigens to induce primary mucosal immune responses and provide local protection at the colonic mucosa was investigated in the present study.

The pipetting and Dermabond® (P&D) method was developed to perform non-invasive IR delivery of Adv (also for HSV-2 challenge) and to prevent leakage of delivered material. Adv-mediated transgene expression increased markedly after mucolytic pretreatment with ethanol and showed pronounced maximum for 3 days. Gene transduction occurred not only within epithelial cells, but also immediately beneath the epithelium. Mucosally-induced immune responses in the rectal mucosa and lymphoid tissues were examined. Antigen-specific IFN-γ-secreting cells appeared early in the iliac lymph nodes (ILNs) and increased over time in the colon and spleen. Lymphoid effector lymphocytes were found to be cytotoxic and able to specifically kill antigen-expressing target cells. There were CD8$^+$ T cell-mediated effector functions, Th1 T-cell responses and locally induced IgA production. The rectal immunization regimen was then validated by applying it in rectal and genital virus challenge models and comparing the protective effect with distant mucosal and systemic non-mucosal immunizations. IR immunization with Adv protected mice from antigen challenge with VV or against HSV-2 infection via the rectal route. CD8$^+$ T cells, IFN-γ and IL-12 appeared to be contributory to anti-HSV immunity. IR immunization also conferred genital protection. As compared to intranasal (distant mucosal) and subcutaneous (systemic) routes, the rectal route was more effective in inducing mucosal immune response and local immunological protection. Thus, rectal

immunization with Adv proved to be a viable vaccination strategy against STD-relevant viral infection.

6.2   The pipetting and Dermabond® method with shortened ethanol treatment

The development of pipetting and Dermabond® (P&D) method for IR delivery, which differed from previous IR catheterization [Wirtz *et al.*, 1999; Wirtz *et al.*, 2002; Lindsay *et al.*, 2003], was based on a number of serious concerns. Since catheters lack flexibility, approximately 10% mice die as a result of colon perforation [Coligan *et al.*, 2002]. Mice can be ill or have rectal bleeding if catheters lacerate colon epithelium. Illness can become apparent 2-3 days later. These confuse observations of morbidity or mortality as a consequence of HSV-2 infection. As careful manipulations might not avoid damage to the epithelial monolayer, direct gene expression in LP can be misinterpreted as across-epithelial gene transfer. In addition, leakage of injected material during and after administration is a major problem for efficient antigen delivery. Previously used methods prevented leakage using an inflated balloon placed in the anorectum [Wirtz *et al.*, 1999]. This is highly likely to cause distortion/rupture and microscopically damage and, thus, is inappropriate.

The P&D method developed in the present study has many advantages. It was

performed by placing a fire-polished pipette tip in the anal canal and pipetting material into the colon. There was no direct contact of delivery device with rectal mucosa, and thereby the method avoided physical damage to the colon epithelium. After pipetting, Dermabond® was immediately applied on the skin of the anal opening. Dermabond® effectively prevented leaks from the rectum and prolonged incubation period to improve better uptake of Adv by the colon mucosa. Also, Dermabond® seal prevented mice from licking the perianal area and obtaining material through the oral route. Therefore, the P&D method completely avoided any physical damage to the colon, ensured that mice were treated with full doses of vaccines, and avoided material uptake via the oral route as well.

For mucus removal from the colon, it was found that the standard 3-hr length of ethanol treatment performed in the previous study [Thomson, 2001] was unnecessary and probably deleterious. After 40 min incubation of ethanol, mucus (and feces) was expelled from the rectum. AB/PAS staining confirmed that mucus was removed from the majority of the colon (see Chapter 3), thus suggesting that the newly-designated time length was sufficient to clear the mucus. Lengthy ethanol treatment might be harmful to epithelial cells. *In vitro* experiments showed that 5-min incubation of 2.5% of ethanol significantly reduced viability of human colon cell line Caco-2, and ~40% cells died when the concentration was 10% [Banan *et al.*, 1998]. After 2-3-hr incubation of T84 human colonic epithelial cell monolayers with 10-30% ethanol, most cells died as assessed by trypan blue staining (data not shown). Thus, a 3-hr ethanol treatment might cause some

epithelial damage and result in poor gene transduction in the epithelium. This might be one of the explanations for previous studies showing that IR-administered Adv could not induce CTL responses in the ILNs after 3-hr ethanol treatment [Thomson, 2001]. Histological analysis in the present study revealed that, after 40 min *in vivo* ethanol treatment, epithelial cells appeared to be morphologically normal, probably due to the protective effect of mucus before being removed. Therefore, the 40 min ethanol incubation was sufficient to remove mucus without causing apparent epithelial cell damage.

The colon is the intestinal tissue of interest for mucus removal and gene transfer. AB/PAS staining showed that ethanol pipetting only removed the mucus from the majority of colon, but not the cecum and distal ileum. Gene transfer studies demonstrated that the transgene was mainly expressed in the colon, very low in the cecum and rarely in the distal ileum. Therefore, the P&D method ensures that mucus was removed mainly from the colon and colonic mucosa was the main site for IR gene transfer.

Overall, the P&D IR delivery method with shortened ethanol treatment can efficiently remove mucus from the colon, prolong vaccine incubation time, and particularly transfer antigen genes to the colon. This allows a better study of the colon as a site of local mucosal immunization for gene-based vaccines.

6.3   The rectal challenge model of HSV-2

6.3.1   The importance HSV-2 animal models

The rectal challenge model of HSV-2 was established to be relevant of human STD infection. Firstly, there are increasing concerns about the rectal mucosa serving as a one of main transmission routes of STDs. Some sexual practices cause repeated mucosal trauma by penetrating the mucus layer and disturbing lumenal environment, and hence, increasing the chance for individuals to be infected [Corey and Spear, 1986a; Corey and Spear, 1986b]. Secondly, HSV-2 infection is identified as a risk factor of other STDs, especially acquired immunodeficiency syndrome (AIDS) which is now worldwide epidemic [Holmberg et al., 1988; Renzi et al., 2003]. The rectal HSV-2 model might be valuable in the development of vaccines against HSV infection and meanwhile reduce susceptibility of transmission of human immunodeficiency virus (HIV). Conceivably, the concept of this model might be also used to establish a rectal challenge model in other species for HIV infection and design anti-HIV vaccines.

The rectal challenge model of HSV-2 was designed to examine the ability of the colonic mucosal immunity to be induced to counteract virus infection. HSV-2 infection proved to be well suited for evaluating the efficacy of vaccines as demonstrated in

previous studies using genital challenge models [McDermott *et al.*, 1984; Gallichan *et al.*, 1995; Parr and Parr, 1997b]. Due to temperature effects on viral replication, HSV pathogenesis locally occurs at the mucosal level, rather than at systemic level, where the temperature is higher [Letchworth and Carmichael, 1984]. Thus, control of HSV-2 infection is reflective of local mucosal immunity. In addition, infection of HSV-2 through mucosal surfaces resulted in neurological sequelae as shown in the genital model [McDermott *et al.*, 1984; Gallichan *et al.*, 1995; Parr and Parr, 1997b] and the rectal model developed in the present study. This allows more direct and dependable assessment of the disease and provides reliable information about the effectiveness of vaccines.

6.3.2 Features of rectal HSV-2 infection

The rectal challenge models of HSV-2 are overall similar to the genital model with respect to clinical courses (see Chapter 3). Rectally-infected mice displayed progressively severe local inflammation, skin lesions and hindlimb paralysis. Viruses replicated within rectal tissues and shed into the intestinal lumen. HSV-2 mainly replicated in the distal part of the colon, which is the limit of rectal swab insertion. Quantitation of rectally-swabbed virus particles paralleled the degree of virus replication inside the tissue and also correlated with disease development (see Chapter 3). Human studies showed that HSV-2 shedding in men who have sex with men was almost exclusively rectal or perianal [Krone *et al.*, 1998]. It was thus believed that shed viruses

175

might be well recovered by rectal swabbing. Since the significant loss of injected fluid in the rectum, rectal wash with PBS was found to be insensitive for the recovery of infectious virus. This issue was also noted in other studies [Zeitlin *et al.*, 2001]. Therefore, rather than rectal wash with PBS, rectal swabbing might be a suitable measure to detect rectal infection of HSV-2.

Other than similarities between rectal and genital models, there are some significant features for the rectal model to be distinct from the other. C57BL/6 mice were found to be resistant to HSV-2 infection at the experimental doses that cause lethal infection through genital tract of Depo-Provera®-treated mice. Only 50% of mice became infected and 20% developed paralysis by the virus at a very high dose 2×10^7 PFU. The mucus layer apparently provided protection against virus infection because ethanol treatment rendered mice susceptible to the challenge of HSV-2. Twenty-five percent of mice developed paralysis at a dose of 2×10^3 PFU, which is 10,000-fold lower than the dose causing similar percentage of lethality in ethanol-untreated mice. Ethanol treatment differs from Depo-Provera® subcutaneous injection to thin the epithelial layer of vaginal walls [Parr and Parr, 1997b]. Ethanol treatment not only removes mucus but might also remove lumenal antibodies including SIgA from the colonic lumen and renders colonic epithelial cells completely exposed to virus attack. Lumenal antibodies play an important role to prevent virus from attacking epithelial cells and, in the absence of lumenal antibodies, the mucosae become vulnerable to viral infection [Bukawa *et al.*, 1995; Hocini and Bomsel, 1999; Alfsen *et al.*, 2001; Silvey *et al.*, 2001]. Therefore, the

preventive role of lumenal antibodies was greatly minimized and, hence, cannot be examined properly in this model.

6.4   Adv-mediated gene transfer in the colonic mucosa

6.4.1   Gene transfer locale

After IR administration of Adβ-gal, a substantial degree of transgene β-gal was expressed in the epithelial cells based on the detection of numbers of positive β-gal staining cells [Thomson, 2001]. The present study showed that gene expression was also immediately beneath the epithelium to a lesser extent (see Chapter 3). This is in contrast to an earlier observation by Foreman *et al.* [1998], who did not find subepithelial gene transduction and assumed that the intact epithelium protected LP cells from being infected. However, Neurath and his colleagues [1999] quantified gene expression following IR infusion Adβ-gal and revealed that isolated LP cells were infected by Adv but expression levels were lower than those in epithelial cells. Whether or not the differences in gene expression correlated with numbers of gene-expressing epithelium and LP cells was not shown by these investigators, probably because of insufficient β-gal expression that could not be visualized since they did not perform mucolytic treatment and might have used an inefficient IR delivery method. Overall, results provided in the

present study suggested that the some viral vectors might gain access to the LP by crossing epithelium and infected subepithelial cells.

The gene expression immediately beneath the intestinal epithelium was found in cells spread diffusely in the colonic LP or within lymphoid follicles. It is possible that Adv entered LP by passing through the epithelium and infecting cells under epithelium. Two pathways were proposed here to be involved in cross-epithelial transport of Adv. One pathway might be the transport of Adv through intestinal epithelial cells (IECs), which might transcytose Adv from apical surface to basolateral membrane. The other pathway might be through M cells. M cells are found in follicle associated epithelium (FAE) overlying both colonic aggregated and isolated lymphoid follicles (ALFs and ILFs). A previous study suggested that M cells were more efficient in Adv uptake compared to conventional intestinal epithelial cells (IEC) because there was a 10-fold increase in gene transfer to M-cell enriched segment of mouse ileum compared to M-cell insufficient areas [Foreman *et al.*, 1998]. It is very likely that M cells serve as a portal for Adv entry. Thus, passage of Adv through IECs might lead to infection of LP cells, while Adv might infect follicle cells after passing through M cells. Future studies might be required to characterize gene transfer immediately beneath the epithelium in association with colonic IECs and M cells.

It remains unclear what type of LP cells are infected by IR administered Adv. By co-localization of β-gal, CD3 and CD4, Neurath and his colleagues [1999] showed that

178

CD4$^+$ LP T cells (examined following cytocentrifugation) were infected by Adv. Since only Percoll$^®$-purified mononuclear lymphocytes were examined, the cell population studied might represent only one of the cell populations that can be infected. Moreover, whether CD4$^+$ LP T cells represent a major population that becomes infected is also a question to consider. *In vitro* studies demonstrated that dendritic cells (DCs) could be infected by Adv for antigen transduction and induced antigen-specific immune responses against tumor and viral infections [Song *et al.*, 1997; Ranieri *et al.*, 1999; Wan *et al.*, 1999; Kikuchi *et al.*, 2000]. It is possible that IR administered Adv might enter the LP and infect colonic DCs which wander beneath the epithelium for antigen sampling. Full characterization of antigen acquisition by colonic immune cells is required to improve our understanding of gene-based vaccines.

6.4.2   Enhanced gene transfer

Adequate antigen delivery to the immune system is essential for immune induction [Healy and Goodnow, 1998]. Without ethanol pretreatment, visualization of β-gal expression could not be achieved (with the X-gal staining) in the colonic epithelium after simple IR infusion of Adβ-gal [Thomson, 2001]. Similarly, other studies were not able to visualize β-gal but only show quantitative gene expression in the colon [Foreman *et al.*, 1998, Wirtz *et al.*, 1999]. Since mucus is increasingly produced in the large intestine and continuously covers the epithelium [Szentkuti and Lorenz, 1995; Matsuo *et al.*, 1997; Atuma *et al.*, 2001], it was speculated that the presence of mucus might render

the host relatively resistant to Adv infection. It was additionally observed that mice were not susceptible to rectal infection of HSV-2 unless the colon was preconditioned with ethanol (see Chapter 3). Thus, removing the mucus layer might be a solution to this problem. The speculation was confirmed by the previous study [Thomson, 2001] and the present study (see Chapter 3) showing that β-gal could be visualized after mucus removal with ethanol treatment. It was further supported by the luciferase assay which demonstrated that gene transfer was at only a limited level without ethanol enema and was remarkably enhanced after such mucolytic treatment (see Chapter 3). It thus can be concluded the presence of mucus might block gene transfer, albeit not completely, and result in insufficient production of catalyzed substrate at low β-gal levels. Therefore, removing mucus is a necessary intervention for viral-vector infection and maximal gene transduction in the colon.

Other than antigen concentration, the duration of antigen exposure duration also is extremely important for the induction of immune responses [Healy and Goodnow, 1998]. Previous study showed that gene expression could be visualized for up to 3 days post-infection of Adv [Thomson, 2001]. By quantitative measurement of luciferase activity after IR administration of AdLuc, the present study confirmed that gene expression was highly expressed within the first 3 days. The 3-4-day presence of highly transduced gene appeared to be one day longer than found in previous studies [Wirtz et al., 1999], in which mucolytic treatment was not performed. The efficient IR delivery method developed in the present study might be the reason for one-day longer gene expression.

This prolonged high-level gene expression might be an important achievement since the duration of antigen presence is highly associated with the induction of immune responses [Healy and Goodnow, 1998].

It was found luciferase activities significantly declined at day 4 and remained at relatively low levels for one week after administration. This might explain why β-gal gene expression became invisible 4 days post-infection as previously shown [Thomson, 2001]. Other investigators [Wirtz et al., 1999] also had similar observations albeit it was at day 3, one day earlier than observed in the present study. The decline in gene expression might be due to the turnover of colonic epithelial cells as previously proposed [Wirtz et al., 1999]. Highly activated state of colonic immune functions such as spontaneous release of IFN-γ from colonic $CD8^+$ intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) might also be a cause [Taguchi et al., 1990]. Long-term gene expression was achieved when self antigen is introduced with Adv following a single intramuscular (IM) injection [Tripathy et al., 1996]; hence specific immune responses induced against Adv encoded foreign antigen as shown in the present study by IFN-γ ELISPOT detected at day 4 might provide a further explanation (see Chapter 4).

6.4.3 Colon as the main site for gene transfer

Previous studies visualized β-gal expression along the entire length of colonic epithelium but more concentrated within the 2 cm colon from the anus [Thomson, 2001].

181

The present study also quantitatively showed that gene expression (luciferase activity) was higher in the distal colon than in the proximal colon (see Chapter 3). There are number of possible explanations. First, mucus was more efficiently removed in the distal colon as compared to the proximal part because mucus was present in the very proximal end. Thus, gene transfer might be better facilitated in the distal half. Second, only 100 µl solution was used for injection and this might cause an uneven distribution of delivered material. Third, uncomfortable colonic dwelling might stimulate intestinal peristalsis to propel delivered reagents to distal colon. Fourth, if M cells are important for gene transfer and are more distributed in the distal segment, more uptake of Adv and increased transgene expression might occur. These proposed mechanisms might also provide possible explanations for the difference in the number of HSV-2 particles within tissues (a 10-fold difference) between the proximal and distal part following rectal challenge.

The colonic mucosa is the main target of IR delivered gene-based vaccines. It was shown that gene expression was substantially low in the cecum and almost undetectable in the small intestine (see Chapter 3). Foreman *et al.* [1998] showed that there was no substantial difference in Adv-mediated gene transduction between small and large intestines. Therefore, minimal gene transfer levels found in cecum and small intestines were probably due to the presence of mucus that prohibited infection of Adv. As a result, local immune responses (IFN-γ production and cytotoxic activity) were induced primarily through the colonic mucosa and the ILNs; very little, if any, was noted in the ileocecum (see Chapter 4). Previous studies showed that there were very low levels of gene

expression in the liver and spleen after IR administration of Adβ-gal [Wirtz *et al.*, 1999]. This further suggests that IR-delivered genes are mostly destined to the colonic mucosa and rarely disseminated to other non-mucosal sites of the body.

6.5   Induction of cellular immune responses

6.5.1   Primary inductive sites

The present study was able to show that IR immunization with Adv could induce antigen-specific cellular immune responses. Both ELISPOT and cytotoxic assays suggested that local immune cells were activated by Adv encoded foreign antigens to produce IFN-γ and exerted cytotoxic activities in response to MHC class I-restricted TCR-specific peptides or antigen-expressing cells. Such responses were induced primarily through ILNs since ILN cells were activated to be able to produce IFN-γ and display CTL responses. IFN-γ-secreting cells were early detected in the colon tissues, thus suggesting that induction of primary immune responses might be also within the colonic mucosa.

It was previously demonstrated that following virus infection, DCs but not macrophages survived for more than 2 days and displayed activities to prime CTL

responses [Bender *et al.*, 1998]. Thus, it was proposed by the present study that DCs might be involved in the induction of such colonic immune responses by involving a number of mechanisms. First, colonic DCs might process apoptotic virally-infected cells or debris and activate $CD8^+$ T cells by cross-priming in the secondary lymphoid organs [Albert *et al.*, 1998b; Carbone *et al.*, 1998; Steinman *et al.*, 1999]. Apoptotic intestinal epithelial cell remnants were identified in the DCs that migrated into MLNs [Huang *et al.*, 2000]. Second, colonic DCs might be infected by Adv and directly present endogenously processed antigens and prime lymphocytes [Koopmann *et al.*, 1997]. Lymphoid tissues appeared to be the major inductive site. Studies of Peyer's patches (PP) showed that PP DCs had the ability to stimulate PP T cells [Kelsall and Strober, 1996; Ruedl *et al.*, 1996; Ruedl and Hubele, 1997]. Possibly, DCs might reside in PP-like aggregated lymphoid follicles (ALFs) in the colon and prime colonic T cells. Other than priming within lymphoid tissues, LP interstitium might also be an inductive site for "in situ" priming as previously proposed [Kelsall and Strober, 1999; Fagarasan and Honjo, 2003]. In the present study, colonic lymphocytes were isolated using the standard LPL isolation protocol which led to a mixture of lymphocytes from LP and lymphoid follicles, and therefore, could not determine at which site T cells were primed. As ALFs and ILFs are well distributed in the large intestine, their roles in the induction of mucosal immunity warrant further study.

It was found that there was a functional dissociation of immune induction between the DLN (ILN) and other sites (colon and spleen). In contrast to the continuingly

increased response in the spleen and rectal tissue, there was an early transient but high frequency of specific IFN-γ-secreting cells in the ILNs. This might suggest an initially localized T-cell clonal expansion in the draining lymphoid tissue [Zinkernagel and Hengartner, 2001]. This favors the view that DLNs are the inductive site for immune responses, and that newly activated lymphocytes leave the DLNs, enter the bloodstream, and ultimately encounter relevant antigens in local tissues [Karrer et al., 1997]. It has been pointed out that although activation and proliferation events occurring in the lymphoid organs are transient, local immune responses declines at a much slower rate as they are maintained by specific T cells that have migrated to the mucosa and have become memory T cells to exhibit long-term effects [Gallichan and Rosenthal, 1996b; Masopust et al., 2001]. Thus, the decline of cytolytic activity in the secondary lymphoid tissues as demonstrated in the present study reflects the induction of immune response and might not be used to interpret immunologic activities in the colonic (local) tissue, at which memory T cells might be generated to maintain antigen-specific killing functions. Therefore, future studies might further explore long-term T-cell activities in colon tissues after IR immunization.

6.5.2   Induction of Th1 T-cell responses

This study demonstrated IR immunization with Adv induced a prevailing Th1 T-cell immune response in an antigen-specific manner. This was suggested by the continuous increase in the frequency of both local and systemic IFN-γ-producing cells in

response to antigens as well as an increased production of antigen-specific IgG2a over IgG1 (see Chapter 4). It is generally believed that at the onset of viral infections, innate cytokines produced by DCs, for example, play a critical role in shaping the downstream adaptive immune responses [Biron, 1999; Moser and Murphy, 2000; Trinchieri, 2003]. Functions of DCs are modulated by the microbes and the microenvironment before they are involved to direct immune responses [Pulendran et al., 2001]. Under the conditions of viral infection, local DCs can be activated to produce the pivotal Th1 type cytokines such as IL-12, which then induces IFN-γ production by T cells [Scott, 1993; Banchereau and Steinman, 1998; Lanzavecchia and Sallusto, 2000a; Moser and Murphy, 2000; MacDonald and Monteleone, 2001; Dalod et al., 2002; Trinchieri, 2003]. This initial progress might be governed by a specific DC subset which has distinct functions from others in activating T cells and shaping cytokine profiles of either Th1 (IFN-γ and IL-2) or Th2 (IL-4, IL-5 and IL-10) dominance [Liu et al., 1998; Maldonado-Lopez et al., 1999; Pulendran et al., 1999; Tanaka et al., 2000; Iwasaki and Kelsall, 2001]. These subsets probably derive from the a common DC precursor, whose differentiation depends upon the nature of antigen that is presented and the microenvironment [del Hoyo et al., 2002; Ardavin, 2003]. It has been revealed that feeding inert antigens in a non-Th-biased environment leads to induction of so-called "default" DC-mediated Th2 pathways, whereas the participation of inflammatory stimuli or Th1 type cytokines results in DC-driven Th1 cell responses prevailing at the mucosa [Holt et al., 1999; Moser and Murphy, 2000; Brimnes et al., 2003]. It has been found that IFN-α/β-producing CD8⁻B220⁺DCs produce IL-12 in viral infections (such as influenza virus and murine cytomegalovirus),

while bacterial infections (such as *Staphylococcus aureus*) appear to involve primarily

$CD8^+B220^-$ DCs [Ohteki *et al.*, 1999; Asselin-Paturel *et al.*, 2001; Iwasaki and Kelsall,

2001; O'Keeffe *et al.*, 2002]. Further studies are needed to determine the prevalence of

different colonic DC subsets and their immunologic roles in antigen uptake and in

orchestrating adaptive immune responses.

6.5.3   Requirement of high antigen dose for immune induction

The results from previous investigations [Thomson, 2001] and current antigen

models (OVA and gB, Chapters 3 and 4) suggested that induction of colonic immune

responses required a high degree of gene transfer. In the OVA model, it was shown that

lower doses (1×10^9 PFU) of AdOVA given IR was not sufficient to induce detectable

levels of primary cytolytic responses in the ILNs. When doses were increased to 2×10^9

PFU, ILN cytolytic activities were detected. Similarly, immunization with 2×10^9 PFU

AdgB generated protection against rectal challenge with 2×10^6 PFU of HSV-2, while

lower doses of AdgB was not successful. The local concentration of antigen is an

important parameter for the induction of immune responses [Healy and Goodnow, 1998].

It has been proposed that there is a high antigen threshold mechanism for the induction of

efficient ·colonic immunity as compared to other mucosal sites, such as in respiratory

tract, and to the periphery [Kelsall and Strober, 1999]. This mechanism might be used to

avoid unnecessary responses to frequent antigen invasion, which is usually readily

controlled by colonic T cells having highly activated status [Taguchi *et al.*, 1990; Saparov *et al.*, 1997].

6.6   Induction of protective rectal immunity

Effective mucosal immune responses should be able to control virus infection and/or proliferation at local tissues and thereby prevent virus spreading to other sites. It was examined in the present study by the rectal challenge model with VV expressing OVA (VV-OVA) after IR immunization with AdOVA. Compared to unimmunized mice, VV infectious particles were undetectable in either colons or ovaries, indicating that Adv-based IR immunization conferred sterile immunity against mucosal infection and effectively limited virus dissemination (see Chapter 4). Other studies using HIV peptides with cholera toxin (CT) showed that mice challenged with VV expressing the same peptide still maintained residual levels of the virus in the ovaries [Belyakov *et al.*, 1998a; Belyakov *et al.*, 1998b]. It seemed to suggest the advantage of using Adv for mucosal vaccine to generate reliable local protection. Further evidence for effective protection was provided by the rectal HSV-2 model developed in the present study (see Chapter 5). It was the first time that IR immunization was effective to render mice resistant to HSV-2 infection through the rectal mucosa. Although a low scored local inflammation and limited virus replication occurred after challenge, these manifestations disappeared in 5-7 days and protected mice did not develop severe disease (lesions or paralysis). This

indicated that mice became infected after rectal challenge of HSV-2 but were able to control the infection quickly and efficiently such that the disease was prevented.

Previous mucosal vaccine studies investigated mucosally-induced systemic immune responses by only assessing serum antibody production or functional splenic T cells; few have examined systemic protection [Hordnes *et al.*, 1995; Belyakov *et al.*, 1998b; Shen *et al.*, 2000]. The present study demonstrated that mice immunized IR with AdOVA developed protective immunity against systemic challenge of OVA-expressing tumors (E.G7-OVA, see Chapter 4), thus suggesting that mucosal immunization was capable of providing systemic protection. Future studies might be conducted to design such vaccine strategies to control systemic infectious disease secondary to mucosal infection. Moreover, IR immunization might be future studied to induce anti-tumor immunity.

6.7   Factors contributory to the local protective immunity

6.7.1   CD8$^+$ T cells

CD8$^+$ T cell-mediated immune responses are the important protective mechanism by which the host controls virus infections. The present study demonstrated that IR

immunization induced mucosal immune responses and protection largely dependent on

CD8$^+$ T cells. Addition of anti-CD8 mAbs abrogated the cytolytic activities. Intracellular

cytokine staining provided evidence that CD8$^+$ T cells were stimulated to produce IFN-γ

in the presence of antigens (see Chapter 4). Further, *in vivo* studies showed that CD8$^{-/-}$

mice immunized IR with AdgB could not survive the lethal challenge of HSV-2 (see

Cheaper 5). Although it was not measured, the cytolytic activity of colonic LP CD8$^+$ T

cells might be mirrored by that of the lymphoid T cells; it is evident that DLN CTLs are

able to migrate to the intestinal mucosa and exert effective cytolytic functions [Klavinskis

et al., 1996; Lefrancois *et al.*, 1999; Masopust *et al.*, 2001].

It was suggested that CD8$^+$ T cells were activated to be able to detect HSV

infection by recognizing the gB peptide expressed on infected cells within two hours of

infection [Mueller *et al.*, 2003]. Anti-HSV killing mechanisms were found to be

associated with perforin or FasL [Topham *et al.*, 2001a] and the effective immunologic

controls attribute to the clonal expansion of CD8$^+$ effector T cells [Butz and Bevan, 1998;

Kalams and Walker, 1998; Barouch and Letvin, 2001]. The essential role of CD8$^+$ T cells

in controlling other virus infections, such as HIV infection, were determined in infected

primates by parenteral infusion of anti-CD8 mAbs, which resulted in dramatically

increased plasma viraemia [Matano *et al.*, 1998; Jin *et al.*, 1999, Schmitz *et al.*, 1999;

Lifson *et al.*, 2001]. Recent studies showed that some CD8$^+$ T cells specific for HSV

were selectively retained in the trigeminal ganglia, functioning as blocking HSV-1

reactivation [Liu *et al.*, 2000; Khanna *et al.*, 2003]. These studies might indicate that by

sensing virus gene expression, $CD8^+$ T cells critically mediate immunosurveillance and exert anti-virus functions.

Most studies asserted that $CD4^+$ T cells were required for direct control of herpes infection similar to $CD8^+$ T cells [Flano et al., 1999; Ghiasi et al., 2000; Keadle et al., 2002]. The present study revealed the presence of $CD4^+$ T cells in the $CD8^{-/-}$ mice did not protect mice from HSV-2 infection. $CD4^+$ T cells are able to produce IFN-γ upon exposure to antigen and exert anti-virus activities [Miskovsky et al., 1994; Milligan and Bernstein, 1995a]. Since they specifically target infected MHC class II expressing cells, $CD4^+$ T cells cannot efficiently attack MHC class I-expressing infected cells. This makes these cells functionally less important than $CD8^+$ T cells in virus clearance [McMichael and Hanke, 2002]. Although they are able to control acute herpesvirus infection in the absence of $CD4^+$ T cells, $CD8^+$ T cells were found to be unable to completely control infection because $CD4^{-/-}$ mice developed lethal recrudescence at a later time [Stevenson et al., 1998]. Recent findings demonstrated that the important role of $CD4^+$ T cells might lie in their professional help to the activation of $CD8^+$ T cells [Bennett et al., 1998; Schoenberger et al., 1998; Whitmire et al., 1999], to maintain effective levels of $CD8^+$ effector T cells [Zajac et al., 1998], and sustain $CD8^+$ memory T cells [Matloubian et al., 1994; Walter et al., 1995; Sun and Bevan, 2003]. Therefore, $CD4^+$ T cells might critically participate in anti-viral immune responses by supporting a full range of $CD8^+$ T-cell effector functions. Also, this information is important for vaccine design against viral infection.

6.7.2   IFN-γ

The present study demonstrated that a large quantity of LP, ILN and spleen cells

secreted IFN-γ in response to antigen and also revealed that IFN-γ played a role as an

important cytokine in the control of viral infection because IFN-γ$^{-/-}$ mice immunized IR

with AdgB could not survived HSV-2 challenge (see Chapter 5). Using animals with

depletion or gene knockout of the IFN-γ or its receptor, a large body of literature has

demonstrated the critical role of IFN-γ in anti-viral infection. Mice deficient in IFN-γ had

immunological failure in the resistance to infections of viruses and intracellular bacteria

[Dalton et al., 1993; Cooper et al., 1993; Graham et al., 1993; Huang et al., 1993; Muller

et al., 1994; Wang et al., 1994]. Mice deficient in IFN-γ receptor were more susceptible

to HSV-1 infection [Cantin et al., 1999b] and developed chronic vascular pathology

(severe large-vessel panarteritis) [Weck et al., 1997]. It was shown that effector CD8^{+} T

cells did not necessarily always kill their virus-infected targets because IFN-γ itself was

able to control virus infection [Guidotti and Chisari, 1999]. Following administration of

recombinant VV encoding IFN-γ in athymic nude mice, IFN-γ expression greatly reduced

the virus growth and enabled the mice to recover from the infection [Karupiah et al.,

1990a; Kohonen-Corish et al., 1990]. Adoptively transferred CTLs non-cytolytically

purged the virus by releasing IFN-γ and other antiviral cytokines, such as tumor necrosis

factor (TNF)-α [Guidotti et al., 1994; Guidotti et al., 1996]. The importance of inducing

IFN-γ production also resides in the ability to control HSV neuronal latency by

efficiently suppressing reactivated HSV-1 (although not by preventing virus reactivation)

[Cantin *et al.*, 1999a; Lekstrom-Himes *et al.*, 2000]. CD8$^+$ T cells responded to early

HSV reactivation in neuron cells by producing IFN-γ as suggested in an *in vitro* study

showing that addition of IFN-γ within 24 after HSV-1 infection in trigeminal ganglia

culture suppressed reactivation of the virus [Liu *et al.*, 2001a]. Through this mechanism,

the virus spreading from the axon to epidermal cells was effectively prevented [Mikloska

and Cunningham, 2001]. The IFN-γ mediated antiviral response is also in CD8$^+$ T cell-

independent manner because Th1 CD4$^+$ T cells as well as natural killer (NK) cells and

NK T cells also demonstrate the ability to produce IFN-γ [Eichelberger *et al.*, 1991;

Maloy *et al.*, 2000]. However, in HSV-2 infection, NK-derived IFN-γ during the early

phase of infection was not essential for control of infection as depletion of NK cells had

no impact on the rate of virus clearance [Milligan and Bernstein, 1997]. Other than direct

antiviral activity, an imbalance of Th1/Th2 paradigm as a consequence of the absence of

IFN-γ might also a working mechanism of IFN-γ. It was shown that following

inoculation of HSV-1, IFN$\gamma^{-/-}$ mice had a Th2-biased response and diminished delayed

type I hypersensitivity responses, which resulted in prolonged infection period and

increased susceptibility to encephalitis as compared to wild type mice [Bouley *et al.*,

1995].

It has been argued that IFN-γ plays a limited role in controlling HSV infections as

IFN$\gamma^{-/-+}$ mice were partially protected [Holterman *et al.*, 1999] and *in vivo* depletion of

IFN-γ only delayed clearance of vaginally-infected HSV-2 [Milligan and Bernstein,

1997]. Interestingly, removal of one of the factors including otherwise perforin, FasL,

TNF did not fully impair anti-virus activities mediated by $CD8^+$ T cells, while combined

disruption of these genes led to uncontrolled infections [Kagi *et al.*, 1994b; Lowin *et al.*,

1994; Guidotti *et al.*, 1996; Fleck *et al.*, 1999; Holterman *et al.*, 1999; Aung *et al.*, 2001;

Chang *et al.*, 2000; Parra *et al.*, 2000]. Thus, the explanation might be that the IFN-γ

response represents only one of important anti-virus mechanisms.

6.7.3   IL-12

IL-12 is an important cytokine serving as a mediator joining innate and adaptive

immune responses [Yang *et al.*, 1995b] and inducing anti-viral Th1 cell responses to

produce IFN-γ [Hsieh *et al.*, 1993; Manetti *et al.*, 1993; Magram *et al.*, 1996] as well as

enhancing IgA production [Boyaka *et al.*, 1999b]. The role of IL-12 in anti-HSV

immunity was investigated in the present study. The majority of $IL-12^{-/-}$ mice following

IR immunization developed paralysis, thus suggesting that IL-12 played a critical role in

anti-HSV responses (see Chapter 5). Belyakov *et al.* [1998b] demonstrated that the

rectally-induced mucosal immune response after IR delivery of HIV peptide gp160 could

be enhanced by local administration of recombinant IL-12, which stimulated IFN-γ

production and CTL responses in wild type but not $IFN-γ^{-/-}$ mice. This implied that IL-

12 elicited Th1 cell responses and $CD8^+$ T cell functions through IFN-γ. Clinical studies

suggested that IFN-γ production depended on the present of IL-12. A recent clinical study

first reported that due to the mutations of IL12B gene, which encodes IL-12 p40 subunit,

12 out of 13 IL-12-deficient patients suffered from intracellular infection of mycobacteria

[Picard *et al.*, 2002]. Addition of exogenous IL-12 into whole-blood cell culture not only significantly increased IFN-γ production but also stimulated patient blood cells to produce a comparable level of IFN-γ compared to normal controls. It was suggested that IL-12 also played an important in maintaining the Th1 cell response. IL-12 exerted its ability by stabilizing the IFN-γ-producing Th1 T-cell clones, which was in contrast to the very transient Th1 cytokine production in the absence of IL-12 (1 week of *in vitro* culture), thus suggesting the necessity of IL-12 to restore Th1 cell responses [Seder *et al.*, 1993; Yang *et al.*, 1995b].

It is well known that DCs are the major source of IL-12 by which they interact with T cells, influence the development of polarized responses [Macatonia *et al.*, 1995] and act in synergy with co-stimulatory molecules to induce IFN-γ production from T cells [Kubin *et al.*, 1994; Murphy *et al.*, 1994]. IFN-γ in turn increases the expression of IL-12 receptor by a positive feedback loop [Wu *et al.*, 2000a]. Thus, upon exposure to antigen, IL-12 cooperates with IFN-γ to stimulate T-cell expansion and differentiation into Th1 T cells which can then suppress Th2 T-cell development [Yang *et al.*, 1995b]. Further evidence showed that $CD8\alpha^{+}$ DCs from IL-12 $p40^{-/-}$ mice, but not from IL-12 competent mice, failed to induce the development of Th1 cell responses, including IFN-γ [Magram *et al.*, 1996; Maldonado-Lopez *et al.*, 1999; Becher *et al.*, 2002; Cooper *et al.*, 2002]. Thus, deficiency in IL-12 production results in the inability of DCs to produce IL-12, leading to the loss of the ability to induce Th1 type cytokine profiles.

In the present study, 20% of IL-12$^{-/-}$ mice survived the HSV-2 challenge. This might imply that IL-12 is not a unique factor in directing anti-viral immunity. Other immune factors, such as IFN-α/β and IL-18 [Cousens *et al.*, 1999; Xing *et al.*, 2000; Freudenberg *et al.*, 2002], might be also involved in such anti-virus responses.

6.8   Induction of humoral immune responses

The present study showed that there were significant levels of IgA production in the colon. This suggested that IR immunization with Adv was able to induce colonic humoral immune responses. The ability of Adv to induce mucosal antibody responses was also shown in previous studies. IN or intratracheal (IT) administered Adv induced local IgA production in the nasal passage and lung [Van Ginkel *et al.*, 1995] or in the genital tract [Gallichan and Rosenthal, 1995]. Although Th1 predominance (as we detected in the present study) was believed to inhibit IgA production in the lung after Adv administration [Yang *et al.*, 1995b], it was recently shown that IL-12 and IFN-γ might be able to enhance IgA production in the intestinal mucosa [Boyaka *et al.*, 1999a; Boyaka *et al.*, 1999b].

Comparing to the ability of antibodies to act against free viruses, it was found that antibodies responded to infected cells less effectively than cell-mediated immune responses did [Burton, 2002]. Antibody-dependent cellular cytotoxicity was detected at

lower levels in the intestinal mucosa of primates than in peripheral blood, and this was probably due to the presence of small numbers of mucosal NK cells [James and Graeff, 1985]. Studies showed also that genital antibody responses proved to be ineffective against HSV infection [Morrison *et al.*, 2001]. Neither IgA nor IgG were sufficient to control virus infection yet did reduce the initial virus infection [Kuklin *et al.*, 1998; Wang *et al.*, 2000]. The role of antibodies became prominent when substantially high concentrations were present [Sanders *et al.*, 1994; Haneberg *et al.*, 1997; Hezareh *et al.*, 2001]. These studies suggested that under normal physiological conditions, IgA mainly provided a preventive role to reduce virus infection and its protective role was limited as compared to cellular immune components. Since rectal HSV-2 is transmitted through abraded body surfaces during sexual behavior, infection of epithelial cells and, particularly, cells immediately beneath the epithelium is not avoidable [Corey and Spear, 1986a; Corey and Spear, 1986b]. In this case, cellular immune responses become more important in controlling virus infection at the mucosal surfaces.

6.9   Enhanced rectal and genital immunity by Adv IR immunization

6.9.1   Mucosal versus systemic immunization

It has long been suspected that systemic, non-mucosal immunization does not

induce reliable mucosal immunity. McDermott *et al.* [1990] showed that parenteral, non-mucosal immunization with attenuated HSV-2 did not elicit mucosal IgG and protective immunity against vaginal challenge of wild type HSV-2 but vaginal immunization stimulated significant levels of IgG in the local tissue and provided protection. Rosenthal's group [Gallichan *et al.*, 1993; Gallichan and Rosenthal, 1995; Gallichan and Rosenthal, 1996b; Gallichan and Rosenthal, 1998] distinctly demonstrated that intranasal mucosal, but not parenteral, immunization with AdgB not only induced higher genital immune responses but also provided genital protection against lethal challenge of HSV-2. Other studies also produced consistent results when comparing various systemic routes with mucosal routes. Intramuscular (IM) immunization of mice with a Sindbis virus-based replicon vector expression HIV-gag did not confer protection against vaginal challenge of VV-gag, while rectal or genital immunization did [Vajdy *et al.*, 2001]. IM prime/boost immunizations of primates with Semliki forest virus (SFV) and modified vaccinia virus Ankara (MVA) vectors enhanced only systemic immune responses but did not protect vaccinated macaques from rectal challenge of simian immunodeficiency virus (SIV) [Nilsson *et al.*, 2001]. Using MHC class I-restricted HIV peptides mixed with a mutant *E. coli* heat-labile toxin (LT), studies showed that systemic immunization was ineffective as compared to IR immunization in the induction of local $CD8^+$ T cytotoxic activities to clear antigen-expressing viruses from rectal tissues [Belyakov *et al.*, 1998b; Belyakov *et al.*, 2001]. The present study provided further evidence that systemic immunization was incompetent for the induction of mucosal immunity. SC immunization with Adv was less effective than IR mucosal immunization in inducing antigen-specific

rectal immune responses (IFN-γ-secreting cells and IgA production) as well as in providing rectal immunity against antigen challenge with VV (see Chapter 5). Furthermore, SC immunized mice were only partially protected from rectal challenge of a lethal dose HSV-2 as compared to IR immunized mice which were fully protected (see Chapter 5). Studies on protection against vaginal challenge of VV and HSV-2 were evident also for the ineffectiveness of SC immunization as compared to IR mucosal immunization (see Chapter 5). Therefore, systemic immunization proved to be an inappropriate regimen for the induction of mucosal immunity.

A number of recent studies [Amara *et al.*, 2001; Amara *et al.*, 2002] claimed that systemic intradermal/IM prime/boosts of rhesus macaques with DNA/MVA89.6 (expressing HIV-1 envelope) generated protective immunity against rectal challenge with a pathogenic simian/human immunodeficiency virus (SHIV) chimera 89.6 (SIV backbone encoding HIV envelope). However, the results might not be interpreted correctly because systemic rather than mucosal immune responses were examined. Reduction in plasma virus load was not surprising since animals were immunized systemically. Histology changes of lymph nodes (LNs, location was not specified) were inspected and it was surprising that LN hyperplasia persisted (or developed) several months after SHIV challenge. This suggested the presence of viral antigens in the host as a consequence of virus replication, thus indicating an ongoing virus infection several months after challenge. Like other investigators studying HIV vaccines [Polacino *et al.*, 1999; Patterson *et al.*, 2001; Muthumani *et al.*, 2003], Amara *et al.* [Amara *et al.*, 2001; Amara

et al., 2002] only examined systemic immunity in response to virus entry into the blood and were not able to demonstrate local mucosal protection against virus challenge and, therefore, did not fully address the question about mucosal protection against virus transmission.

It has been argued that systemic immunization might induce a certain degree of mucosal immune response [Bouvet *et al.*, 2002]. Antigen might diffuse from the systemic vaccination site to mucosal tissues or mucosal lymphoid tissues (such as MLNs), where it can be taken up by MLN DCs. DCs from the systemic compartment might enter mucosal lymphoid tissues to present antigen. However, antigen density and antigen-bearing DC numbers in mucosal lymphoid tissues might be low and unable to induce significant mucosal immune responses. Studies have showed convincingly that systemic immunization induces insufficient numbers of activated lymphocytes expressing α4β7 (receptor for homing of lymphocytes to intestinal LP), thereby accounting for the poor contribution to the effector function in intestinal mucosae [Quiding-Jabrink *et al.*, 1997; Csencsits *et al.*, 2001; Campbell and Butcher, 2002]. Therefore, as compared to mucosal immunization, systemic immunization is not a suitable regimen to induce mucosal protection.

6.9.2 Local versus distant mucosal immunization

The common mucosal immunologic system (CMIS) has been the guiding

principle for mucosal vaccine design. It is known that mucosally activated lymphocytes can enter a mucosal site distant from the immunization site. The present study also supported this theory by showing specific IFN-γ-secreting cells and IgA production in the colon after IN immunization with AdOVA as well as partial protection against rectal and genital challenge of HSV-2.

The present study also suggested that colonic immune responses induced via a distant mucosal route were less effective than via the rectum as a local mucosal route. The difference became significant when VV-OVA was used to rectally challenge these mice in which IN immunization did not completely suppress the virus replication but IR immunization did (see Chapter 5). Further evidence was provided showing that protection against rectal challenge of HSV-2 at a dose of 2×10^5 PFU was partially provided by IN immunization with AdgB but fully by IR immunization (see Chapter 5). Gallichan *et al.* [2001] showed that most IN immunized mice lost protection from vaginal challenge of HSV-2 when doses were increased to 2×10^5 PFU, thus suggesting that distant mucosal immunization did provide protection but to a limited extent. Similar observations were made also by other investigators. Belyakov *et al.* [1998b; 2001] demonstrated that neither IN nor intragastric (IG) but IR immunization of primates with HIV-peptides was able to induce significant protection against rectal challenge of antigen-expressing viruses. Vajdy *et al.* [2001] showed that triple IN immunizations with HIV-gag-expressing replicons did not confer protection against vaginal challenge of gag-expressing VV, while IR or intravaginal (IVAG) immunizations generated almost full local protection. In human

studies, IN immunization was also found to be less effective than the local IR or IVAG

route in the induction of antibody responses [Crowley-Nowick *et al.*, 1997; Kozlowski *et*

al., 1997; Kozlowski *et al.*, 1999; Kozlowski *et al.*, 2002].

A few studies showed that local mucosal immunization induced less immune

response than did distant mucosal immunization. In comparison to IN or oral

immunization of mice with DNA or protein with CpG oligodeoxynucleotides as an

adjuvant for Th1 T-cell response, it was shown that IR immunization induced weaker

serum IgG and fecal IgA and no vaginal lumenal IgA (local IgG not shown) unless CT

was incorporated as a powerful mucosal adjuvant [McCluskie and Davis, 2000]. Other

investigators showed also that IR or IVAG routes were inferior to IN immunization in

inducing rectal and genital immune responses [Staats *et al.*, 1997; Johansson *et al.*, 1998].

These studies did not fully examine gene transfer nor cellular responses and protection,

the latter of which is the most important parameter to validate a vaccine strategy. As

suggested in the present study, the thresholds for antigen uptake and immune responses at

the rectal mucosa might be relatively high. If antigens are given at lower doses or antigen

delivery systems are inefficient, it might be difficult to induce colonic immune responses

and, thus, unable to address the question on whether rectal immunization is effective.

Moreover, in order to compare efficacy of different immunization strategies against

pathogens, the dose for challenge test should be at a significant high level such that

discrepancy in protection between experimental groups can become obvious.

When comparing local immune responses, it is more appropriate to perform comparisons before pathogen challenge because local lymphocytic infiltration or expansion after challenge is not associated with protection [McBride *et al.*, 1989]. Vajdy *et al.* [2001] found that IM and IN immunized mice which were unprotected from vaginal challenge with VV had demonstrated 5- to 10-fold higher frequencies of IFN-γ-secreting cells in the ILNs but not in spleen 5 days post-challenge than IR or IVAG vaccinated mice. This paradox occurred because the comparison was made after but not before challenge. In the present study, it was shown that viable VV particles were already eliminated from the mucosa within 5-6 days after challenge in AdOVA IR immunized mice (see Chapter 5). Vajdy *et al.* [2001] showed VV replication in both IM and IN immunized but not in IR or IVAG immunized mice. As a result, viral antigens should not be significantly produced after local mucosal immunization. Thus, higher numbers of IFN-γ-secreting cells in IM and IN immunized animals might reflect the presence of viral antigens as a consequence of uncontrolled ongoing VV replication. By contrast, IR and IVAG immunized mice efficiently controlled local immunity and, thus, massive lymphocytic expansion was not required.

The insufficiency of IN immunization in providing rectal protection might reflect compartmentalized immunologic networks that limit the movement of cells within the CMIS from the upper respiratory tract to the genital and rectal tract. The compartmentalization of CMIS might also exist within the GI system. From the results of the present study showing that at day 4 antigen-specific LP T cells were detected in the

colon and ILNs but barely found in the ileocecum or MLNs, it might be that local activated T cells did not enter the small intestine or its DLNs, at least during the early immunologic priming phase. Instead, the present study discovered a CMIS flow from rectum to genital tract, which was probably due to the lymphocytic priming in the ILNs shared by both rectal and genital mucosae. Previous studies demonstrated preferential migration of MLN blasts into small intestines [McDermott *et al.*, 1989a] and induction of both rectal and genital immunity by the targeted iliac lymph node (TILN) immunization [Klavinskis *et al.*, 1996; Lehner *et al.*, 1996]. It is probable that DLNs might link to their mucosal sites in each sub-compartmentalized network, playing a central role in the generation of mucosal immune responses in tissue "terminals". Thus, it might be appropriate to propose ILNs as an independent mucosal-associated lymphoid tissue, namely genito-rectal associated lymphoid tissue for both colonic and genital tissues [Lehner, 2003]. Since the small intestine and colon are not drained by the same LNs, they might belong to different grouped networks. As the interaction of MAdCAM-1 with lymphocyte α4β7 adhesion molecules particularly occurs in the gut but not in inflamed pulmonary or bronchial sites [Rose *et al.*, 1998; Butcher, 1999; Lefrancois *et al.*, 1999], there exits more mechanisms of cell homing selectivity. Each sub-compartmentalized network might govern cell migration with a special homing mechanism different from others [Brandtzaeg *et al.*, 1999b]. For the maintenance of effector memory cells in mucosal tissues, additional mechanisms were suggested to ensure immigrated cells to proliferate and avoid apoptosis in their preferential homing sites [Bode *et al.*, 1997]. Altogether, lymphocytes do not enter a distant mucosal site arbitrarily. Future studies are

required to identify specific CMIS homing mechanisms and improve our understanding in regard to the design of effective mucosal vaccines.

The genital immune responses induced by immunization through upper respiratory and oral routes as shown in previous studies [Gallichan *et al.*, 1993; Gallichan and Rosenthal, 1995; Gallichan and Rosenthal, 1996b; Kantele *et al.*, 1998] might be due to the accessibility of the cells to the airway and genital tract without the need of $\alpha4\beta7$ expression [Brandtzaeg, 1997]. The source of repopulating lymphocyte is not absolutely homogenous in regional lymphoid and tissue [Brandtzaeg *et al.*, 1999b]. There might be a certain (low) level of promiscuous cell accumulation to peripheral and mucosal tissues because various degrees of regional mucosal immune responses at a distant site are always seen regardless of immunization route [Brandtzaeg, 1997; Kozlowski *et al.*, 1997; McDermott and Bienenstock, 1979; Quiding-Jabrink *et al.*, 1997; Balmelli *et al.*, 1998; Kantele *et al.*, 1998]. This might be particularly the case for the antibody response because of antibody diffusion (transudation) throughout the body [Menge *et al.*, 1993, Czerkinsky *et al.*, 1995, Bergquist *et al.*, 1997, Ferko *et al.*, 1998, Kleanthous *et al.*, 1998, Kilhamn *et al.*, 2001]. Therefore, it is possible that similar levels of mucosal immune responses at one mucosal site might be induced through a distant mucosal and non-mucosal site [Kuklin *et al.*, 1997]. The present study demonstrated that IN immunization did not induce significantly better protection than SC immunization (see Chapter 5), thus suggesting that IN and SC primed lymphocytes enter rectal mucosa without a special homing mechanism different from each other. Currently, understanding

of this issue is still poor and a more comprehensive approach is needed to reveal the insight of CMIS and lymphocyte homing.

Rectal mucosa has been particularly investigated in previous studies using Adv for cytokine therapy because of the low levels of gene transduction in the local mucosal system after administration at systemic routes [Wirtz et al., 2002; Lindsay et al., 2003]. The present study found that lower levels of rectal immune responses were induced by immunization at a distant site (see Chapter 4). T-cell unresponsiveness was previously shown as a result from insufficient or appropriate activations and adequate delivery antigens induced T-cell response [Ohashi et al., 1991; Karrer et al., 1997]. It was observed by the present study that lower immunization doses (less than 2×10^9 PFU of AdOVA or AdgB) rarely induced immune responses or provided a suboptimal mucosal protection. Perhaps antigen given at a distant site results in an inadequate antigen load at the other site(s) including associated lymphoid tissues and leads to insufficient immune induction. Further, lymphocytes that emigrate to a different sub-compartmentalized network are possibly in inadequate numbers and might not be activated to reach effective levels [Bode et al., 1997]. It has been suggested that when antigens are present at low levels, B cell activation is more dependent on T-cell help [Zinkernagel and Hengartner, 2001]. Thus, the low levels of rectal IgA production and IFN-γ-secreting cells detected in the present study after IN or SC immunization might be owing inadequate local antigen load and also the insufficient help from low numbers of local activated T cells [Slifka and Ahmed, 1998].

In addition to antigen presentation and regional site-specific migration mechanisms, there might be another account to explain why protection is better induced by local vaccination. Newly activated T cells home into the site of immunization independent of antigen presence [Topham *et al.*, 2001b]. Such T-cell tropism was found to be associated with local expression of CD62E, an adhesion molecule recognizing CD62E ligand-1 expressed on activated T cells. After having departed from DLNs, activated cells migrate via the blood circulation specifically to the site of antigen injection at which CD62E is present on the endothelial cells of blood vessels [Reinhardt *et al.*, 2003]. By this mechanism, activated cells efficiently accumulate at the immunization site to a much greater extent than at distant sites where even antigen might be present.

Taken together, these data suggested that mucosal protection was best induced by local mucosal immunization and sub-optimally via a distant mucosal or non-mucosal site. This might involve the mechanisms of antigen loading, CMIS site-specific homing and antigen-independent lymphocyte tropism to the immunization site. Importantly, local mucosal immunization might minimize immune responses induced at a mucosal site that does not serve as a transmission route and, thus, can minimize any potential adverse effects [Fujihashi *et al.*, 2002].

6.10 Genital protection against virus infection

6.10.1 IR vaccination induced genital protection

Previous studies demonstrated that IN immunization with AdgB provided 80% protection against vaginal challenge with HSV-2 at a dose 2-fold greater than the LD_{100} (2×10^4 PFU), but markedly reduced at the does of 20-fold greater than the LD_{100} (2×10^5 PFU) [Gallichan and Rosenthal, 1998; Gallichan et al., 2001]. The present study showed that IR immunization provided full protection from vaginal challenge of HSV-2 at the dose of 20-fold greater than LD_{100} and remarkably reduced duration and degree of virus shedding (see Chapter 5), thus suggesting that IR immunization might be more potent. Induction of genital immunity through the rectal route has been highly expected by World Health Organization for the control of worldwide spreading HIV infection. So far, only a few investigators have embarked on this task but yielded variable results. Neither DNA vectors nor CpG-based vaccine used at the rectal mucosa of mice showed effective in generating vaginal immune responses [McCluskie et al., 1999; McCluskie and Davis, 2000]. IR immunization of guinea pigs with VV expressing HSV glycoprotein D reduced virus shedding following HSV-2 vaginal challenge [Bernstein, 2000]. Multiple (3 times) immunizations of mice·with Sindbis virus-based replicon induced vaginal protection against recombinant VV at a lower dose (1×10^7 PFU) [Vajdy et al., 2001]. Thus, the present study might shed new light on the vaccination against genital STD infection.

Since the genital tract and the colon are shared by the same ILNs, it was suggested that lymphocytes primed in the DLNs eventually homed to both mucosal sites [Lehner, 2003]. By transferring DLN cells from vaginally immunized mice with attenuated HSV-2, McDermott *et al.* [1989] demonstrated that DLNs cells provided protection against HSV-2 infection and revealed that DLN CTLs were important in the migration to vaginal tissues and to exert protective activities. Immunization with horse ferritin plus aluminum hydroxide as an adjuvant at the subserous and presacral spaces of the pelvis, from which lymph drains mainly to the ILNs, also induced anti-ferritin IgA and IgG antibodies in the vaginal fluid [Thapar *et al.*, 1990a]. Lehner and his colleagues [1996] were subsequently inspired to specifically aim their study towards immunization at the ILNs, the so called targeted iliac lymph node (TILN), with a bolus injection in the proximity of ILNs. TILN immunization of macaques with immunogenic gag antigen of SIV induced higher CTL responses in the ILNs than did rectal or vaginal immunization, and CTLs were detected also in the LP of both sites [Klavinskis *et al.*, 1996]. More significant protection was seen in animals boosted by TILN than by either rectal or genital route following oral priming [Klavinskis *et al.*, 1996]. Using the TILN technique, Lehner *et al.* [1998] also confirmed that B cells, CD4$^+$ and CD8$^+$ T cells from the rectum, colon and vaginal cervix mucosa preferentially migrated from ILNs but not from other LNs irrelevant to colonic or genital mucosa. This indicated that generation of ILN response was the key to provide effective immune responses in either distal mucosa. From this point of view, protection against vaginal challenge of HSV-2, as demonstrated in the present study, might be due to the induction of an efficient ILN immune response

209

(see Chapter 4). This might also explain similar results found in other studies showing that genital protection against VV challenge was achieved after IR immunization with replicons [Vajdy *et al.*, 2001]. Failure to generate genital immune responses by IR immunization with DNA or protein [McCluskie *et al.*, 1999; McCluskie and Davis, 2000] might be a result of an inefficient immune response in the ILNs and might be due to insufficient gene transfer to the rectal mucosa using these vaccine approaches.

6.10.2 Inability of IVAG immunization to induce genital protection

Adv-mediated gene transfer to the genital tract was relatively low unless mice were pretreated by Depo-Provera®. Mice were not protected without Depo-Provera® treatment probably due to the low levels of antigen load in the genital immune system. However, IVAG immunization after Depo-Provera® pretreatment did not confer protection either. Similar observations were shown by Kaushic *et al.* [2003], who demonstrated that C57BL/6 mice immunized IVAG with thymidine kinase (TK)-deficient HSV-2 (TK⁻ HSV-2) were more susceptible to genital HSV-2 infection if they were pretreated with Depo-Provera®. It was further suggested that Depo-Provera® might suppress immune responses due to a significant reduction in local IgA and IgG production [Kaushic *et al.*, 2003]. It was found in the present study that mice treated with Depo-Provera® prior to IR immunization did not succumb to rectal or genital HSV-2 infection (another Depo-Provera® treatment was applied before challenge, data not shown). The possible explanation might be that Depo-Provera® had a negative influence

on the genital immune system and offset genitally-induced (humoral) immune responses

IVAG immunization with other antigen delivery systems was found to be capable of inducing genital immune responses. Primary IVAG immunization with large doses of horse ferritin plus aluminum hydroxide for 5 consecutive days was able to induce anti-ferritin IgA and IgG responses in vaginal fluid [Thapar *et al.*, 1990b], but was less effective than those induced by pelvic immunization targeting the ILNs [Thapar *et al.*, 1990a]. Other studies showed that immune responses could be induced locally when mice were immunized IVAG with attenuated HSV-2 [McDermott *et al.*, 1984; McDermott *et al.*, 1990; Milligan and Bernstein, 1995a; Parr and Parr, 1998]. Gallichan *et al.* [1995] found that IVAG immunization with Adv induced lower antibody responses than did IN immunization. Similar findings were found for the lower levels of local IgA responses induced by IVAG immunization with protein+CT (triple immunizations) than IN immunization alone [Wu *et al.*, 2000b] or combined with an enteral route (oral) [Bergmeier *et al.*, 1995]. It was thus suspected by the present study that genital immune responses might be induced by IVAG immunization with Adv but probably not efficient enough to provide protection. Overall, vaginal tract might not be an effective inductive site for Adv-based vaccines to induce genital immunity.

The ineffectiveness of IVAG immunization with Adv and other means (protein-based) might not necessarily be generalized. Vaccine efficacy depends highly upon the route and antigen delivery system. Since anatomic differences exist, and most

importantly, the immunological constituents usually differ from one site to another, one formula might be effective at one site but not at other sites. McDermott *et al.* [1984; 1987; 1989; 1990] showed that attenuated TK^- HSV-2 applied in the vaginal tract of BALB/c mice (no Depo-Provera® pretreatment) induced protective immunity against vaginal challenge of wild type HSV-2. IgG was found to be the major contributory factor to the genital protection [McDermott *et al.*, 1990; Parr and Parr, 1997a; Parr *et al.*, 1998; Parr and Parr, 1998]. Antigen-specific $CD4^+$ T cells dominated in the DLNs and were the major source of IFN-γ production as compared to LN $CD8^+$ T cells [Milligan and Bernstein, 1995a]. On the basis of current observations on vaginal vaccination against HSV-2 infection, infectious viruses or bacteria were able to induce robust vaginal immune response, while non-replicating vectors or antigens must be combined with adjuvants to achieve high immune responses [Thapar *et al.*, 1990b; Kozlowski *et al.*, 1997]. Therefore, using attenuated HSV-2 as a vaccine might be a better choice for genital immunization.

6.11 Implications of the rectal immunization and challenge models

6.11.1 Rectal versus oral-gastric route

The oral-gastric tract has been the most common route for intestinal

administration. However, previous studies showed that oral-gastric immunization was not able to induce efficient rectal mucosa immunity [Belyakov *et al.*, 1998b; Kaneko *et al.*, 2000; Belyakov *et al.*, 2001; Sharpe *et al.*, 2002]. This might be due to several reasons other than the "local versus distant" theory. First, it was found that gene transfer to the colon was very efficient after IR but not intragastric (IG) administration. After IG administration of Adv, gene transfer was not focused at one location but dispersed along the entire length of GI tract and gene expression at each segment was substantially low (see Chapter 5). Second, higher percentage of regulatory γδ T cells are located in the small intestine than in the colon [Floochi, 1990]. The colon is exposed to vast numbers of bacteria but lacks the function of absorption of nutrition, whereas the small intestine, especially the duodenum and jejunum, contains much less flora but takes up large amounts of nutrients. Due to the distinct working environments, it is crucial for the small intestine to maintain a tolerant status and colon to be less "immuno-suppressed". Third, the liver is a major depot for the absorption of antigen derived from the small intestine through the portal vein. It is evident that the liver is also responsible for the induction of oral tolerance due to the presence of resident γδ T cells and DCs [Gorczynski *et al.*, 1996; Thomson *et al.*, 2002]. Injection of antigen directly into the portal vein can induce a systemic tolerance [Yang *et al.*, 1994a]. It was shown that gene expression in the liver was substantially low after IR administration of Adv [Wirtz *et al.*, 1999], thus suggesting that gene transfer to the liver is minimal through the rectal mucosa. Taken together, whereas the oral-gastric route might be ideal for tolerance induction, the rectal route might provide preferable conditions for vaccines against infection.

6.11.2 Vaccination against HSV-2

HSV-2 invades the nervous system and establishes latent infection, causing patients to suffer recurrent infection at mucosal sites. Recurrence is however controlled by the mucosal immunity. Thus, powerful vaccines are required to induce protective mucosal immunity against HSV invasion. Adv-based immunization might be used to achieve the goal of preventing HSV spreading and minimizing HSV recurrence.

The IR immunization model developed in the present study might be applicable for vaccination using attenuated HSV-2. It was demonstrated by the present study that removing mucus facilitated viral vector-based gene transfer and enhanced the effectiveness of IR immunization. It was shown also that removing mucus increased the susceptibility of mice to become infected by HSV-2. Since attenuated HSV-2 was shown to be immunogenic and safe [McDermott *et al.*, 1984; McDermott *et al.*, 1987; Gurwith *et al.*, 1989], IR immunization with attenuated HSV-2 would be an interesting approach for the induction of anti-HSV-2 immunity at both rectal and genital mucosae.

6.11.3 Vaccination against HIV infection

Current HIV vaccine strategies and antiviral therapies applied in clinical or pre-clinical studies are not effective. In an attempt of protein-based vaccine, envelope vaccines using recombinant HIV gp120 protein were found disappointing in a phase II

trials in the US and Thailand [Connor *et al.*, 1998]. Specific antibodies were not elevated significantly and were not able to neutralize viruses. The highly active antiretroviral therapy (HAART) was shown efficient in treat HIV viremia but not sufficient to eradicate live viruses from the $CD4^+$ T cells [Siliciano *et al.*, 2003].

Effective control of HIV infection depends highly on the induction of mucosal immunity [Czerkinsky *et al.*, 1999; Lehner *et al.*, 1999; Belyakov *et al.*, 2001], especially $CD8^+$ T cells which play a key role in anti-HIV immunity [Belyakov *et al.*, 1998a; Kaul *et al.*, 2000]. Adv administered mucosally induced $CD8^+$ T cell-mediated immune responses against viral infections as shown in the present study and in others [Witmer *et al.*, 1990; Hanke *et al.*, 1991; Gallichan *et al.*, 1993; Zheng *et al.*, 1993; Gallichan and Rosenthal, 1996b; Gallichan and Rosenthal, 1998]. In comparison with other vaccination schemes using modified vaccinia virus Ankara (MVA), DNA or proteins as vaccines, Adv used either alone or as a boost vector in primates has been shown to induce the most effective immune responses to challenge of SHIV [Shiver *et al.*, 2002]. Adv has now been considered as a good candidate for HIV vaccination [Letvin *et al.*, 2002; Robinson, 2002]. Targeting intestinal mucosa as an immunization site might be beneficial. Recent studies in primates and humans indicated that the GI tract was a reservoir harboring HIV viruses in addition to serving as a transmission route [Veazey *et al.*, 1998; Siliciano *et al.*, 2003]. Therefore, immunization at the rectal mucosa using Adv would be an effective vaccination strategy to induce mucosal immunity and eliminate HIV from the GI tract.

6.11.4 Vaccination for male individuals

The rectal immunization strategy might represent a solution to genital STDs that are a major concern in public health. The present study showed that immunization via the rectal route induced specific immune responses in the ILNs and conferred protective immunity in both rectal and genital mucosae. This suggested that separate immunization at each site might not be necessary. It also implies a potential vaccination strategy against viral infections at the GU tract for the male population who might not be practically vaccinated in the urinary tract. It was recently reported that condom use reduced HSV-2 transmission from men to women but not from women to men [Wald *et al.*, 2001], indicating the necessity of vaccination of men to acquire GU mucosal immunity. However, human studies indicated that rectal or genital mucosal immune responses (antibodies) were only induced by immunization at each site [Kozlowski *et al.*, 1997; Kozlowski *et al.*, 1999]. Further studies on large animals or humans might be required to confirm whether efficient immune responses in the male urinary mucosa can be generated.

6.11.5 Vaccination against cancer development

Viral infection has been found to be closely associated with the development of GI cancers. The present study demonstrated IR immunization with Adv elicited immune responses against both viral infection and tumor growth. The induction of such immune

216

responses is important to prevent the development of malignancies in the presence of STDs [Schwartz *et al.*, 1991] which include highly aggressive mucosal melanomas that occur at upper aerodigestive tract, anorectum, and male and female genital tract [Scholefield *et al.*, 1989; Batsakis and Suarez, 2000]. Mucosal melanomas are more refractory than skin melanomas to therapeutics because of anatomic inaccessibility by conventional intervention [Batsakis and Suarez, 2000]. Thus, Adv-based immunotherapy at the rectal mucosa might be sufficient and advantageous in preventing cancer development in the rectal and genital tracts.

6.11.6 The rectal challenge model

The rectal challenge model of HSV-2 requires mucus removal from the mucosal surfaces, thereby mimicking the clinical course of STD acquisition as a result from sexual practices. Thus, this new experimental model might be of informative value for the development of mucosal vaccines against HSV-2 and other STDs.

6.12 Concluding remarks

IR delivery of Adv efficiently introduced encoded heterologous genes to the colon after mucolytic treatment. The transgene was not only produced in the epithelium but also

immediately beneath the epithelium. The gene product was highly expressed for 3 days and remained for approximately one week after administration. IR immunization of antigen-encoded Adv induced potent antigen-specific mucosal immune responses, including $CD8^+$ T cell-mediated IFN-γ secretion and cytotoxic activities, Th1 T-cell responses and IgA production. As compared to distant mucosal and non-mucosal systemic routes, local mucosal immunization induced stronger rectal immune responses and better protective immunity against rectal challenge of pathogenic viruses. In addition, this rectal immunization regimen also conferred better genital protection against virus challenge.

In conclusion, immunization via the rectal mucosa with Adv stimulated the mucosal immune system to create a immunological barrier at both rectal and genital mucosae. This provided a new avenue for the design of mucosal vaccines against STD infection.

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