GENERATION AND CHARACTERIZATION OF THE CELLULAR IMMUNE RESPONSE TO A *CLOSTRIDIUM PERFRINGENS* ANTI-SIV MUCOSAL VACCINE

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Most new human immunodeficiency virus (HIV) infections are acquired through vaginal or rectal mucosa, and gut mucosal tissue is a primary target of HIV infection. To generate mucosal immunity against HIV or its simian counterpart simian immunodeficiency virus (SIV), the Gram positive bacterium *Clostridium perfringens* was used to develop a vaccine that delivers SIV p27 to the gut and induces local T cell immunity.

Under *in vitro* conditions, *Clostridium perfringens* expressing SIV p27 (*Cp*-p27) was found to induce dendrite cell (DC) maturation and stimulate p27-specific T cell responses. To improve intracellular delivery of p27 to DCs and thereby enhance immune priming, *Cp*-p27 variants expressing p27 conjugated with protein transduction domains (PTDs) at the 5' end were constructed. While internalization of p27 by DCs and gut epithelial cells was improved following exposure to the PTD-*Cp*-p27 variants, cellular p27-specific immune stimulation was not significantly improved compared with wild-type *Cp*-p27.

The *Cp*-p27 vaccine was then tested *in vivo* in mice for its ability to prime gut mucosal T cell responses. First, an adjuvant optimization study with three mucosal adjuvants, cholera toxin (CT), mutant *E. coli* heat-labile enterotoxin (LT(R192G)), and unmethylated cytosine-phosphate-guanine oligodinucleotides (CpG ODNs) was performed to determine the best T cell immune response in the gut. While the combination of CpG ODNs and (LT(R192G)) induced the highest

T cell immune response, (LT(R192G)) alone provided the best multifunctional $CD8^+$ T cell response in the gut.

Oral *Cp*-p27 vaccination was then tested for induction of T cell immunity *in vivo* in a prime-boost model by combining *Cp*-p27 with systemic immunization with an adenovirus expressing p27 (Ad-p27). *Cp*-p27 vaccination primed a strong multifunctional T cell immune response in gut lamina propria, although it could not stimulate a systemic immune response. In contrast, Ad-p27 vaccination stimulated strong systemic immunity but limited gut mucosal immunity. By sequentially delivering *Cp*-p27 and Ad-p27, immunity in both the gut and systemic tissues was achieved.

Altogether, this study demonstrates that *Cp*-p27 can deliver p27 to gut T cells through dendritic cells to prime a strong, multifunctional immune response in the gut effector tissue.

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Ad: adenovirus

Ad5: adenovirus serotype 5

ADP: adenoside diphosphate

AIDS: acquired immune deficiency syndrome

APC: antigen presenting cell

BGS: bovine growth serum

BMDC: bone marrow-derived dendritic cell

CAF: $CD8^+$ cell antiviral factor

cAMP: cyclic adenosine monophosphate

cfu: colony-forming unit

CPE: *Clostridium perfringens* enterotoxin

Cp-p27: *Clostridium perfringens* expressing SIV p27

CpG: cytosine-phosphate-guanine

CT: cholera toxin

CTB: cholera toxin B subunit

CTL: cytotoxic T lymphocyte

DC: dendritic cell

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DTE: dithioerythritol

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

env: envelope

FAE: follicle-associated epithelium

FBS: fetal bovine serum

FCS: fetal calf serum

FITC: fluorescein isothiocyanate

GALT: gut associated lymphoid tissue

gag: group-specific antigen

GC: germinal center

GI: gastrointestinal

GTP: guanosine triphosphate

HIV: human immunodeficiency virus

HPLC: high performance liquid chromatography

IFN-γ: interferon gamma

IFR: intrafollicular region

IL: interleukin

KIR: killer immunoglobulin-like receptor

LP: lamina propria

LPDC: lamina propria dendritic cell

LPS: lipopolysaccharide

LT: *E. coli* heat-labile enterotoxin

LTNP: long-term non-progressor

LT(R192G): arginine-192 to lysine mutant *E. coli* heat-labile enterotoxin

M cell: microfold cell

MHC: major histocompatibility complex

MIP-1β: macrophage inflammatory protein 1-beta

MLN: mesenteric lymph node

NEMO: nuclear factor kappa B essential modulator

NF-κB: nuclear factor kappa B

NK: natural killer

ODN: oligodinucleotide

PBS: phosphate buffered saline

PE: phycoerythrin

pfu: plaque-forming unit

PI3-kinase: phosphoinositide 3-kinase

plc: phospholipase C gene

pfoA: perfringolysin O gene

pol: polymerase

PP: Peyer's patch

PPDC: Peyer's patch dendritic cell

PTD: protein transduction domain

R-PE: R-phycoerythrin

RNA: ribonucleic acid

SED: sub-epithelial dome

sfc: spot-forming cell

SHIV: simian-human immunodeficiency virus

SIV: simian immunodeficincy virus

TLR: Toll-like receptor

TGF-β: transforming growth factor beta

TNF-α: tumor necrosis factor alpha

1.0 INTRODUCTION

1.1 INFECTION AND CONTROL OF HIV

1.1.1 Global HIV Infection

Since its description in 1981 [1-4], the acquired immune deficiency syndrome (AIDS) has been responsible for the death of 20 million people around the world [5]. In December 2007, 33.2 million people were estimated to be infected with the causative agent of AIDS, human immunodeficiency virus (HIV), with 2.5 million new infections of HIV estimated to have occurred in 2007 alone [5]. Highly active antiretroviral therapy drugs can extend the lives of many infected individuals. However, much of the HIV epidemic is due to infections in countries where access to health care and medicines is limited [5]. In some rural areas of Zimbabwe, life expectancy has been reduced by 19 to 22 years since the onset of the HIV epidemic [6].

The HIV epidemic has severe public health and economic consequences. In South Africa, an estimated $1342 is spent per person per year by the public sector for HIV antiretroviral therapy and health-care treatment, resulting in an economic burden of the equivalent of several billion US dollars each year [7]. Infection with HIV enhances susceptibility to other infectious diseases such as tuberculosis, Kaposi's sarcoma, cryptosporidiosis, and *Pneumocystis carinii* pneumonia, further enhancing the cost of health care per capita [8]. The morbidity and mortality associated with HIV infection also lowers the economic productivity of regions affected by HIV. This scenario is most severe in areas where

HIV is widely prevalent, which are usually areas where access to treatment is limited. The World Bank estimates that the annual national income in a country with an HIV infection prevalence of 10% would decrease by up to one-third [9]. In 2005, 8 of 26 sub-Saharan African countries reported prevalence rates of at least 10% [5].

Controlling the HIV virus through vaccination is thus a major goal of international organizations; however, it has proven to be an incredibly difficult goal to achieve. To aid in the development of such a vaccine and the construction of novel vaccine vectors, the monkey counterpart of the virus, simian immunodeficiency virus (SIV), is used as a model. Studies of SIV infection in susceptible animal species such as rhesus macaques have helped to guide human studies, drive vaccine development, and shed light on the tissues and immune responses important in HIV infection.

1.1.2 HIV Life Cycle

HIV infection of a susceptible cell begins with binding of the virus's envelope protein gp120 to CD4, a surface protein predominantly expressed on immune cells such as macrophages and the $CD4^+$ subset of T cells [10, 11]. This initial binding to CD4 is followed by a conformational change of gp120 and its associated transmembrane glycoprotein gp41, enabling binding of the envelope protein to a co-receptor protein [12-14]. The two major co-receptors for gp120 are CCR5 and CXCR4 [15-17]. Following entry of the viral core through fusion of the viral membrane with the host cell membrane, the HIV RNA genome is reverse transcribed into DNA through the action of the reverse transcriptase enzyme carried in the virion [18]. This DNA is then integrated into the host cell genome [19, 20]. New viruses can be produced from infected cells by induction of the HIV long terminal repeat promoter, which drives transcription of the HIV genes and genome, resulting in production of HIV proteins and new full-length

copies of the HIV RNA genome [21, 22]. Generally, the structural polyproteins gag-pol and env accumulate at the host cell plasma membrane and interact with the viral genetic information to package the genome into virions [23, 24]. Complete virions then bud from the plasma membrane [25, 26]. Final maturation of the virion occurs after budding from the cell and involves cleavage of the remaining gag polyprotein into a proline-rich protein (p6), a nucleic acid binding protein (p7), matrix (p17), and capsid (HIV p24, SIV p27) [23].

1.1.3 Natural History of HIV Infection

The natural history of HIV disease can be divided into three major phases: i) acute infection (3 to 6 weeks in humans); ii) chronic, asymptomatic infection (1 to 10 years); and iii) symptomatic infection and the onset of AIDS (12 to 18 months). Immediately following infection of cells with the virus, a rapid decline in $CD4^+$ T cells is observed in multiple tissues as infected cells are eliminated from the host by a number of mechanisms including super-antigen-induced death and direct effects of the virus on the cell membrane and genetic material [27-29]. HIV or SIV DNA/RNA becomes detectable in the blood within the first month after viral exposure, and viremia peaks around 4 to 8 weeks [30-34]. Individuals are often unaware of their infection status at this stage since the typical manifestations of acute HIV infection include only mild symptoms such as fever, rash, and swollen lymph nodes. However, the results of this initial phase of infection likely determine the remainder of HIV/SIV disease progression [35]. It is during the acute stage that viral reservoirs are established, ensuring chronic infection of the host [35, 36].

Viremia in the acute phase is limited by the action of viral-specific $CD8^+$ T cells which control viral replication through cytolytic and non-cytolytic actions [37-49]. However, by this time the infecting strain(s) of virus has completed several replication cycles and accrued

mutations that allow some viruses to escape immune control and persist. The controlled but not ablated infection establishes a viral load setpoint that is maintained throughout the chronic phase of infection with only a gradual increase. Virus replication is limited by the continued action of $CD8^+$ T cells as well as HIV-specific neutralizing antibodies that arise around the time of chronic infection establishment [50-52]. The severity of the setpoint viral burden is inversely associated with development of disease and the duration of the chronic phase [53-56].

The $CD4^+$ T cell level in the blood progressively declines during the chronic phase. It is the loss of $CD4^+$ T cells that ultimately drives the collapse of the host's immune system, for they are required for the formation and maintenance of $CD8^+$ T cell and antibody immune responses [57, 58]. When $CD4^+$ T cell levels fall below a critical level (approximately 200 cells/mL blood), the host loses the ability to control HIV viral replication and AIDS disease is imminent. Since $CD4^+$ T cells are necessary for formation of immunity against new threats to the body, as $CD4^+$ T cell levels decline the ability to fight other infections is also weakened. Therefore, AIDS patients are susceptible to and often succumb to opportunistic infections. Because HIV infection immediately diminishes the capacity for immune response formation, a vaccine that provides immunity before HIV exposure and infection is desirable.

1.2 HIV AND THE MUCOSA

Much of the research on immunity regarding HIV and SIV has been examined by analyzing the systemic immune response in lymphatics, organs such as the spleen and liver, and the easily-monitored blood. However, it appears that immunodeficiency virus infection is primarily a disease of mucosal immune tissue [59, 60]. The mucosal immune system protects

the tissues lining environment-exposed body cavities, including nasal, bronchial, gastrointestinal, rectal, and urogenital tracts. The major routes of HIV transmission are rectal and urogenital tissue (i.e. vaginal, cervical, and potentially foreskin tissues) [61]. Mucosal tissue is well-suited for infection by HIV because it contains a large population of activated $CD4^+$ T cells that express high levels of CCR5, the major co-receptor for viral entry [62, 63]. Infection and replication of virus has been observed in both urogenital and intestinal mucosa in humans and macaques [64-67]. Thus, to control the virus infection and replication, protective immune responses must be induced at mucosal sites.

The intestine is a primary target for HIV and SIV infection. The reason for this is not fully defined, but likely the nature of gut resident cells and the ability for virus or virally-infected cells to enter the gut mucosa contribute to infection of gut tissue. Intestinal gut lamina propria tissue contains a majority of the body's $CD4^+$ T cells, and about 70% of these express CCR5 [68-70]. Arthos *et al*. recently demonstrated that lymphocytes, including natural killer (NK) cells and $CD4^+$ and $CD8^+$ T cells, can bind to HIV gp120 via the $\alpha_4\beta_7$ gut homing molecule, suggesting a method by which virus is preferentially delivered to the gut following transmission [71]. Since envelope proteins remain associated with the host cell membrane following fusion [72], infected cells as well as free virus may bind to $\alpha_4\beta_7$ and thus be transported to the gut.

In both monkeys and humans, SIV or HIV infection leads to a profound loss of lymphoid tissue in the gut, primarily through the depletion of $CD4^+$ T cells via direct effects of the virus on infected cells and activation-induced cell death of bystander cells [29, 36, 37, 73]. Lymphocyte repopulation of the gut is decidedly absent throughout infection [29, 74-77]. The long-term effects of such a disruption to the major mucosal surface of the body include malabsorption of nutrients, increased gut inflammation, weight loss, diarrhea, increased permeability of the

epithelium, and enhanced susceptibility to enteric pathogens [73]. In addition, infected cells that are not eliminated are maintained as viral reservoirs throughout infection [39, 78, 79].

Destruction of gut $CD4^+$ T cells occurs within days of infection, before an adaptive immune response can form [36]. HIV patients known as long-term non-progressors (LTNPs) who exhibit low levels of HIV RNA and sustain healthy levels of $CD4^+$ T cells in the blood also maintain gut $CD4^+$ T cells and show low levels of HIV replication in the gut [80]. These observations suggest that $CD4^+$ T cell depletion is due to the direct effects of replicating virus on cells, killing them either through lysis or bystander effects. If $CD8^+$ T cells able to selectively destroy infected $CD4^+$ T cells through cytolytic and non-cytolytic mechanisms were to exist in the gut mucosa, ablation of this uncontrolled gut viral replication may be achieved. Therefore, because of its significance in HIV and SIV infection, the formation of such an immune response in the intestine should be a major function of an HIV vaccine.

1.3 PROTECTIVE IMMUNITY AND CORRELATES OF PROTECTION AGAINST HIV/SIV

Knowing the characteristics of an immune response that is effective against a given pathogen creates a framework for rational vaccine design against the pathogen and guides the evaluation of vaccine trials during vaccine development. Despite years of research and many advances in the field, the type of immunity required for protection against and/or control HIV or SIV infection has not been fully defined. Undoubtedly the correlates of immune protection are multiple and complex. A majority of the current understanding about the type of immunity that is most effective against HIV or SIV has come from LTNP HIV patients, individual animals who

control SIV infection, and animals or humans who have been exposed to virus but remain uninfected.

Both antibody-producing B cells and antiviral $CD8^+$ T cells have been implemented in control of HIV or SIV. While these two types of immune responses are not mutually exclusive, there is often a tendency to form one or the other type. The direction in which the immune response is swayed depends upon the cytokine environment in which it is formed. A Th1-type environment, dominated by interferon gamma (IFN-γ) and interleukin (IL)-12, promotes the generation of mature $CD8^+$ T cell responses. A Th2-type environment, dominated by IL-4, promotes B cell response maturation. Much of the cytokine production driving Th1- or Th-2 responses occurs in activated $CD4^+$ T cells, although other cells also play vital roles.

1.3.1 Neutralizing Antibodies: Immune Correlate of Protection from Infection

B cells are referred to as plasma cells when they are activated to produce antigen-specific antibody. Antibodies are able to bind to free virus to prevent viral attachment and infection of target cells. Virus opsonized by antibody can be internalized by phagocytes and destroyed intracellularly. Unfortunately, such internalization of virus has also been implemented in viral spread to new target cells [81, 82]. Opsonization can also target virus for direct lysis through the complement cascade, which has also been correlated with viral dissemination [82, 83]. The most effective antibody function against HIV is neutralization of free virus. Antibodies that bind to virus and block the interaction of gp120 with the CD4 receptor or a coreceptor are able to prevent infection of host cells [52]. Antibodies against gp41 can also be neutralizing by inhibiting fusion of the viral and host membranes [84]. Since these actions prevent entry of virus into cells, effective neutralizing antibodies form the immune correlate of protection from SIV or

HIV *infection*. Neutralizing antibodies have been found in LTNP but have proven to be extraordinarily difficult to induce in humans or animals [85-88].

The specificity and quality of antibody responses against HIV and SIV change throughout the course of infection. During the first 6 to 8 months after infection, both SIV infected macaques and HIV infected patients demonstrate a gradual maturation of the antibody response that includes changes in the antibody titer, antibody avidity, and dependence upon the natural conformation of envelope glycoprotein for antibody recognition of envelope [89]. Regardless of whether individuals show control of viral infection or rapid progression to AIDS, antibodies change during this maturation phase from being of low titer to being of high titer, having low avidity to having high avidity, and being dependent upon natural envelope conformation to possessing conformational independence [89-91]. When mature, these qualities of antibody responses are maintained throughout infection, and mature antibody responses appear to help control SIV and HIV viral titers during chronic infection [92-98].

The extent to which antibodies can continue to control virus and thus promote long-term nonprogression to AIDS is dependent upon the specificity of antibody to the viral envelope glycoprotein. Structural properties of HIV envelope glycoproteins ensure that a large portion of gp120 and gp41 can evade antibody responses. Antibodies that are specific to the many variable loops, buried residues, and glycan-shielded regions of the envelope proteins are much less effective at neutralizing than are antibodies specific for the few vulnerable regions that tend to be conserved among diverse viral isolates [99-102]. Envelope proteins that cannot successfully bind and fuse with CD4 serve as decoy antigens against which antibodies are formed [103, 104]. These antibodies can bind to the non-functional protein but fail to prevent viral entry into cells. Indeed, one of the requirements of an effective neutralizing antibody is its ability to recognize

the functional trimeric form of envelope protein [105, 106]. Despite these challenges to the immune system, when an effective neutralizing antibody response is formed, antibodies alone can prevent infection of cells and thus block HIV infection at the site of transmission [107-109].

1.3.2 Cytotoxic T Lymphocytes: Immune Correlate of Protection from Disease

SIV- or HIV-specific cytotoxic T lymphocytes (CTLs) recognize cells displaying viral antigen on MHC class I, which generally denotes viral infection of that cell. Upon recognition, CTLs induce apoptosis of the infected cell by one of two mechanisms. First, signal transduction initiated by the binding of FasL on CTLs to Fas on target cells engages the caspase cascade, leading to apoptosis [110, 111]. Second, and most commonly, CTLs degranulate, releasing perforin and granzyme proteins from intracellular lytic granules into the space between the CTL and its target [112]. Monomeric perforin inserts into the target cell plasma membrane and can polymerize to form pores through which granzymes can enter. Granzymes are serine proteases which can cleave cellular proteins to initiate the caspase cascade. Most CTLs are $CD8^+$ T cells, although $CD4^+$ CTLs have also been described [111, 113].

The $CD8^+$ CTL response to HIV or SIV appears to play a major role in controlling viral infection. Since CTLs are effective only after virus has infected cells, this cellular response is the immune correlate of protection from *disease*. However, recent evidence suggests that CTLs directed against capsid protein could feasibly also serve to prevent productive infection of $CD4^+$ T cells. This was demonstrated by Sacha *et al.* using an *in vitro* infection system. Gag-specific CTLs were able to detect and eliminate SIV-infected cells within 2 hours after infection, before viral integration and *de novo* viral protein expression [114]. While the full implications of this remain to be defined, a large body of other research makes it clear that CTLs directed against HIV/SIV are important in controlling infection.

Evidence for a CTL correlation of protection from disease comes from both monkeys and humans. Primary SIV infection is controlled by CTLs in rhesus macaques [43], and depletion of $CD8^+$ T cells eliminates the ability of these monkeys to control viral load [44, 45]. Macaques with strong vaccine-induced CTL responses can control viral load and maintain $CD4^+$ T cells following challenge with SIV [47]. Resistance to HIV infection has been correlated with enhanced levels of HIV-specific $CD8^+$ T cells in mucosal tissue of exposed, seronegative individuals [46]. Protection from establishment of a productive infection has been also been correlated with local mucosal CTLs [48, 49]. Clearly, CTLs are important in systemic and mucosal immune responses to HIV/SIV.

A mucosal CTL response may be effective at limiting early infection and thus preventing a sustained infection [36]. In the first 3 to 4 days after exposure to SIV, the number of virally infected cells in vaginal and rectal mucosa is low [115-118]. This is the first site at which CTLs may be able to prevent the establishment of infection. If not checked and given a large enough and sufficiently concentrated target population, this small level of infection can seed a larger infection in draining lymph nodes and other lymphoid tissue, including the gut [115, 116, 118]. An effective CTL immune response in such lymphoid tissue may be able to effectively limit the infection [119]. Such an effect has been observed in monkey models of infection. Monkeys who demonstrated the presence of antigen-specific $CD8^+$ T cells in the colon displayed lower levels of virus in the blood [120]. A separate study observed a delay in detectable serum SIV when immunization of monkeys generated colonic high avidity SIV-specific $CD8^+$ T cells, which was interpreted to mean that the mucosal SIV-specific CTL response controlled dissemination and establishment of viral infection [121]. When vaccine-induced anti-SIV CTLs were present in the

small intestine of monkeys, establishment of a productive mucosally transmitted infection was abrogated or was significantly less severe [79, 122].

1.3.3 Role of Multifunctional T cells in Immune Control of HIV Infection

The quantitation of T cell responses is usually evaluated using an IFN-γ ELISpot assay, with the assumption that IFN-γ production signifies an effective T cell capable of having positive effects on HIV control [123]. Indeed, IFN-γ has been shown to interfere with viral replication of other viruses, promotes an antiviral environment in the infected cell, and promotes Th-1 type immune response formation [124-127]. However, the correlation between IFN-γ production and health of HIV-infected individuals is weak and in many cases inverse, and IFN-γ levels in vaccinated individuals do not correlate with protection [128-130]. The production of IFN-γ is only one of several features of activated, effective antigen-specific anti-viral T cells. IL-2 and tumor necrosis factor alpha (TNF-α) production are also key determinants of T cell survival and anti-viral effectiveness, respectively. IL-2 drives T cell proliferation and differentiation [131, 132]. TNF-α enhances the production of IFN-γ, supports Th1-type response formation, and can trigger apoptosis of virally infected cells through death domain signaling [133, 134]. As mentioned above, the cytotoxic capacity of $CD8^+$ T cells is imperative in HIV virus control. Cytotoxicity can be assayed by the detection of CD107a and b on the surface of T cells after they have released the granzyme and perforin molecules from their lytic granules [130, 135]. Growing evidence supports the concept that evaluation of the multifunctionality of T cells more accurately reflects effective anti-viral immunity in HIV or SIV infection than IFN-γ production alone. In other words, cells that display more than one of the "functions" of IFN-γ production, TNF-α production, IL-2 production, and degranulation via CD107a/b surface expression are

more often detectable in individuals with low viral loads and healthy levels of $CD4^+$ T cells [40, 42, 136-141].

The reasons for the superiority of multifunctional $CD8^+$ T cells in HIV infection are being delineated. Investigation of the functionality of human $CD8^+$ T cells at various stages of infection with viruses that persist for varying lengths of time indicate that the initial $CD8^+$ T cell response to viral infection is dominated by IFN-γ production, with most $CD8^+$ T cells exclusively producing IFN-γ but not IL-2 [132, 142]. These cells are unable to proliferate in the absence of antigen-specific $CD4^+$ T cells [138, 143]. The same type of response persists in infections with uncontrolled viral replication [142]. However, chronic infection in which virus is maintained at a low level is associated with $CD8^+$ T cells that produce both IL-2 and IFN-γ [142]. These dual-producing $CD8^+$ T cells show proliferative capacity after antigen-specific stimulation independent of $CD4^+$ T cells [131, 144-146]. $CD8^+$ cells producing IL-2 and IFN-γ simultaneously have been observed in HIV LTNPs and 30-40% of patients who maintain low HIV viral loads while receiving anti-retroviral therapy [40, 142]. In addition to IL-2 and IFN-γ co-production, the $CD8^+$ T cells from these patients also display populations of $CD8^+$ T cells with additional functions such as cytotoxicity and TNF-α production, which are at lower levels or absent in uncontrollers [40, 138]. Such a multifunctional response is thought to be desirable to achieve through vaccination.

The quality of response is important not only in $CD8^+$ T cells but also in $CD4^+$ T cells. In these cells, the co-production of IL-2 and IFN-γ is also correlated with control of viral infection, whereas IFN-γ-only producing cells are associated with high viral loads [137, 144, 147-149]. The importance of multi-function $CD4^+$ cells is highlighted by the fact that only when HIV-infected patients have detectable levels of IFN-γ^+IL-2^+ $CD4^+$ T cells do they also have IFN-γ^+IL-

2^+ $CD8^+$ T cells [142]. Like $CD8^+$ T cells, the capacity to secrete TNF-α in addition to IL-2 and IFN-γ has been associated with control of HIV infection [136]. Notwithstanding, the significance of vaccine-induced multifunctional $CD4^+$ T cells is less defined than for $CD8^+$ T cells. It is not clear whether vaccine-induced activation of $CD4^+$ T cells would aid in limiting infection or would aid in establishment of infection by supplying ideal targets (i.e. activated $CD4^+$ T cells) for HIV infection.

1.3.4 Other Functions of the Immune System Controlling HIV Infection

1.3.4.1 *Physical Barriers*

An important aspect of the immune system often neglected is the fact that many infections are fended off simply because infectious agents cannot overcome the body's anatomic barriers (skin, keratinous layers, sebum, cilia, mucus) and physiologic barriers (temperature, enzymes, pH). Letvin *et al*. observed that a cohort of mucosally exposed, uninfected rhesus macaques displayed no detectable systemic or mucosal anti-SIV immunity using the most sensitive standard techniques to assess cellular immunity [150]. Furthermore, the presence of local mucosal IgA did not correlate with protection. Despite their resistance to repeated mucosal infection, these animals were readily infected intravenously. These findings, along with others [151-153], suggest that factors other than the adaptive immune response provide resistance to primate lentivirus infection. Intact tissue that is impenetrable by virus at the site of exposure may be sufficient to prevent infection. How to maintain intact mucosal tissue in the face of human physical interactions that spread HIV and inherently disrupt the mucosa is the challenge of this approach to preventing HIV infection.

1.3.4.2 *Innate Immunity*

HIV and SIV can counteract the efforts of cellular and humoral adaptive immune responses by mutating. The error-prone nature of reverse transcriptase allows for inaccurate insertion of nucleotide base-pairs at a rate of 3 x 10^{-5} per cycle of replication, errors are maintained because reverse transcriptase lacks proof-reading ability, and recombination between different viral quasispecies is promoted by reverse transcriptase at a rate of over two recombination events per replication cycle [154-160]. Given the fact that HIV can produce on the order of 10^9 to 10^{10} new virions every day [161, 162], the mutation rate of virus in an infected individual is extremely high. These activities, as well as direct effects of HIV/SIV accessory proteins, contribute to immune escape from both humoral and CTL viral-specific immune responses [163-165]. However, other types of immune responses are not dependent upon antigen specificity and can provide immune control of HIV/SIV either before adaptive immunity forms or after virus has escaped established adaptive immune responses. Inflammatory responses that occur immediately after viral exposure encourage the action of cells comprising the innate immune system, including phagocytes and natural killer cells.

Innate immune responses have been suggested to inhibit HIV/SIV infection and disease upon initial exposure to virus, and yet they remain largely uncharacterized. Of particular interest to HIV/SIV infection is the natural killer (NK) cell. NK cells cytotoxically eliminate non-self cells similar to $CD8^+$ T cells and express activating and inhibitory killer immunoglobulin-like receptors (KIRs) on their surface, which recognize specific MHC class I molecules. Recognition of non-self cells occurs through activating KIRs, with ensuing signal transduction leading to the release of cytolytic granules. Inhibitory KIRs counteract this action by preventing the cytotoxic activity of NK cells and ensure that cytotoxicity is directed only against cells with abnormal expression of MHC class I molecules. However, many virally infected cells display

downregulation of MHC class I, and thus these cells become targets for NK cells. Mounting evidence suggests a role for NK cells in control of HIV viral load and disease progression. This includes the observation that certain HIV-control-associated genotypes encode Bw4-80I, the ligand of a particularly strong inhibitory KIR [166-170]. Lack of inhibitory KIR binding by downregulation of Bw4-80I upon HIV infection may escalate NK cell killing of infected cells. The activating KIR KIR3DS1 is also able to bind Bw4-80I [166, 170-172], possibly promoting NK cell activation against these cells in the absence of an effective inhibitory KIR signal. A broader understanding of the function and regulation of NK cell killing in the face of HIV infection may provide new avenues for HIV prophylaxis research.

In addition to cytolytic activity, $CD8^+$ cells exhibit non-cytolytic control of HIV replication. This phenomenon does not require MHC antigen presentation or even expression of HIV protein by target cells and is active against cells infected with diverse viral isolates [173-178]. Indeed, $CD8^+$ T cells demonstrating HIV suppressive activity but lacking T cell receptors specific for HIV have been identified [179, 180]. Non-cytolytic control occurs through the repression of HIV replication by inhibiting transcription from the long terminal repeat promoter through action at a location immediately downstream of the transcription initiation site [175, 177, 181]. The identity of this $CD8^+$ cell antiviral factor (CAF) remains unknown, but it is has been observed to exist in both a secreted form and a cell membrane-associated form. CAF activity is a correlate of protection from disease progression insomuch as CAF activity in untreated HIV-positive patients inversely correlates with viral load, and CAF activity in lymphoid tissue positively correlates with control of viral replication [182, 183]. Whether CAF activity can be primed through prophylactic intervention will also be a question that future

research should address. Some studies have suggested that this may be the case, but how this occurs has not been delineated [184-188].

1.4 HIV VACCINE DEVELOPMENT

Thus far, the understanding of CAF activity and innate immunity suggests that these types of responses cannot be achieved through traditional vaccination since antigen-specificity and lasting memory for these activities do not appear to be inducible. However, vaccines that generate antibody and CTL adaptive immune responses against HIV/SIV are both being pursued.

1.4.1 Challenges of a Neutralizing Antibody-Inducing Vaccine

Effective prevention of SIV infection via a vaccine-induced antibody response has been demonstrated in the rhesus macaque model [189-194]. Since antibodies can limit viral infection before SIV or HIV enters host cells, this sort of sterilizing immunity is a primary goal for HIV vaccine development [189, 195]. However, protective antibody responses have been difficult to achieve through vaccination, with killed or attenuated strains of SIV being the most consistently successful strategies [194].

There are many concerns about employing killed or attenuated HIV for vaccination purposes. The potential for recombination of the attenuated strain with circulating strains has limited the development of this approach [196, 197]. In addition, long-term studies of monkeys and patients infected with attenuated strains have displayed eventual progression to disease, possibly via mutations acquired through the natural error-prone replication of the viruses [194, 196, 198]. Using killed HIV as a vaccine strategy has also been met with both safety and technical concerns. Currently there exist no approved culture methods for generating HIV

virions for vaccine use. There are also technical challenges in the ability to achieve purified virus with intact envelope glycoprotein, and traditional chemical and heat-based methods of viral inactivation tend to denature the trimeric conformation of HIV envelope proteins that is imperative for successful protective antibody response formation [196]. Novel strategies for virus inactivation are helping to overcome this problem [192, 199, 200]. However, to date the killed SIV vaccinations that elicit protective immunity in animal models have required the use of strong adjuvants not appropriate for human use [190-193, 201].

Alternative vaccination strategies for producing the envelope trimer and inducing neutralizing antibodies include DNA vaccines and non-replicating virus-like particles [196, 202-205]. These approaches show much promise and are safe for use in humans but thus far cannot fully protect against viral infection in animal models [196]. Thus, while a vaccine-induced neutralizing antibody response would undoubtedly provide the ideal situation of sterilizing immunity against HIV, current technical and knowledge limitations have prevented the successful formation of this type of response. The potential for an antibody vaccine certainly exists, and as technology and further understanding of the structure of the HIV envelope protein advance a neutralizing antibody-inducing vaccine is likely to be developed.

1.4.2 Rationale for a Cellular Immunity-Inducing Vaccine

Since an effective $CD8^+$ CTL response can control HIV and SIV infection and possibly prevent productive infection, a vaccine that induces appropriate T cell responses against HIV/SIV may be adequate to control infection at both the individual and population levels. Based on experiments in the monkey model and the observation that control of HIV replication in acute infection occurs through CTL immune responses, a vaccine-induced T cell immune response should be able to control early infection and prevent the establishment of a productive,

persistent infection [36, 73, 206]. However, even if this sterilizing immunity does not result, a cellular response that can limit the infection, similar to the level of infection observed in LTNPs, may significantly alter the course of the HIV epidemic [206-212]. Presumably, the resultant lower viral load in a T cell-limited infection would not only provide longer, healthier lives for those who contracted HIV, but also less virus would be transmitted to new individuals [207, 213, 214].

1.4.3 Systemic Vaccine Development

The overwhelming majority of currently licensed vaccines against infectious diseases are delivered systemically, generally intramuscularly or subcutaneously. It thus comes as no surprise that most of the HIV vaccines in completed or current human trials are also systemic vaccines. Early HIV vaccines focused on eliciting antibody responses against the HIV envelope proteins included vaccination strategies such as recombinant whole protein administered with adjuvant and recombinant vaccinia virus expressing HIV protein. By 1993, new strategies began to be tested as novel vectors and antigens (e.g. gag, pol) showed more promise than conventional vaccination approaches. Vectors derived from pox viruses (i.e. canary pox, modified vaccinia Ankara), plasmid DNA, and non-replicative HIV virus-like particles were introduced into clinical trials, and cellular immunity in addition to humoral responses grew to be standard in vaccine evaluation. The possibility of utilizing two separate vaccines in a prime-boost regimen to create better immune responses than a single vaccine began to be explored in clinical trials in the late 1990s, with encouraging results. To date, over 60 clinical trials have been completed worldwide. As of January 2008, the AIDS Vaccine Advocacy Coalition reports 29 ongoing human trials, which include vaccines using pox-based vectors, adeno-associated virus, DNA, adenovirus, lipoprotein, protein, and peptide.

Adenovirus (Ad) has been employed by many researchers as a vaccine vector for efficient delivery of HIV or SIV proteins through intramuscular inoculation. The resultant systemic cellular and humoral immunity tends to be quite strong and multifunctional, even with only a single dose [215, 216]. This is due partly to the broad tropism of adenovirus, which is able to enter a number of cell types from a multitude of lineages [217]. Naturally, more than 50 serotypes of these DNA viruses cause generally mild disease in respiratory, gastrointestinal, urogenital, and ocular tissues. Of these serotypes, Ad serotypes 5 and 35 have been widely explored for their use as vaccine vectors. By deleting genes essential for Ad replication (e.g. E1 and/or E3) and replacing them with a vaccine antigen gene, Ad can be manipulated to carry vaccine antigen genes, which are expressed in the cells which Ad infects.

Many reports argue for the notion that pre-existing immunity to Ad lowers the effectiveness of Ad-vectored vaccination [218-222]. Preliminary results from a recent proof-of-concept clinical trial also suggest that pre-existing immunity may be a factor in Ad-vectored vaccine-inducible immunity [207]; however, the overarching result of this trial was that systemic immunization using the Ad-vectored vaccine did not afford protection from HIV or lower the viral setpoint after infection. Nevertheless, Ad remains one of the most well-defined vector strategies currently being used for HIV vaccine development, and future improvements may overcome the limitations of current Ad vectors. In addition, priming the immune system using a rare Ad serotype vector, a separate vector, or an alternative route of administration before boosting with a systemic Ad-based vaccine have been noted to aid in overcoming the limitation of pre-existing immunity that would otherwise compromise immune response formation stimulated by Ad-vectored vaccination [222-228].

1.4.4 Mucosal Vaccine Development

Mucosal vaccines have the potential advantages of being painless, easy to administer on a large scale and also generally less expensive to produce, store, and deliver than current systemic vaccine technologies [229]. In light of the fact that most HIV infections are transmitted through rectal and vaginal mucosal contact, it is imperative for a vaccine to provide mucosal immunity. Vaccination at a mucosal tissue stimulates local immunity in that tissue and usually also induces systemic immune responses detectable in the blood, spleen, and peripheral lymph nodes. This is in contrast to systemically delivered vaccines, which are generally incapable of or limited in the ability to stimulate an immune response in mucosal tissues. Thus, mucosal immunity is thought to best be induced by antigen delivery directly to the mucosa [229].

Some systemically delivered viral vectors appear to enable mucosal immune response formation against HIV or SIV, most notably in the gut, rectal, and genital mucosa [216, 230]. However, the strength of these responses is generally poor. For example, some level of mucosal immunity can be detected after systemic inoculation with Ad-vectored vaccines, but at levels approximately 10-times less than in systemic samples [216, 230]. Individuals inoculated intramuscularly with a pox-based vector have also demonstrated some mucosal immune responses [231, 232]. It is generally accepted that direct mucosal stimulation achieves more effective mucosal immunity [229], although again this is dependent upon the vaccine strategy utilized. Ad-vectored vaccines delivered mucosally have been explored; however, although Ad typically causes mucosal disease, mucosal immunity has not been detected following oral inoculation [227]. Alternative vectors that are more adept at antigen delivery to mucosal tissues are necessary for successful mucosal vaccination.

The development of a mucosal SIV or HIV vaccine has been pursued for many years. Vaccines targeted to the nasal, oral, rectal, and urogenital mucosa and to mucosal draining lymph nodes are under investigation [233, 234]. Antigen delivery has been tested using attenuated live virus, killed virus, recombinant virus, DNA, dendritic cells and peptides. Recently the potential for recombinant microorganisms to be used as vectors has been investigated. HIV vaccine vectors have been designed using organisms such as *Salmonella enterica,* the Bacillus of Calmette and Guerin, *Shigella flexneri*, and *Listeria monocytogenes* [235-238]. Many of these novel vaccine strategies have been shown to induce strong humoral or cellular immunity in mucosal compartments, including the gut, more effectively than systemically delivered vaccines.

Most mucosally administered HIV vaccines are in pre-clinical stages of development. In the vaccine pipeline are vaccines vectored by Venezuelan equine encephalitis, attenuated vesicular stomatitis, herpes simplex, and Sindbis viruses, which may be effective when administered mucosally [229, 239, 240]. One ongoing clinical trial (C86P1 through St. George's University of London, Richmond Pharmacology, and Novartis Vaccines) uses a protein-plus-adjuvant vaccine administered intranasally as a prime to an intramuscular boost. This mucosal-systemic prime-boost strategy is a popular strategy being characterized in pre-clinical trials utilizing a multitude of vaccine vectors [228, 241-246]. The only other mucosa-targeted vaccine to have reached clinical trials utilizes *Salmonella enterica* serovars *typhimurium* and *typhi* that include a Type-III secretion system to deliver gag proteins to the cytoplasm following invasion of gut macrophages upon oral ingestion. Phase I clinical trials have shown a single dose of the *S. typhimurium* vaccine to induce mild gastrointestinal symptoms and nearly no systemic immunity to HIV gag [247]. In the SIV model, monkeys primed with *S. typhimurium* and boosted with a systemic vaccine demonstrated SIV-specific $CD8^+$ T cells in the colon and blood, as well as

somewhat lower severity of infection than a systemic-only vaccinated animal following intrarectal SIV challenge [248].

Mucosal vaccines against any infectious disease have historically been difficult to generate. Of the over 55 licensed vaccines in the United States, only 7 are delivered mucosally, 6 through the oral route and one intranasally. By and large, the effectiveness of these mucosal vaccines is wholly dependent upon the production of humoral, not cellular, responses. This underscores the difficulty the scientific community has experienced in developing mucosally delivered vaccines that induce antigen-specific $CD8^+$ T cell responses. Ongoing studies of novel vaccine vectors may prove to overcome this challenge.

1.5 MUCOSAL IMMUNOLOGY

The major obstacle in mucosal vaccine development is the challenge of induction of immunity in the mucosa. Whereas the systemic immune system readily responds to most foreign molecules with responses that eliminate the foreign particle, the mucosal immune system is more selective in the molecules to which it produces responses that destroy foreign particles. The necessity of the mucosa to co-exist with environmentally acquired non-pathogenic molecules (i.e. food) and organisms (i.e. beneficial commensal bacteria) indicates that an intricate system exists to discern between safe and toxic antigens acquired at mucosal surfaces.

1.5.1 Anatomy of Mucosal Immune Tissue

The various immune inductive sites of the mucosal immune system show similarity in overall structure. A single layer of epithelial cells separates interstitial tissue from the external environment. As shown in Figure 1, the underlying tissue just beneath the barrier consists of

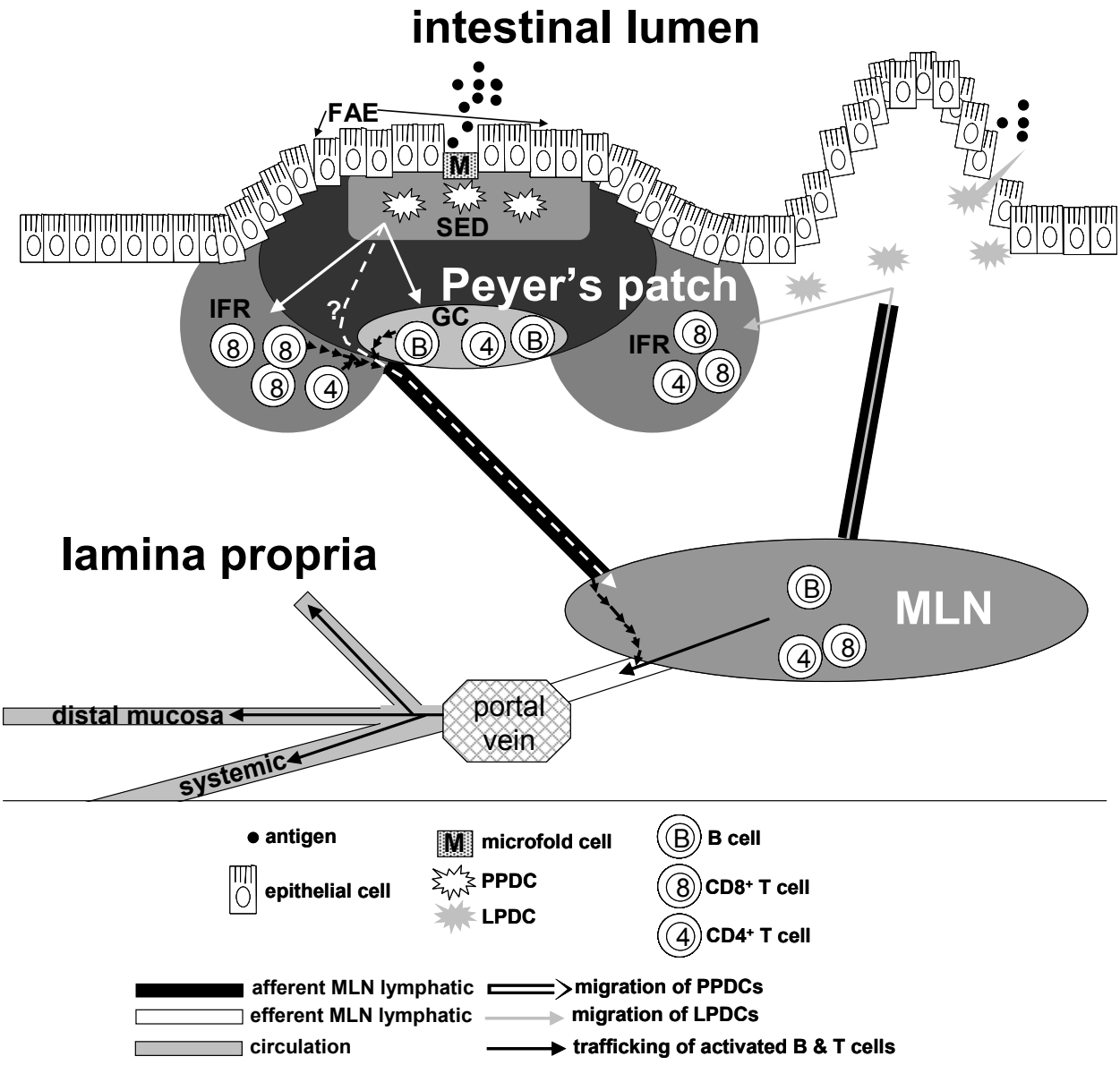


Figure 1. Gut associated lymphoid tissue (GALT) anatomy and immune priming
The primary inductive tissue structure of the GALT in the terminal ileum of the small intestine is the Peyer's patch (PP). Antigen is transcytosed by microfold (M) cells of the follicle associated epithelia (FAE) and is delivered to dendritic cells (DCs) in the sub-epithelial dome (SED). DCs can migrate to intrafollicular regions (IFRs) or germinal centers (GCs) to prime cells in the PP; some evidence also suggests that PPDCs can migrate via afferent lymphatics to prime cells in the mesenteric lymph node (MLN). DCs from the lamina propria also acquire antigen (see text for details) and can migrate to PPs or MLN to prime immunity. Primed B and T cells travel via lymphatics to enter circulation via the portal vein and are then delivered to the gut effector tissue (lamina propria), distal mucosal sites or systemic sites.

large numbers of antigen presenting cells (APCs), particularly dendritic cells (DCs). As mentioned above, generation of a protective immune response in one mucosal area is able to afford protection at other mucosal sites [249-251]. For example, an oral vaccine against typhoid has been shown to induce mucosal immune responses in saliva and vaginal secretions in human volunteers [250]. Mucosal tissue therefore does not rely on systemic immune responses to populate its effector sites with B and T cells. Interactions between the two immune systems do occur, however. This is believed to be mediated by lymph nodes of the mucosal immune system serving as crossover points and may also involve lymphocytes trafficking through the liver [252, 253].

The gut associated lymphoid tissue (GALT) comprises inductive immune tissue that collect antigen from the mucosal surface and lack afferent lymphatics. Peyer's patches (PPs), isolated lymphoid follicles, and the appendix make up the GALT, and it is in these tissues that most gut mucosal B and T cell immune responses are primed. PPs are the major GALT structure in the small intestine, where they are concentrated in the terminal ileum in humans.

1.5.2 Generation of Mucosal Immune Response in GALT

Generation of a protective immune response in the GALT is able to afford protection at other mucosal sites (see Figure 1). Microfold (M) cells in the follicle-associated epithelium (FAE) transport antigen from the lumen to the sub-epithelial dome (SED) where DCs serve as the major antigen presenting cell [254]. Peyer's patch DCs (PPDCs) have also been observed to acquire antigen from apoptotic epithelial cells [255]. Following antigen uptake, DCs mature and can migrate to present antigen to naïve B and T cells in the intrafollicular region (IFR) and B-cell rich germinal center (GC) areas of the PP. Activated B and T cells primed in the PP can be imprinted with the $\alpha_4\beta_7$ mucosal homing marker, and thus the cells localize to effector sites such

as the lamina propria. Also, DCs from the PP may travel to the mesenteric lymph node (MLN), the regional lymph node draining the gut. Intra- and subepithelial DCs in the small intestinal lamina propria can also acquire antigen from the lumen directly by extending dendrites across the FAE barrier, and then migrate to PPs or MLN to prime B and T cells [256]. T cells primed in the MLN can travel to local mucosal effector sites, e.g. lamina propria. In addition, efferent lymphatics may serve as conduits for activated T cells to travel from the gut to distal mucosal effector sites as well as the systemic immune system after entering the bloodstream through the thoracic duct. Lymphocytes from the gut can also drain via portal blood to the liver, where regulation of immunity may occur [253].

The normal reaction to oral antigen presented by gut DCs is tolerance via the generation of secretory IgA and either Th3 or T-regulatory cells. The major cytokines present during tolerance induction include IL-10 and transforming growth factor beta (TGF-β), and low levels of co-stimulation occur between DCs and $CD4^+$ T cells [252]. An immune response is generated only under inflammatory conditions, such as those generated by pathogenic organisms. Under these circumstances, completely mature DCs provide high levels of co-stimulation and produce IL-12 [257]. Both Th1 and Th2 responses can result. The natural tendency for oral antigen to produce tolerance complicates the formation of mucosal immune responses with vaccines.

1.5.3 Mucosal Adjuvants

Adjuvants are used in mucosal vaccines to overcome tolerance and direct the immune response towards either Th1 or Th2 immune responses. To date, no mucosal adjuvants have been licensed for use in prophylactic vaccines in the United States. The major mucosal adjuvants in various development stages fall into two categories, bacterial toxin-based and Toll-like receptor (TLR)-stimulating.

The heat-labile enterotoxin (LT) produced by certain species of enterotoxigenic *E. coli* has been known for years to be a potent mucosal adjuvant. The A subunit of LT is the catalytic subunit responsible for LT toxicity as well as immune enhancement. Dickinson and Clements created a mutant form of LT by substituting alanine at residue 192 of the A subunit with glycine [258]. The resultant LT(R192G) is capable of inducing both Th1 and Th2 responses and in SIV vaccines enhances SIV-specific CTL levels [259, 260]. Furthermore, the safety of LT(R192G) has been demonstrated in mice, non-human primates, and humans [259-261]. Incorporating LT(R192G) into an oral vaccine is expected to prevent tolerance and encourage a Th1 response.

Cholera toxin (CT) is a related bacterial toxin that serves as a mucosal adjuvant. It is produced by *Vibrio cholerae* and, like LT(R192G), is known to help overcome mucosal tolerance when administered orally with protein [262-264]. Although the CT B subunit (CTB) is not approved for use as an adjuvant, CTB is delivered with inactivated whole cell *V. cholera* in a safe and widely-used oral vaccine against cholera [265]. Safety mutation versions of CT and recombinant CTB have been created and tested for decreased toxicity and sustained immunogenicity with somewhat less success than LT(R192G) [266, 267]. Nevertheless, this potent mucosal adjuvant may be applicable for human use in the near future, and it serves as the classical oral adjuvant. Orally delivered CT is known to be transcytosed by M cells and is thought to promote maturation of DCs in the SED, driving their migration to T and B cell priming areas in PPs [266, 268]. Both CT and LT have also been shown to induce the rapid migration of murine DCs to the PP FAE and subepithelial connective tissue of villi where they are thus situated to acquire antigen from the lumen [266, 269].

A leading TLR-stimulating mucosal vaccine is a mimic of bacterial DNA. TLR9 helps the mucosa to differentiate between the presence of safe host DNA and pathogenic bacterial

DNA by recognizing differences in the methylation patterns of cytosine-phosphate-guanine (CpG) dinucleotides. Accurate identification of foreign DNA depends upon the fact that bacterial DNA contains far fewer methylated CpG motifs than host DNA and that host DNA is not normally found in endosomes, the location of TLR9 molecules [270, 271]. Three different classes of CpG oligodinucleotides (ODNs) have been described based on their different primary sequence motifs, secondary and tertiary structures, backbone, and stimulatory effects on B cells and DCs [272]. All three classes result in the induction of Th1-promoting cytokines by DCs and promote Th1 cellular responses [273]. In the gut, TLR9 expression has been observed in villus enterocytes and Paneth cells typically found in villus crypts, as well as PPDCs, which can mature in response to CpG ODNs [274, 275]. The administration of CpG ODNs with a systemic vaccine has been tested in human clinical trials [272, 276, 277]. In mice, CpG ODNs delivered with oral vaccines have helped stimulate Th1-type mucosal immune responses against a variety of antigens using numerous vector systems [278-283].

1.5.4 Encouraging Mucosal Immune Response Formation

Many factors influence the immunostimulatory nature of antigen that interacts with the GALT. Proteins tend to be poorly immunogenic, but the nature, dose, and frequency of their delivery can dramatically influence the propensity for immune activation to result following their ingestion. Particulate or denatured protein is less likely to induce tolerance than soluble or intact protein, probably because particulates and unfolded proteins are more readily engulfed by M cells and better delivered to DCs [229, 284-287]. Low levels of antigen administered repeatedly do not encourage immune responses, whereas low doses can prime immunity if not administered frequently [229]. A single high dose of antigen encourages tolerance, whereas medium to high doses of antigen administered repeatedly can result in immunity [229]. Of course, the

administration of adjuvant with the antigen also drives immune response formation and overcomes tolerance. In summary, mid- to high-level doses of particulate or denatured protein that can be delivered to DCs repeatedly are most likely to induce strong mucosal immunity.

Protein transduction domains (PTDs) are cationic peptides that enable the proteins on which they are located to efficiently enter target cells through a receptor-independent mechanism [288-290]. It is hypothesized that PTDs function by preferentially adhering to negatively charged molecules on the outer membrane of cell, thus enhancing the number of PTD-containing proteins in position for internalization. PTDs have been designed that specifically target proteins to certain cell types. The PTD peptide known as PTD-5 enhances protein uptake by many cell types, including epithelial cells and DCs [291]. Another PTD, 8K, is especially proficient at directing proteins into DCs (P.D. Robbins, personal communication). Incorporating either of these PTD peptides as a fusion to orally administered antigen should increase delivery of the antigen to PPDCs either directly or indirectly via enhanced uptake by M cells. In addition, PTD-fused antigen may be better internalized by the lamina propria epithelial cells or DCs that are important for immune response formation in the MLN.

Activation of $CD8^+$ CTL responses requires presentation of antigen on MHC class I molecules. DCs possess the unique ability to present exogenously acquired antigen, which normally is loaded onto MHC class II molecules, in the context of MHC class I. While the details of this cross-presentation are not fully understood, it is known that some phagocytosed antigen interacts with the proteasome and is thus directed for MHC class I loading [292-295]. In this way, increased internalization of protein by DCs through PTD-fusion can encourage presentation of epitopes on MHC class I. PTD sequences may also promote the delivery of protein directly to the cytoplasm of cells, which engages the MHC class I pathway, thus

enhancing DC cross-presentation and increasing induction of CTLs [296, 297]. Therefore, presentation of protein epitope of MHC class I via either pathway can be enhanced through the fusion of PTD to the protein.

1.6 *CLOSTRIDIUM* PERFRINGENS EXPRESSING SIV P27 AS A VACCINE VECTOR

1.6.1 Exploiting *Clostridium perfringens* as a Vaccine Vector

In addition to the microorganisms mentioned above, *Clostridium perfringens* has also been considered as a vehicle for antigen delivery to the GALT. *C. perfringens* is a Gram-positive spore-forming rod-shaped anaerobic bacterium [298]. In adult humans, ingested vegetative *C. perfringens* that survive the upper gastrointestinal (GI) tract conditions enter the small intestine where bile salts help to induce sporulation of the bacteria. Spores and cytoplasmic inclusion bodies are protected in the mother cell as sporulation ensues and the bacteria travel through the small intestine. The spore and inclusion bodies are released from the mother cell when *C. perfringens* cells lyse at the terminal ileum of the small intestine, which is where PPs are found in high frequency in humans. The ability for *C. perfringens* to naturally travel through the intestine, deliver protein to the vicinity of PPs, and exit the host without colonizing or causing infection makes it an attractive vector for further exploration.

Rare isolates of *C. perfringens* cause food poisoning and nonfoodborne GI disease because they carry *C. perfringens* enterotoxin (CPE) encoded by the *cpe* gene [299]. Knocking out the *cpe* gene renders such isolates non-pathogenic for GI disease and thus provides a bacterial vehicle safe for human consumption [300]. The *cpe* gene can be carried chromosomally or episomally and is under control of the unique *cpe* promoter. The *cpe*

promoter is activated only during sporulation [301, 302]. The strength of the *cpe* promoter is demonstrated in the fact that CPE production in the human intestine accounts for up to 15% of total bacterial protein, which accumulates in cytoplasmic inclusion bodies inside the mother cell. This ensures that the protein expressed from the *cpe* promoter remains untouched by proteases and bile salts of the intestinal lumen which would degrade extracellular or secreted protein. These properties make the *cpe* promoter and its natural regulation in *C. perfringens* an exquisite tool for expressing large amounts of protein that can be delivered intact in particulate form to the site of many PPs in the small intestine terminal ileum. Both the large dose and particulate nature of protein delivered in this manner are believed to be associated with the induction of an antigen-specific mucosal immune response and resistance to mucosal tolerance.

Oral ingestion of *C. perfringens* not carrying the *cpe* gene may be considered safe for humans [300]. Nevertheless, *C. perfringens* encodes several other toxins that may damage the host if orally ingested bacteria surpass the mucosal barrier and enter the underlying tissue. For example, gas gangrene can result if type A *C. perfringens* carrying the perfringolysin O (*pfoA*) and phospholipase C (*plc*) genes enters an open wound [303]. The actions of these two exotoxins collectively lead to the creation of pores in cell membranes and necrotization of tissue. Phospholipase C, also known as α-toxin, has never been shown to cause gangrene following oral delivery of type A *C. perfringens*, and inoculation of rabbit ileal loops with the toxin does not result in cytopathic effects [300]. Perfringolysin O, also known as θ-toxin, is also unlikely to cause disease when acquired orally. Of the hundreds of thousands of cases of *C. perfringens* type A-related gastrointestinal disease, none have included gangrene. The only possible examples of α- and/or θ-toxin-induced disease resulting from gut *C. perfringens* infecting extraintestinal tissue might occur following surgery that involves opening of the intestine or via

metastasized cancerous cells. While literature indicates that there is a very low potential for orally acquired type A *C. perfringens* to exert harmful effects through the actions of α- and/or θ-toxin, to ensure the safety of *C. perfringens* for its use as an oral vaccine vector, *C. perfringens* strains with inactivation of both of these toxins have been created [304, 305]. The triple toxin-depleted *C. perfringens* is now being developed as a vaccine vector against HIV and SIV.

In addition to the natural ability to deliver intact protein to the locale of GALT, *C. perfringens* has the benefits of being an inexpensive vaccine vector that can be delivered without the requirement of needles. Both of these qualities are important to possess when a vaccine is most needed in resource-poor areas; indeed, the bulk of the burden of HIV is in countries defined as low- or middle-income [5]. In summary, the use of *C. perfringens* as an inexpensive, non-toxic vaccine vector is appealing because of the inherent ability of *C. perfringens* to express viral protein from *cpe* promoter in a sporulation-regulated manner that allows for production of a large quantity of protein which is naturally delivered intact to a region of concentrated inductive GALT. Non-toxic, *cpe*-negative *C. perfringens* has been reengineered to produce HIV and SIV proteins under control of the *cpe* promoter for use in development of vaccines against HIV and SIV [306].

1.6.2 Initial Characterization of *C. perfringens* Expressing SIV p27 as a Vaccine Delivery System

Generation of *C. perfringens* expressing SIV p27 (*Cp*-p27) has been achieved using the pJRC200 plasmid, which contains the *C. perfringens cpe* gene including its promoter [307]. Restriction enzyme digestion of the plasmid with *BstB I* and *Bsu36 I* removes all cytotoxic portions of the *cpe* gene and retains the initial 36 nucleotides necessary for efficient transcription from the *cpe* promoter and a small number of C-terminal nucleotides required for stabilization of

trascribed mRNA. The SIV p27 gene from the molecular clone SIV-17e [308] was inserted into the digested vector to achieve a plasmid that, upon electroporation into *cpe*-negative type A *C. perfringens*, allows for expression of SIV p27 explicitly as a result of sporulation. p27 can be detected in inclusion bodies of sporulating recombinant *Cp*-p27 using immunogold stain electron microscopy. Additionally, expression of p27 by sporulating *Cp*-p27 has been quantitated to be 20-30μg/mL sporulated culture (10^8 cfu) or approximately 70μg p27/mg *C. perfringens* protein using semi-quantitative Western blot. In comparison, reports of other bacteria-based expression systems include protein production of 2.5μg *Helicobacteri pylori* urease/10^8 cfu *Salmonella typhi* [309]. The expression rate of p27 accounting for about 7% of the total *C. perfringens* protein is one of the highest known to be reported in a bacterial vaccine construct. Indeed, the expression rate is also respectable when compared with bacteria commonly used for protein expression. In one example of an efficient expression system, *E. coli* expressed human metallothionein 2A as 10-15% of total protein [310].

Murine bone marrow-derived DCs (BMDCs) accumulated p27 when incubated with lysates of sporulating *C. perfringens* expressing p27 [306]. Furthermore, incubation of ligated murine intestine with the same *C. perfringens* preparation enabled DC in PPs to take up p27, and oral delivery of this *Cp*-p27 vaccine resulted in the presence of p27 in the lumen of the terminal ileum within 90 minutes of administration [306]. These results suggest that the *Cp*-p27 vaccine can deliver p27 to PPDCs in the terminal ileum of mice. The resulting gut and systemic immune response remained to be fully characterized and is the object of the study described herein.

1.7 STATEMENT OF THE PROBLEM AND AIMS OF THE STUDY

Since most HIV infection is transmitted through vaginal or rectal mucosal tissue and the gut mucosa is an immediate target following HIV/SIV infection, vaccine-induced mucosal immunity against the virus is important to control HIV infection. The *Cp*-p27 oral vaccine is designed to stimulate immunity in the gut, and the resulting immunity may be transferred to other mucosal sites such as vaginal and rectal tissue. Previous studies have demonstrated that oral inoculation of *Cp*-p27 can deliver a large quantity of SIV p27 to the terminal ileum, which contains a concentration of PPs patches that are rich with antigen presenting cells such as DCs. The central hypothesis formed from these previous findings is that ***Cp*-p27 can deliver p27 to gut DCs and thereby prime mucosal and systemic humoral and cellular immunity against SIV.** To address this hypothesis the present study was undertaken to generate and characterize an anti-p27 cellular response upon oral administration of the *Cp*-p27 vaccine in mice. The Specific Aims and research plans were as follows:

Specific Aim 1: Characterize the ability of *Cp*-p27 vaccine to induce DCs to stimulate p27-specific T cells *in vitro*

Hypothesis: DCs exposed to Cp-*p27 will mature and gain capacity to present p27 epitopes on MHC to T cells*

Approach: Murine bone marrow-derived and purified Peyer's patch DCs were exposed to *Cp*-p27 and examined for maturation characteristics including co-stimulatory molecule expression and cytokine production. The function of DCs was assessed through ELISpot to detect IFN-γ produced by p27-specific T cells following restimulation by vaccine-exposed DCs.

Specific Aim 2: Improve *Cp*-p27 vaccine to stimulate stronger DC and T cell responses *in vitro* by using PTDs conjugated to p27

Hypothesis: Vaccines containing p27 conjugated to PTD sequences will increase internalization of p27 and thus drive production of stronger immune response

Approach: *C. perfringens* strains expressing p27 conjugated to PTD-5 and 8K PTD sequences were constructed and evaluated for their effects on cellular internalization. Efficiency of uptake of conjugated or unconjugated p27 by DCs and intestinal epithelial cells were tested using quantitative protein immunoblotting. These PTD-conjugated *C. perfringens* strains were then compared with wild-type *Cp*-p27 for effects on DCs and resulting T cell-stimulating capacity.

Specific Aim 3: Evaluate *Cp*-p27 as an oral vaccine *in vivo* in mice

A. Optimize mucosal adjuvants for use with *Cp*-p27

Hypothesis: Combinations of strong mucosal adjuvants will improve immune response to Cp-*p27 vaccination*

Approach: The mucosal adjuvants CT, LT(R192G), and CpG ODNs were administered to mice orally with *Cp*-p27. The resulting cellular immunity in gut tissues was assayed with IFN-γ ELISpot. The functionality of p27-specific T cells generated through use of leading adjuvants/adjuvant combinations was also assayed using intracellular cytokine staining and surface staining followed by flow cytometry.

B. Determine the priming and/or boosting capacity of *Cp*-p27 when combined with a systemically delivered adenovirus expressing p27

Hypothesis: Cp-*p27 priming can enhance mucosal and/or systemic immunity induced by boosting with adenovirus expressing SIV p27*

Approach: *Cp*-p27 was administered to mice orally as a prime or boost to an intramuscular adenovirus vaccine expressing SIV p27. SIV p27-specific cellular immune responses in systemic and gut tissues were assayed through IFN-γ production via ELISpot, and p27-specific humoral responses were assayed with ELISA. In addition, the functionality of gut T cell responses in inductive and effector tissues was characterized with multi-color flow cytometry.

2.0 *IN VITRO* DENDRITIC CELL RESPONSE TO *CP-P27*

2.1 PREFACE

The study described in this chapter is a collaborative effort between Dr. Phalguni Gupta and Dr. Jay K. Kolls and constitutes a manuscript currently being revised following peer review. Dendritic cell cultures and experiments were performed by Ruth Helmus in Dr. Gupta's laboratory, Bio-Plex assays were performed by Amy Magill in Dr. Kolls' laboratory, and animals were cared for by the University of Pittsburgh Division of Laboratory Animal Resources. These results were presented as poster abstracts at AIDS Vaccine 2003 (Development of a novel *Clostridium perfringens*-based oral vaccine against SIV. Chen, Y., R. Helmus, B. McClane, J. Clemens, R. Hoffman, and P. Gupta.) and the 2005 Conference on Retrovirology and Opportunistic Infections (A novel *C. perfringens*-based SIV vaccine induces maturation of dendritic cells and enables dendritic cell priming of T cells. R. Helmus, Y. Chen, T. Wehrli, and P. Gupta.)

2.2 ABSTRACT

The induction of both systemic and mucosal immunity is a high priority in the development of an anti-HIV vaccine. Dendritic cells (DCs) at inductive mucosal sites such as gut Peyer's patches are important mediators of mucosal immune priming. In this study, the interaction of a vaccine using *Clostridium perfringens* expressing SIV capsid p27 (*Cp*-p27) with murine DCs was investigated. Both bone marrow-derived DC (BMDCs) and freshly isolated Peyer's patches DCs (PPDCs) responded to exposure to *Cp*-p27 by upregulating maturation markers and producing pro-inflammatory cytokines. Furthermore, the mature dendritic cells stimulated p27-specific IFN-γ production by T cells, demonstrating that the p27 antigen was efficiently delivered to, processed by, and presented on MHC by BMDCs and PPDCs. These findings suggest that *Cp*-p27 vaccine-mediated delivery of p27 to DCs could induce immunity against SIV.

2.3 INTRODUCTION

The major sites of exposure to and transmission of human immunodeficiency virus (HIV) are mucosal surfaces. An immune response induced by delivery of antigen to one mucosal site can stimulate immune responses at other mucosal sites as well as systemic immune responses [48, 49, 238, 249, 311]. Thus, an effective vaccine against HIV or its non-human primate counterpart simian immunodeficiency virus (SIV) should target a mucosal site. One important mucosal tissue that can be targeted by oral vaccination is the gut. In addition to its role in mucosal immunity, the gut has been shown to be a major site of viral replication and cell destruction early in SIV/HIV infection in both non-human primates [64, 312, 313] and humans [74, 75, 314] and likely serves as a reservoir for virus throughout infection [78, 79]. It is, therefore, important for an HIV or SIV vaccine to provide immunity in the gut by inducing immune responses through the gut associated lymphoid tissue (GALT).

The main inductive immune tissues of the GALT are Peyer's patches (PPs). Dendritic cells (DCs) in terminal ileum PPs are adept at capturing and processing antigens for presentation to naïve T cells. DCs possess the unique ability to present exogenously acquired antigens that are normally loaded onto MHC class II molecules in the context of MHC class I, which is necessary for inducing a $CD8^+$ cytotoxic T lymphocyte (CTL) response (reviewed in [315, 316]). The $CD8^+$ CTL response to HIV or SIV appears essential for limiting viral infection, as displayed by numerous studies of both systemic and mucosal immune responses to HIV/SIV [43-49, 79]. Thus, to generate an effective anti-SIV $CD8^+$ CTL response, a vaccine must cause DCs to present SIV antigen to $CD8^+$ T cells and induce priming of anti-SIV $CD8^+$ CTLs.

It has previously been shown that *Clostridium perfringens* expressing SIV p27 (*Cp*-p27) can deliver a large amount of viral antigen to the terminal ileum where PPs are concentrated [306]. The bioengineered *C. perfringens* has the natural ability to generate high levels of antigenic protein using the strong CPE gene (*cpe*) promoter during sporulation and then shield this protein in the mother cell until reaching the PPs. To study the effectiveness of the *Cp*-p27 vaccine to induce a DC-mediated immune response, the phenotype, cytokine profile, and T-cell stimulatory capacity of DCs exposed to the vaccine were investigated. The effects of the vaccine were examined using both systemic (bone marrow-derived DCs) and gut mucosal (freshly isolated Peyer's patch DCs) DCs. The results demonstrate that the *Cp*-p27 vaccine can efficiently stimulate a DC-mediated p27-specific immune response *in vitro*, which suggests that *in vivo* immunization would result in p27-specific $CD8^+$ CTL responses. This is the first analysis of the immune response of an extracellular bacteria-based oral vaccine via its target mucosal DCs.

2.4 MATERIALS AND METHODS

Animals

Female Balb/c mice were purchased from Charles Rivers Laboratories, Inc. and housed in a pathogen-free facility in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee and federal regulations. Animals were used between 6 and 8 weeks of age.

Antibodies

FITC-α-CD40 (clone L3T4), FITC-α-CD40 (HM40-3), FITC-α-CD80 (16-10A1), FITC-α-CD86 (GL1), FITC-α-I-A^d (39-10-8), R-PE-α-CD8a (53-6.7), R-PE-α-CD11c (HL3), unconjugated α-I-A^d (AMS-32.1), and unconjugated α-H-$2D^d$ (34-5-8S) antibodies against mouse antigens and α-human CD3 (UCHT1) were purchased from BD Pharmingen. PE/Cy5-α-CD3ε (145-2C11), PE/Cy5-α-CD40 (1C10), PE/Cy5-α-CD80 (16-10A1), and PE/Cy7-α-CD86 (GL1) antibodies against mouse antigens were purchased from BioLegend. Isotype control antibodies were purchased from the same manufacturers according to the fluorescent conjugate used.

Vaccine

Construction of the *Clostridium perfringens* vaccine expressing SIV p27 has been described previously [306]. Sporulating cultures were achieved by overnight culture of modified Duncan-Strong medium [299] inoculated with a fresh 8h culture grown in fluid thioglycolate broth (Difco), with all growth performed at 37°C and in the presence of 10μg/mL

chloramphenicol. Sporulation of at least 90% of all bacteria in cultures was confirmed by phase-contrast light microscopy. Sporulated bacteria were isolated and washed twice with PBS by centrifugation at 9700xg at 4°C for 10 minutes per centrifugation. Isolated sporulating bacteria were sonicated, and expression of p27 was confirmed in sporulating cultures of the transformed bacteria by Western blot.

BMDCs

Mouse bone marrow-derived dendritic cells (BMDCs) were prepared as described previously [306]. Briefly, bone marrow cells flushed from the femurs and tibias of mice were seeded and cultured at $8x10^6$ cells in 6-well plate wells in 4mL RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 1% penicillin-streptomycin, 0.025M 2-mercaptoethanol, and 4ng/mL of both GM-CSF and IL-4. At day 2, nonadherent cells were removed and 50% of the supernatant was replaced with fresh cytokine-containing medium. Cells (40-60% CD11c-positive) were used on culture day 5 or 6.

Isolation of PPDCs

Peyer's patch dendritic cells (PPDCs) were isolated as described by Iwasaki *et al.* [317] with modifications. Aside from enzymatic incubations, isolation of cells was performed on ice. Peyer's patches (PPs) were aseptically removed from small intestines and incubated at 37°C with stirring for 15-30 minutes in Hank's balanced salt solution with 10% heat-inactivated FBS, 145µg/mL 1,4-dithioerythritol, 25mM HEPES, and 5mM EDTA. After washing with PBS, PPs were incubated for 15-30 minutes with stirring at 37°C in RPMI 1640 containing 10% heat

inactivated fetal bovine serum, 1% penicillin-streptomycin, and 1mg/mL collagenase D (Roche). The medium was passed through a 70μm-pore nylon mesh strainer and a 5mL syringe pestle was used to crush PPs through the same strainer. The cell mixture was then passed through a 40μm-pore nylon mesh strainer and recovered by centrifugation at 4°C. Cells were blocked with anti-CD16/CD32 antibody at 1:100 dilution, washed, and then incubated at 4°C for 15 minutes with MACS CD11c MicroBeads (Miltenyi Biotec) using 10μL beads per 10^7 cells. Cells were then enriched for $CD11c^+$ cells by passing through MS Columns (Miltenyi Biotec) following the manufacturer's protocol. Cells were then cultured at $1x10^6$ cells/mL in 96-well plate wells in RPMI 1640 containing 10% heat-inactivated FBS, 1% penicillin-streptomycin, 3mM L-glutamine, 1mM sodium pyruvate, and 50μM 2-mercaptoethanol. Isolated cells were routinely 65-75% CD11c-positive as detected by flow cytometry.

Treatment of cells with vaccine

BMDCs that had been grown for 5-6 days ($1\text{-}2x10^6$ cells per 4mL) or freshly isolated PPDCs ($2x10^5$ cells per 200μL) were incubated with ~$2x10^5$/mL sporulated, sonicated *C. perfringens* expressing p27, empty vector *C. perfringens* control, purified p27 protein at a concentration equivalent to that expressed by the vaccine bacteria. As a positive control 0.5μg/mL LPS were added to BMDC, and 10μM unmethylated CpG oligodinucleotides were added to PPDCs. Treatments were removed after 2h via washing and centrifugation, and the cells were returned to culture for 22 (BMDCs) or 4 (PPDCs) additional hours. Supernatant of cultures was then collected and stored at -20°C, and cells were harvested and utilized for further assays.

Flow cytometry

DCs were surface stained for CD11c, CD40, CD80, CD86, and MHC class II (I-A^d). BMDCs were also assayed for phagocytosis by incubating cells for 30 minutes in media containing 1mg/mL FITC-dextran (40,000 kDa molecular weight; Sigma). Cells were then surface stained at 4°C. After staining, all cells were fixed, and data were collected on using a Coulter Epics XL-MCL flow cytometer. Cytometry data were analyzed using FlowJo version 7.2.2.

Bio-Plex

DC supernatants were analyzed for cytokines using the Bio-Plex Mouse Cytokine Th1/Th2 Bio-Plex Panel kit from Bio-Rad. 50µL samples were assayed following the manufacturer's instructions, and beads were analyzed using a Bio-Plex Luminex system.

Western blot

Cells were lysed and separated on a 15% SDS-PAGE gel. Protein was transferred to nitrocellulose and blotted with monkey anti-SIV serum (a gift from Michael Murphey-Corb), washed, and blotted with horseradish peroxidase-conjugated goat anti-monkey antibody (Nordic Immunological Laboratories). Protein bands were detected with SuperSignal West Pico Chemiluminescent Solution (Pierce). Blots were analyzed via densitometry using Quantity One software (Bio-Rad).

ELISpot assay

Detection of interferon-gamma (IFN-γ) was performed using mouse IFN-γ ELISpot Kits from Mabtech. Precoated anti-IFN-γ ELISpot plates were activated with 5 washes with PBS and blocked at room temperature for ≥30 minutes with RPMI containing 10% FCS. $2x10^5$ SIV p27-specific splenocytes derived from mice inoculated subcutaneously with SIV p27 in Freund's adjuvant were plated in wells. These cells were placed at 37°C for 1h, and then BMDCs or PPDCs exposed to SIV p27-expressing *C. perfringens* or vector control *C. perfringens* were added to ELISpot plate wells at a 1:5 or 1:25 DC:splenocyte ratio, respectively, in a final volume of 200μL ELISpot media (RPMI-1640 with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 0.1mM non-essential amino acids, 2mM L-glutamine, 10mM HEPES, and 1mM sodium pyruvate) per well. Control wells included splenocyte or DCs only, and media-only control wells were also included for each plate. In the case of MHC-blocking experiments, DCs at a concentration of $4x10^5$ cells/mL were pre-incubated for 2h 1:50 (v/v) with the appropriate antibody (α-I-A^d for MHC class I blocking, α-H-$2D^d$ for MHC class II blocking, and α-human CD3 as a control) before being added to splenocytes. The cells were then incubated at 37°C for 24 to 36h. Detection of IFN-γ spot-forming cells (sfc) was performed according to the manufacturer's protocol. Briefly, cells were removed and plates were washed 5 times with PBS. 100μL of 1μg/mL R4-6A2-biotin detection antibody in PBS with 0.5% FCS was added to each well, and plates were incubated at room temperature for 2h. Antibody was discarded and plates were washed 5 times with PBS, then 100μL of 1:1000 streptavidin-ALP in PBS with 0.5% FCS was added to each well, and plates were incubated at room temperature for 1h. Plates were washed as before, and 100μL of 0.45μm-filtered BCIP/NBT-plus substrate solution was added to each well. Spots were allowed to develop for about 20 minutes at room

temperature, and then plates were washed extensively with tap water. The underdrain was removed and the back of well membranes was also washed. Excess water was blotted away using paper towel, and plates were left to dry in the dark at room temperature. When dry, sfc were counted on an automated ELISpot reader. Background sfc values from media- and cell-only control wells were removed as appropriate, and sfc were normalized to 10^6 cells.

Statistics

Except where noted, *p* values were determined using 1-tailed two-sample Student's t-test with unequal variance.

2.5 RESULTS

2.5.1 Maturation of BMDCs Exposed to *C. perfringens* Expressing SIV p27

In order to prime an effective immune response against an antigen, a DC exposed to the antigen must mature. To test the ability of the *C. perfringens* SIV vaccine to stimulate maturation of DCs, murine bone marrow-derived DCs (BMDCs) were exposed to *C. perfringens* expressing SIV p27 or empty vector *C. perfringens* or other stimuli. Following exposure to *Cp*-p27, BMDCs showed an increased surface expression of CD80, CD86, CD40, and MHC class II, similar to stimulation with LPS as a positive control (Figure 2). BMDCs exposed to control

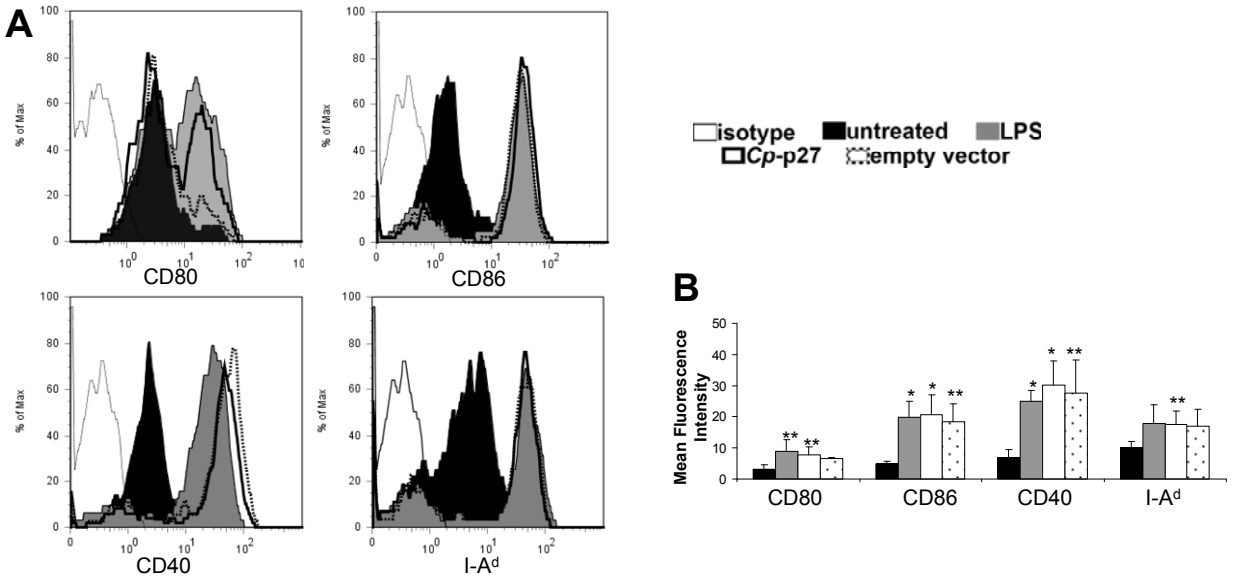


Figure 2. Maturation of bone marrow-derived dendritic cells (BMDCs) in response to *C. perfringens* SIV p27 vaccine *(Cp*-p27)
BMDCs were cultured without stimulus (filled grey curve/bar), with LPS (dotted curve/bar) as a positive control, with empty vector (black filled bar) control *C. perfringens*, or with *Cp*-p27 (unfilled solid dark curve/bar). Unfilled solid light curve represents isotype control. *A*, Representative flow cytometric histograms from 3 or 6 independent experiments showing the level of expression of maturation markers CD80, CD86, CD40, and MHC class II (I-A^d). All histograms are pregated on $CD11c^+$ cells. *B*, Average mean fluorescence intensity + standard error of the mean of maturation marker expression in response to stimuli as determined by flow cytometry. Values of *p* were determined by Student's t-test against untreated cells. **p*<0.05; ***p*<0.10.

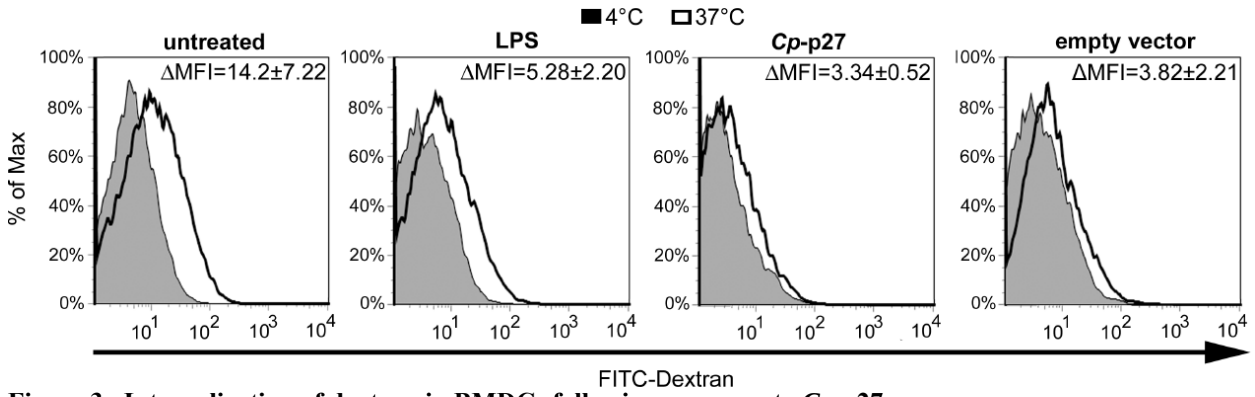


Figure 3. Internalization of dextran in BMDCs following exposure to *Cp*-p27
BMDCs were cultured without stimulus, with LPS as a positive control, or with *Cp*-p27. Following culture, cells were assayed for phagocytic ability by culture with FITC-conjugated dextran at 4°C (grey filled curve) or 37°C (unfilled curve). Flow cytometric histograms show FITC-dextran internalization in the $CD11c^+$ cell gate representative of 3 independent experiments. Average difference in mean fluorescence intensity (ΔMFI) ± standard error of the mean is shown, with ΔMFI calculated by subtracting the 4°C MFI from the 37°C MFI.

bacteria carrying an empty expression vector also displayed increased expression of the maturation markers, as is expected due to the presence of peptidoglycan in the *C. perfringens* cell wall. To confirm maturation, BMDCs exposed to stimuli were incubated with FITC-labeled dextran, and phagocytosed FITC signal was detected via flow cytometry. BMDCs exposed to LPS, *Cp*-p27, or empty-vector *C. perfringens* displayed lower dextran internalization than untreated BMDCs, indicating a loss of phagocytic capacity (Figure 3).

The matured BMDCs in all treatment groups produced high levels of a number of cytokines. Specifically, proinflammatory cytokines known to play important roles in developing Th1 responses (IFN-γ, TNF-α, IL-12 p70) were all produced in quantities at least 3.5-fold higher than untreated cells (Table 1). IL-4 was reduced in vaccine-treated cells compared with untreated cells; however, the Th2 and T-regulatory mediator IL-10 was increased, albeit to a much lesser degree than in positive-control LPS-treated cells. BMDCs exposed to empty-vector *C. perfringens* produced less IL-5, IL-10, IL-12 (p70), and TNF-α than *Cp*-p27-exposed BMDCs. IFN-γ and IL-4 levels were similar.

Table 1. Cytokine profile of culture supernatants from BMDCs exposed to no stimuli, *Cp*-p27 vaccine, empty-vector control *Cp* vaccine or LPS

	IL-2		IL-4		IL-5		IL-10	
	(pg/mL)	fold increase	(pg/mL)	fold increase	(pg/mL)	fold increase	(pg/mL)	fold increase
none	1.53±0.40		1.85±0.90		0.21±0.06		3.19±1.34	
***Cp*-p27**	20.78±1.80	7.05	0.47±0.10	0.25	2.02±1.59	9.49	137.21±58.94	43.01
control *Cp*	35.81±14.32	23.40	0.47±0.06	0.26	0.41±0.11	1.95	69.09±27.02	21.66
LPS	2.80±0.53	1.83	1.67±0.31	0.90	5.73±1.55	26.98	570.70±168.2	178.90

	IL-12 (p70)		TNF-α		IFN-γ	
	(pg/mL)	fold increase	(pg/mL)	fold increase	(pg/mL)	fold increase
None	0.80±0.27		2.28±0.31		0.08±0.05	
***Cp*-p27**	14.34±5.48	17.89	135.25±83.53	59.19	0.31±0.13	3.73
control *Cp*	4.82±1.96	6.02	10.54±3.87	4.61	0.40±0.16	4.75
LPS	100.50±67.14	125.43	2366.91±964.14	1035.85	0.42±0.18	5.03

Changes observed in BMDCs following exposure to the *Cp*-p27 vaccine are in accordance with a mature DC phenotype activity [318]. These results suggest that the BMDCs exposed to the vaccine should be able to prime T cells to form a productive immune response.

2.5.2 Functional Capacity of *Cp*-p27 Vaccine-Exposed BMDCs

It has been demonstrated previously that p27 protein is internalized by BMDCs when they are exposed to the *Cp*-p27 vaccine [306]. To track the fate of the internalized p27, a polyclonal anti-SIV serum was used in Western blots to probe cell lysates of BMDCs over a time course following exposure to the vaccine. Within 8 hours after exposure, these p27 levels were markedly decreased or undetectable in the BMDCs (Figure 4 *A*).

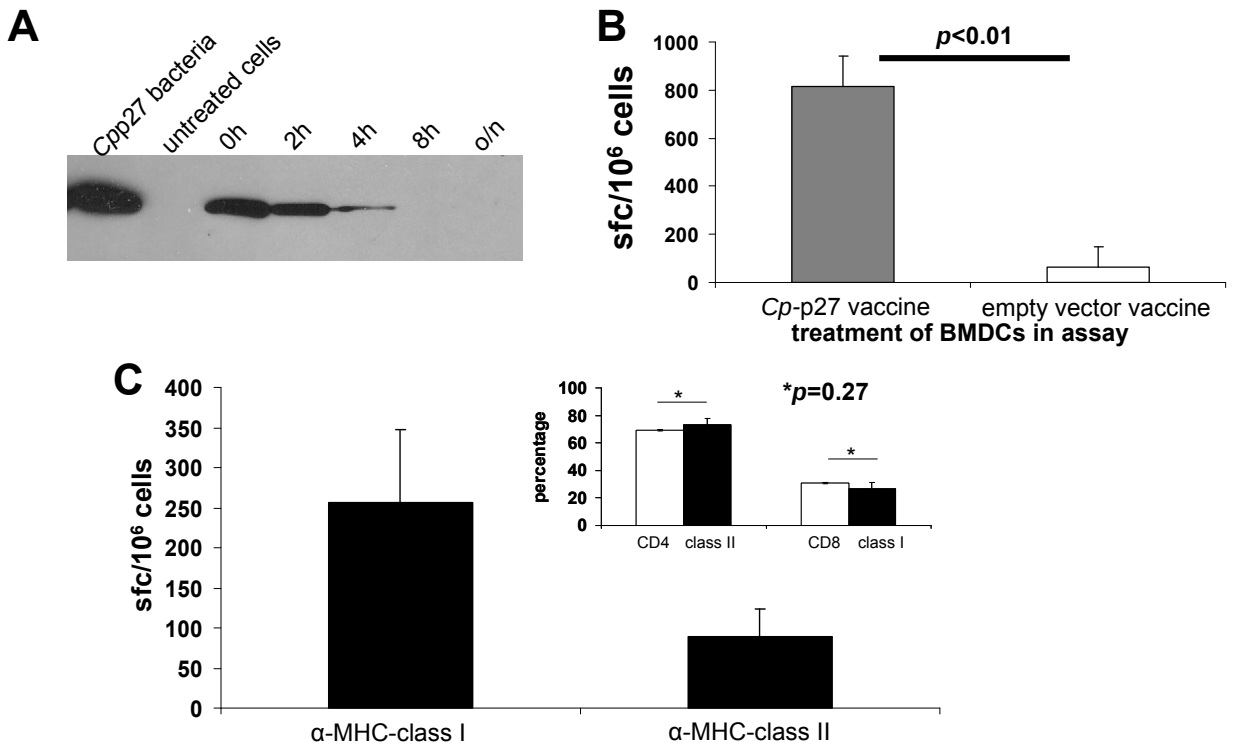


Figure 4. SIV p27 delivered by *Cp*-p27 processing and presentation by BMDCs
A, Western blot of lysates from *Cp*-p27 bacteria and BMDCs at multiple times following exposure to *Cp*-p27. *B*, IFN-γ ELISpot results representative of 5 independent experiments with vaccine-treated BMDCs cultured with p27-specific murine splenocytes. Error bars indicate standard error of the mean of samples assayed in triplicate. *p*-value determined by Student's t-test. *C,* IFN-γ ELISpot results representative of 3 independent experiments with vaccine-treated BMDCs cultured with p27-specific murine splenocytes in the presence of antibody against MHC class I or MHC class II. The inset shows the percentage of $CD4^+$ or $CD8^+$ T cells in murine splenocytes (white bars; n=6) compared to the percentage of MHC class II- or class I-mediated IFN-γ production in the ELISpot assays (black bars). Error bars indicate standard error of the mean. *p*-value determined by 2-tail heteroscedastic Student's t-test.

Next the fate of p27 was investigated by using vaccine-exposed BMDCs as antigen presenting cells in an ELISpot assay to determine if degraded p27 epitopes could be displayed on BMDC MHC molecules. For this purpose, the vaccine-exposed and matured BMDCs were used to restimulate p27-specific mouse splenocytes in an IFN-γ ELISpot assay. Splenocytes cultured with the vaccine-exposed BMDCs displayed p27-specific IFN-γ production, indicating that the BMDCs were indeed displaying p27 epitopes on MHC molecules and were capable of stimulating an immune response against p27 (Figure 4 *B*). The IFN-γ ELISpot response stimulated by *Cp*-p27-exposed BMDCs was about 12.8 times that stimulated by BMDCs

exposed to empty vector *C. perfringens*. It should be noted that the low background IFN-γ response detected by ELISpot in BMDC-only wells was similar regardless of whether BMDCs were treated with *Cp*-p27 or the empty vector control *C. perfringens*.

By blocking MHC class I- or II-mediated epitope presentation with antibodies against these molecules, p27 epitopes were detected to be presented in the context of both MHC class I and class II (Figure 4 *C*). More IFN-γ response was detected in assays with MHC class I blocking, indicating that the BMDCs presented more antigen in the context of MHC class II. However, the percentage of $CD4^+$ or $CD8^+$ T cells used in the assay were similar to the percentage of ELISpot sfcs resulting from MHC class II- or MHC class I-mediated expression, respectively (Figure 4 *C* inset). These findings indicate that presentation of p27 peptides on BMDCs after exposure to the vaccine is biased neither towards MHC class I nor towards class II.

2.5.3 Immune Response to *Cp*-p27 Vaccine in PPDCs

BMDCs, representative of systemic myeloid DCs, have many differences from the Peyer's patch DCs (PPDCs) that are the target for the development of mucosal immunity via oral vaccination. The DC subpopulations resident in PPs occur at different frequencies than in systemic compartments, including higher proportions of lymphoid and $CD4^-$ DCs [319-322]. DCs native to the PPs differ from other DCs in their reaction to antigen and subsequent cell-stimulatory ability. For example, PPDCs produce significantly more IL-10 following antigen-induced maturation and are more inclined to stimulate a Th2-type T cell response [317, 323, 324]. In order to better assess the vaccine's potential to prime a gut immune response and to determine if these cells behave differently in response to the vaccine than BMDCs, the BMDC

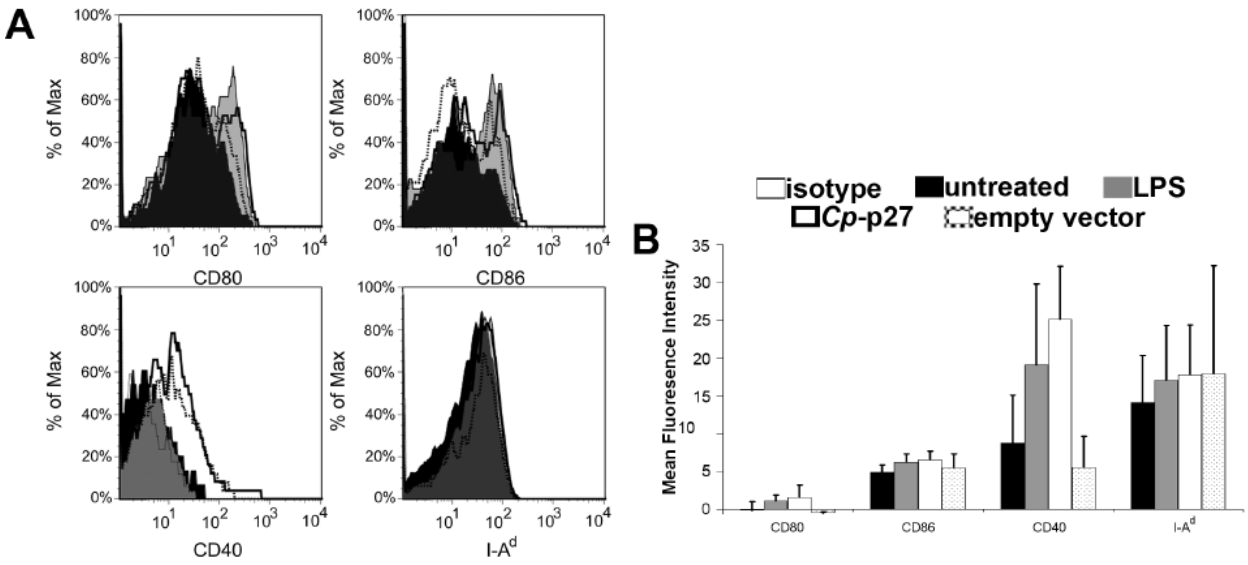


Figure 5. Maturation of Peyer's patch dendritic cells (PPDCs) in response to *Cp*-p27
PPDCs were cultured without stimulus (filled grey curve/bar), with CpG ODNs (dotted curve/bar) as a positive control, or with *Cp*-p27 (unfilled solid dark curve/bar). Unfilled solid light curve represents isotype control. *A*, Representative flow cytometric histograms from 2-5 independent experiments showing the level of expression of maturation markers CD80, CD86, CD40, and MHC class II (I-A^d). All histograms are pregated on $CD11c^+$ cells. *B*, Average mean fluorescence intensity + standard error of the mean of maturation marker expression in response to stimuli as determined by flow cytometry.

Table 2. Cytokine profile of culture supernatants from PPDC exposed to no stimuli, *Cp*-p27 vaccine, empty-vector control *Cp* vaccine or CpG ODN

	IL-2		IL-4		IL-5		IL-10	
	(pg/mL)	fold increase	(pg/mL)	fold increase	(pg/mL)	fold increase	(pg/mL)	fold increase
None	0.95±0.04		0.51±0.14		0.10±0.02		1.29±0.16	
***Cp*-p27**	1.61±0.28	1.69	1.79±0.61	3.54	26.48±2.46	268.10	163.82±18.74	126.99
control *Cp*	1.05±0.09	1.11	1.23±0.38	2.44	0.20±0.09	2.00	5.74±4.45	4.45
CpG ODN	2.25±0.60	2.37	0.85±0.43	1.68	0.13±0.03	1.30	11.79±5.01	9.14

	IL-12 (p70)		TNF-α		IFN-γ	
	(pg/mL)	fold increase	(pg/mL)	fold increase	(pg/mL)	fold increase
none	0.64±0.08		1.89±0.21		0.70±0.13	
***Cp*-p27**	308.57±23.31	485.93	1806.96±150.94	956.69	39.69±13.53	56.80
control *Cp*	3.59±2.09	5.66	18.34±12.05	9.71	0.84±0.34	1.20
CpG ODN	12.93±4.43	20.36	3.41±0.97	1.80	1.61±0.35	2.30

experiments were repeated using DCs isolated from the PPs of mice. Vaccine-treated PPDCs showed enhanced surface expression of maturation markers and production of pro-inflammatory cytokines, similar to BMDCs (Figure 5 and Table 2). Interestingly, the percentage of PPDCs expressing CD40 in response to the positive control unmethylated cytosine-phosphate-guanine oligodinucleotides (CpG ODN) was similar to that of unstimulated cells; however, the mean fluorescence intensity of the small percentage of CD40-expressing cells was distinctly higher in CpG ODN-stimulated PPDCs than unstimulated PPDCs. In contrast to BMDC cytokine responses, PPDCs treated with *Cp*-p27 displayed much higher levels of expression of IL-5, IL-10, IL-12(p70), TNF-α, and IFN-γ in comparison to untreated PPDCs as well as CpG ODN- and empty vector *C. perfringens*-treated PPDCs. In an ELISpot assay, the *Cp*-p27-exposed PPDCs also displayed the ability to stimulate p27-specific IFN-γ (Figure 6). In this assay, background control wells of just PPDCs showed identical numbers of IFN-γ spot-forming cells (sfc) (which was at or near zero) regardless of whether they were treated with *Cp*-p27 or empty vector *C. perfringens*. Thus, although PPDCs respond to antigen differently than other DCs, these data suggest that the *C. perfringens*-p27 vaccine would be able to generate an anti-SIV-p27 immune response in the gut if successfully delivered to PPDCs.

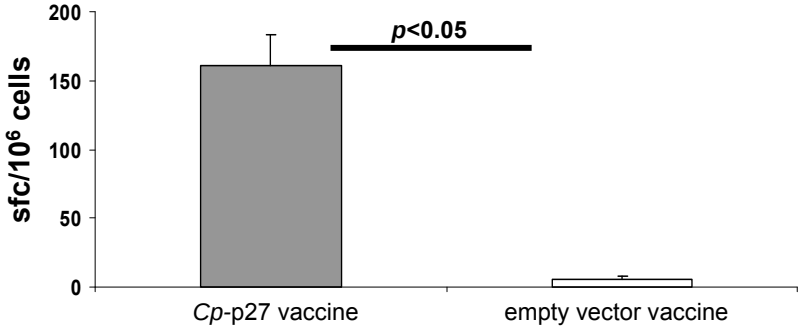


Figure 6. SIV p27 delivered by *Cp*-p27 vaccine is presented as epitopes by PPDCs
Shown are IFN-γ ELISpot results representative of 5 independent experiments with vaccine-treated PPDCs cultured with p27-specific murine splenocytes. Error bars indicate standard error of the mean of samples assayed in duplicate. *p*-value determined by Student's t-test.

2.6 DISCUSSION

The present study investigated the phenotype and functionality of murine DCs following exposure to *C. perfringens*-SIV p27 vaccine. Both bone marrow-derived and freshly isolated PP DCs demonstrated characteristics of maturation following exposure to vaccine including upregulation of costimulatory and MHC molecules and loss of phagocytic capacity (Figures 1 and 4). Once internalized into BMDCs, p27 was degraded, and the antigen became undetectable within 8 hours (Figure 3a). The matured dendritic cells expressed high levels of pro-inflammatory and Th1- and memory-promoting cytokines (Tables 1 and 2) and stimulated an IFN-γ response in p27-specific cells (Figures 3b and 5). IFN-γ was produced in the presence of antibodies against either MHC class I or MHC class II (Figure 3c), indicating that p27 epitopes are effectively presented in both contexts, and the DCs can thus stimulate both $CD4^+$ and $CD8^+$ T cells. This study demonstrates the ability of p27 in the context of the *Cp*-p27 vaccine to induce murine DCs to stimulate a p27-specific T cell response.

The goal of the *C. perfringens*-p27 vaccine examined in this study is to successfully deliver SIV p27 antigen to DCs of the PPs and induce an immune response against the antigen. Since the p27 antigen is present in inclusion bodies of the bacterial cell, the p27 is protected from destruction by stomach acid and by degrading enzymes of the small intestine as the bacterial vector travels through the duodenum and jejunum until it reaches the terminal ileum. This would allow for delivery of high amounts of intact viral antigen to the site of the gut that has been shown in humans and monkeys to be rapidly infected and depleted of lymphocytes following infection of the individual by HIV or SIV, respectively [64, 74, 75, 312-314]. A vaccine-induced

immune response in these tissues is likely to be important in controlling the virus soon after infection, and the *Cp*-p27 is an attractive possible tool for inducing such a response. DC maturation and antigen presentation is the first requirement for the formation of a DC-mediated immune response.

The evaluation of HIV and SIV vaccines by characterizing their interactions with and effects upon DCs has been undertaken with leading vaccine vectors. For example, a study of the mechanism of the ALVAC canary-pox vector's induction of innate immune activation included investigation of its effects upon murine BMDCs [325]. ALVAC is used as a vector for many HIV vaccines previously and currently in clinical trials. BMDCs exposed to ALVAC displayed enhanced expression of costimulatory molecules CD40, CD80, and CD86. In the current study, BMDCs exposed to *Cp*-p27 demonstrated upregulation of these same markers as well as MHC class I. Whereas ALVAC treatment was reported to induce less costimulatory molecule upregulation as compared to a positive control TLR4 agonist, *Cp*-p27 treatment in the current study displayed equal or enhanced levels of most maturation indicators compared with the TLR4 agonist LPS. This may be due in part to the presence of peptidoglycan in *C. perfringens*, which might interact with DCs via TLR2 as has been suggested for another Gram positive organism, *Staphylococcus aureus* [326-329]. Both ALVAC- and *Cp*-p27-treated BMDCs secreted cytokines including TNF-α and IL-12. Additionally, *Cp*-p27-treated BMDCs and PPDCs secreted IFN-γ, which has been suggested to be essential for PPDCs to mediate optimal resistance against oral pathogens [330].

The most promising bacteria-based anti-immunodeficiency virus vaccines to date utilize *Salmonella enterica* serovar *Typhimurium* [247, 331] and *Listeria monocytogenes* [332, 333]. Like these vaccines, *Cp*-p27 is designed to deliver intact protein to gut cells. However *Cp*-p27

does not involve infection or colonization of the gut mucosa since the protein is delivered in the context of a non-replicative bacterial spore. Human monocyte-derived DCs exposed to HIV gag-expressing *L. monocytogenes* stimulated cytotoxicity against target cells [334]. Although the evaluation of this *Listeria* vaccine in mucosal DCs was not reported, the vaccine has been shown to stimulate gut mucosal as well as systemic immunity in animal models when delivered orally, indicating that the *in vitro* response to the vaccine in systemic-type DCs (e.g. monocyte-derived DCs) corresponded to mucosal *in vivo* immunostimulatory capacity of the vaccine[332]. In the current study, murine systemic DCs exposed to *C. perfringens* expressing SIV p27 caused IFN-γ production by p27-specific cells via both MHC class II and class I, indicating that stimulation of CTLs was likely resulting from vaccine-exposed DCs. Murine gut mucosal DCs exposed to the vaccine also stimulated p27-specific IFN-γ production. In light of the findings with the *L. monocytogenes* vaccine, the current results encourage further exploration into the *in vivo* capacity of the orally-delivered *Cp*-p27 vaccine to stimulate systemic and mucosal immunity via DCs.

Dendritic cells exposed *in vitro* to free antigen or vaccine-delivered antigen have also been used themselves as vaccines in many non-human primate and human studies (reviewed in [335]). When treated *in vitro*, these DCs demonstrate upregulation of costimulatory molecules and MHC class II, pro-inflammatory cytokine production, and capacity to trigger antigen-specific IFN-γ production. These characteristics correspond to immunostimulatory capacity of the DC-vaccines when administered *in vivo*. The enhancement of DC-mediated IFN-γ observed in these types of studies is of the same magnitude as that detected in this study with *Cp*-p27-exposed DCs (i.e. approximately 10-fold enhancement of IFN-γ sfc when comparing vaccine-exposed DCs with DCs exposed to vector control vaccine) [336, 337].

The results of the current study demonstrate that *in vitro* exposure of both systemic and gut mucosal DCs to the *Cp*-p27 vaccine leads to effective stimulation of a p27-specific immune response. Previously it was shown that oral delivery of the vaccine to mice resulted in high levels of p27 in the terminal ileum [306]. Together, these findings suggest that the vaccine should be effective at priming an immune response in Peyer's patches *in vivo* when orally delivered. Given the importance of the gut in HIV and SIV infection, it is vital to pursue development of vaccine vectors such as ours that may induce effective anti-viral immunity in the gut. Experiments are underway to investigate the immune response generated against SIV p27 when animals are fed the *Cp*-p27 vaccine.

3.0 USE OF PROTEIN TRANSDUCTION DOMAIN CONJUGATION TO SIV P27 TO ENHANCE IMMUNE RESPONSE AGAINST VIRAL ANTIGEN

3.1 PREFACE

The study described in this chapter was performed by Ruth Helmus in Dr. Phalguni Gupta's laboratory. Dr. Bruce McClane graciously provided the pJRC200 plasmid. Bio-Plex assays were performed by Amy Magill in Dr. Kolls' laboratory, and animals were cared for by the University of Pittsburgh Division of Laboratory Animal Resources. Dr. Paul D. Robbins provided guidance for PTD sequence selection. These results were presented as a poster abstract at the 2005 Keystone Symposia on HIV Vaccines (Protein transduction domain fusion enhances dendritic cell SIV p27 internalization and stimulatory function in response to a *C. perfringens*-based SIV vaccine. R. Helmus, Y. Chen, T. Wehrli, and P. Gupta.)

3.2 ABSTRACT

Protein transduction domains (PTDs) are cationic peptides that improve the delivery of their attached cargo into the cytoplasm of mammalian cells. The *Cp*-p27 vaccine consists of a *Clostridium perfringens* bacterium expressing SIV p27 and is designed to deliver p27 to dendritic cells (DCs) of the small intestine. By conjugating PTD peptide sequences 8K and PTD-5 to the N-terminus of p27 in *Cp*-p27, it was hypothesized that enhanced p27 delivery would result, leading to more p27 epitope display on MHC class I, and thereby improving the induction of CD8+ T cell immune responses that have been associated with control of SIV. Although *C. perfringens* expressing p27 conjugated to PTD sequences resulted in higher protein internalization, no difference was observed between PTD-conjugated and unconjugated strains in the ability to induce DCs to stimulate p27-specific IFN-γ by splenocytes. In addition, no improvement in systemic immunity was observed following vaccination with the 8K-conjugated strain when compared with the unconjugated *Cp*-p27 vaccine. In summary, a higher immune response by the PTD-conjugated *Cp*-p27 strains was not induced despite an increase in protein internalization by DCs.

3.3 INTRODUCTION

In order to stimulate an immune response in the gastrointestinal tract, antigen must be delivered in sufficient quantity to antigen presenting cells (APCs). The major APCs of the intestine are dendritic cells (DCs), which are found in the subepithelial dome (SED) of Peyer's patches (PPs) and interspersed in lamina propria tissue. PPDCs acquire antigen when it is transcytosed to the SED through specialized epithelial cells known as M cells [254]. Lamina propria DCs extend their dendrites across the epithelial layer to directly capture luminal contents [256, 338] and can also acquire antigen from intestinal epithelial cells [339, 340]. DCs are generally required for the formation of productive mucosal immune responses [341, 342], although intestinal epithelial cells may also function as APCs [343].

DCs possess the unique ability to present exogenously acquired antigens, which normally are loaded onto MHC class II molecules, in the context of MHC class I. Antigen presentation on MHC class I is required for the formation of $CD8^+$ T cell responses, including $CD8^+$ CTL responses, which are important in control of HIV and SIV infection [40, 42, 136-140]. Enhancing the delivery of vaccine antigen to DCs is expected to increase the number of DCs presenting antigen and thus the number of resulting antigen-specific effector cells.

Protein transduction domains (PTDs) are peptides that enable the proteins to which they are bound to efficiently enter target cells through a receptor-independent mechanism [288-290]. PTD-mediated uptake delivers antigen to the cytoplasm, which engages the MHC class I pathway, thus enhancing DC cross-presentation and increasing induction of CTLs [296, 297]. Unique PTDs have been designed with the ability to specifically target proteins to certain cell

types. Among these are the arginine-rich PTD-5 peptide (RRQRRTSKLMKR) and the lysine-rich 8K peptide (KKKKKKKK). Both of these positively charged cationic peptides show strong binding to glycosaminoglycans such as heparan sulphate [288]. Thus, through electrostatic interaction with the plasma membrane, the PTDs with their attached protein cargo are more efficiently transduced into cells than unattached protein [288]. PTD-5 and 8K have been successfully used to deliver fluorescent proteins and active enzymes to cells and tissues [288, 291, 344]. PTD-5 enhances protein uptake by many cell types, including epithelial cells and DCs [291]. 8K also transduces protein into epithelial cells [288] and is especially proficient at directing proteins into DCs (Dr. Paul.D. Robbins, personal communication). Incorporating either of these PTD peptides as an N-terminal conjugation to orally administered antigen should increase delivery of the antigen directly to gut DCs or to DCs indirectly via enhanced uptake by M cells or epithelial cells.

To evaluate the potential for PTD conjugation to enhance immunity against SIV p27 through vaccination with *Clostridium perfringens* expressing SIV p27, PTD-p27 conjugate strains of *C. perfringens* were examined for immune stimulatory capacity under *in vitro* and *in vivo* conditions. The responsiveness of DCs to the various *C. perfringens* vaccine strains expressing PTD-conjugated SIV p27 and the resultant ability of DCs to stimulate p27-specific cellular responses were determined.

3.4 MATERIALS AND METHODS

Construction and growth of *C. perfringens* expressing SIV p27 and PTD conjugates

Construction of *C. perfringens* expressing SIV p27 (*Cp*-p27) using the pJRC200 plasmid has been described in Chapter 2 and published elsewhere [306]. For PTD conjugate strains in which p27 was expressed with the N-terminal fusion of either PTD-5 (RRQRRTSKLMKR) or 8K (KKKKKKKK), primers encoding the appropriate PTD sequence, *BstB I* and *Bsu36 I* enzyme cut sites, and a di-glycine (GG) linker were used in the initial p27 amplification step (see Table 3) and the resulting PCR product was cloned. The empty vector *C. perfringens* used in these experiments contained the pJIR418 plasmid, the parent of pJRC200. Recombinant plasmids and *C. perfringens* strains were confirmed by sequencing to contain the desired genetic information. Sporulating cultures were achieved, isolated, and quantitated for p27 expression as described in Chapter 2.

Table 3. 5' primers used for cloning of SIV p27 into *C. perfringens*

peptide for conjugation to p27	5’ to 3’ primer sequence
PTD-5	*TTCGAA*ATGAGACGCCAGCGTCGCACGAGCAAACTGATGAAACGA**GGCG GCC**CAGTACAACAAATAGGTGGTAAC
8K	ACTGTACTACTC*TTCGAA*ATGAAGAAGAAGAAGAAGAAGAAGAAG**GGCG GC**CCAGTACAACAAATAGGTGGTAAC
none	*TTCGAA*CCAGTACAACAAATAGGTGG

3' primer for all constructs was 5’--*CCTAAG*GACATTAATCTA GCCTTCTG--3’
Italics: *BstB I* recognition site
Underline: PTD overhang
Bold: diglycine linker

Animals

Female Balb/c mice were purchased from Charles Rivers Laboratories, Inc. and housed in a pathogen-free facility in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee and federal regulations. Animals were used at 6 to 8 weeks of age.

Cell culture

Bone marrow-derived DCs (BMDCs) were generated and grown as described in Chapter 2. The DC2.4 cell line was cultured in DMEM containing 10% FBS, 1% penicillin-streptomycin, and 2mM L-glutamine. Adherent cells grown in $75cm^2$ flasks were trypsinized and reseeded ($1\text{-}2x10^6$ cells per flask) every 3-4 days. For uptake experiments, cells were seeded at day 0 at $1x10^6$ cells per 100mm diameter round culture dish and grown in a volume of 10mL for use on culture day 4. CaCo2 cells were cultured in DMEM containing 10% FBS, 1% penicillin-streptomycin, 0.76% sodium bicarbonate, 0.1mM non-essential amino acids, and 2mM L-glutamine. Cultures were fed on day 3 or 4 of culture by removing ¾ media and replacing with fresh media. Adherent cells grown in $75cm^2$ culture flasks were trypsinized and reseeded ($1.5x10^6$ cells per flask) on day 6-8 of culture. For uptake experiments, cells were seeded on day 0 at $1x10^6$ cells per 100mm diameter round culture dish and grown in a volume of 10mL for use on culture day 7, with feeding at day 3. All cells were grown at 37°C in the presence of 5% CO_2.

BMDC treatment and assays

BMDCs were treated with bacteria and surface stained as described in Chapter 2. Dextran uptake experiments were also performed as described in Chapter 2. Cells were

examined using a Coulter Epics XL-MCL flow cytometer or FACS Canto flow cytometer. Cytometry data were analyzed using FlowJo version 7.2.2. DC supernatants were analyzed in the laboratory of Dr. Jay K. Kolls for cytokines using the Bio-Plex Mouse Cytokine Th1/Th2 Bio-Plex Panel kit from Bio-Rad. 50μL samples were assayed following the manufacturer's instructions, and beads were analyzed using a Bio-Plex Luminex system.

p27 uptake experiments

Optimal exposure time and treatment concentration was determined for both DC2.4 and CaCo2 cells grown in culture dishes to be 2 hours with 100mg p27 expressed by sonicated *C. perfringens* strains all grown to similar concentrations and with similar sporulation percentages. After this incubation period, cells were extensively washed with Hanks' buffered salt solution and trypsinized (1mL per dish) for 1-5 minutes at 37°C. Trypsin was neutralized by the addition of RPMI containing 10% FBS, and all cells were removed from the dish and pelleted at 700rpm. Cell samples from each pellet were counted and assessed for viability using trypan blue exclusion. For CaCo2 cells, viability was confirmed with propidium iodide staining and analysis via flow cytometry. Pellets were washed with HBSS and stored at -20°C until Western blot analysis. For Western blots, a minimal volume of loading buffer containing SDS was added to each cell pellet, and this mixture was boiled for 10 minutes. Samples were separated on a 15% SDS-PAGE gel which contained low mass p27 standards ranging from 25ng to 250 ng). Protein was transferred to nitrocellulose and blotted with monkey anti-SIV serum (a gift from Michael Murphey-Corb), washed, and blotted with horseradish peroxidase-conjugated goat anti-monkey antibody (Nordic Immunological Laboratories). Protein bands were detected with SuperSignal West Pico Chemiluminescent Solution (Pierce). Blots were analyzed via densitometry using

Quantity One software (Bio-Rad), with valid measurements falling within the linear portion of a standard curve.

Each uptake experiment contained 5 or 6 culture dishes per *C. perfringens* strain tested, and cells from each dish were loaded into separate lanes on the same SDS-PAGE gel. The average mass of p27 internalized was calculated for each *C. perfringens* strain in each experiment. For both cell lines used, the experiment was performed at least 3 times with similar internalization trends observed each time.

Dendritic cells ELISpot assay

Detection of interferon-gamma (IFN-γ) was performed using mouse IFN-γ ELISpot Kits from Mabtech as described in Chapter 2. To determine the percent of response due to MHC class I epitope presentation, MHC-blocking experiments were conducted as described in Chapter 2. The number of sfc from α-H-2D^d-treated samples was divided by the sum of sfc from α-H-2D^d-treated samples and α-I-A^d-treated samples and then multiplied by 100 to determine the percentage of response due to MHC class I.

Vaccination

Mice were inoculated using an infant enteral feeding tube inserted down the esophagus into the stomach, where a total volume of 500μL was delivered. Two mice per group received either *Cp*-8K-p27 or *Cp*-p27, each expressing similar levels of p27 and each delivered with 25μg LT(R192G) adjuvant (provided by J. D. Clements), or PBS as a control. Vaccine was administered 3 times at 2 week intervals. 19 days after the final inoculation mice were sacrificed

and spleens were collected. Spleens were gently crushed with glass stoppers to release splenocytes, which were passed through nylon mesh, pelleted with centrifugation at 4°C at 1200 rpm for 5 minutes, treated with 3-5mL red blood cell lysis buffer (Sigma) for 5 minutes with a gentle shake after 3 minutes, and washed with RPMI containing serum. $2x10^5$ splenocytes were assayed using mouse IFN-γ ELISpot Kits from Mabtech in a volume of 200μL ELISpot media (RPMI-1640 with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 0.1mM non-essential amino acids, 2mM L-glutamine, 10mM HEPES, and 1mM sodium pyruvate). Samples were stimulated with two separate pools of SIV mac239 15-mer peptides (NIH AIDS Research and Reference Reagent Program) covering the majority of SIV p27 with peptides 5265 through 5298 of SIV gag with each peptide at a concentration of 5μg/mL. Each sample also included a background control in which a concentration of DMSO equivalent to that in the peptide pools was added to the well. As a positive control, each sample was stimulated with 1mg/mL concanavalin A. All treatments for all samples were plated in triplicate. Cells were incubated at 37°C for 24h. Detection and quantitation of IFN-γ sfc was performed as described in Chapter 2. These results were compared with IFN-γ sfc values determined from mice who received 10^9 pfu of Ad-p27 delivered in 50μL into the quadriceps muscle using a 26G needle and were sacrificed 2 weeks later.

Statistics

Values of *p* were determined using 1-tail two-sample Student's t-test with unequal variance.

3.5 RESULTS

3.5.1 Construction of *C. perfringens* Expressing PTD-conjugated SIV p27

C. perfringens expressing p27 conjugated to either PTD-5 or 8K were constructed as described in *Materials and Methods* (section 3.4) using the standardized pJRC200 expression plasmid cloning method established during the engineering of *Cp*-p27 [306]. Expression of p27 with each PTD conjugate was confirmed by Western blotting in sporulated cultures of each conjugate strain (Figure 7). The level of p27 expression was not significantly affected by inclusion of either PTD conjugate (Figure 7).

3.5.2 Maturation of BMDCs Exposed to PTD-Conjugated *C. perfringens* Strains

C. perfringens strains expressing each PTD conjugate were then evaluated for their abilities to stimulate dendritic cell (DC) maturation. Like *Cp*-p27, *Cp*-PTD-5-p27 and *Cp*-8K-p27 conjugate strains enhanced expression of CD80, CD86 and CD40 costimulatory molecules, as well as MHC class II (Figure 8 *A&B*). Phagocytosis of dextran was decreased in DCs after exposure to conjugate strains, similar to wild type *Cp*-p27 (Figure 8 *C*).

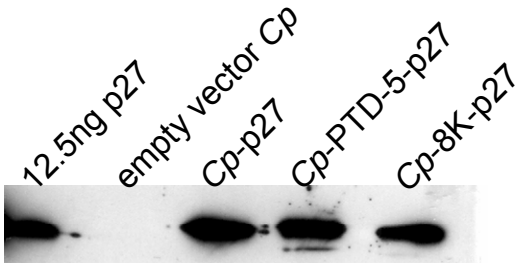


Figure 7. Expression of PTD-conjugated p27 from *C. perfringens*
Samples of sporulated *C. perfringens* expressing unconjugated p27 (*Cp*-p27), p27 conjugated to PTD-5 (*Cp*-PTD-5-p27), or p27 conjugated to 8K (*Cp*-8K-p27) were probed via Western blot using SIV-specific antisera. Control lanes contained 12.5ng purified p27 or *C. perfringens* carrying an empty expression plasmid (empty vector *Cp*).

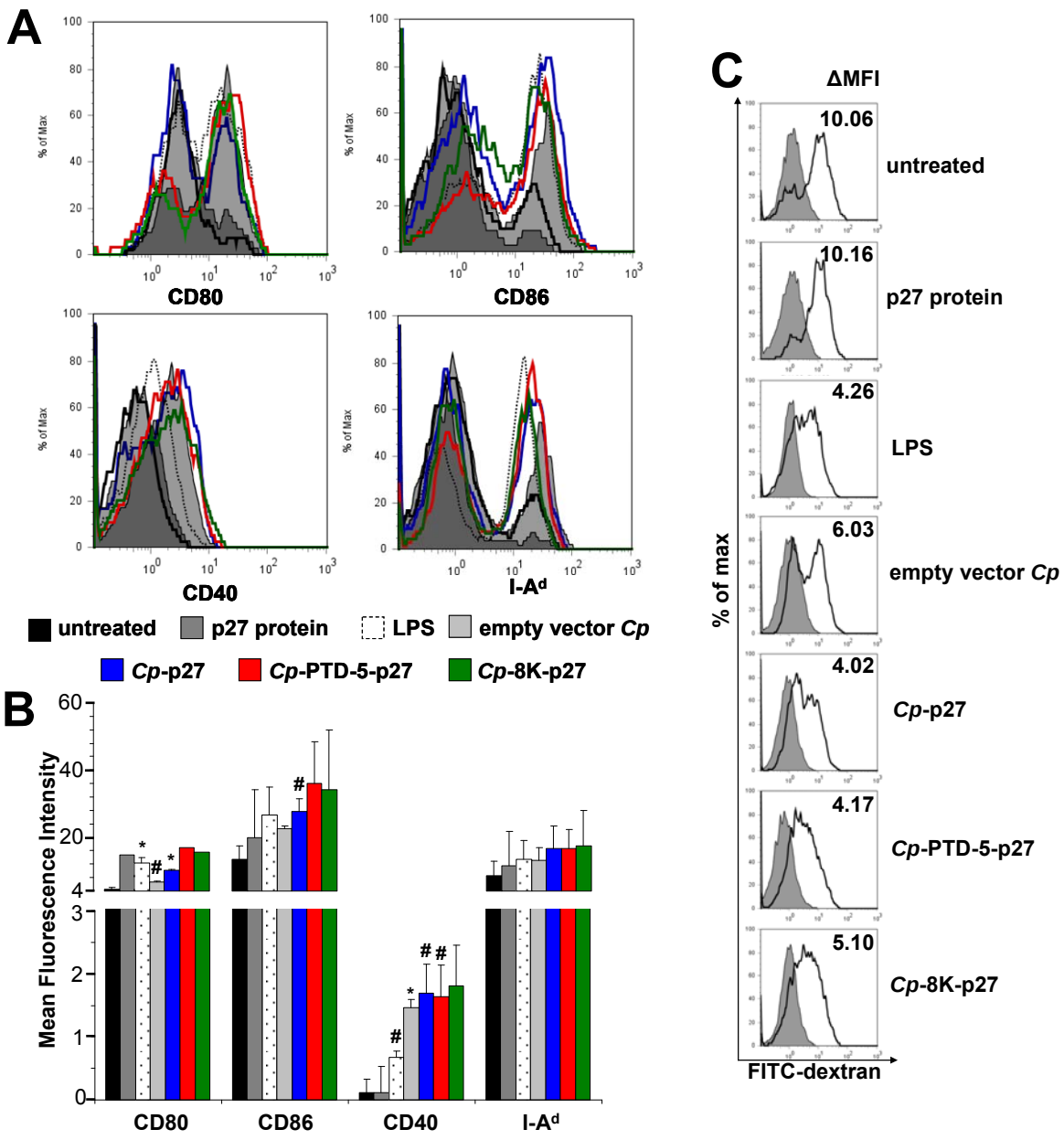


Figure 8. Dendritic cell maturation following exposure to *C. perfringens* expressing PTD-conjugated SIV p27
Bone marrow-derived dendritic cells were exposed to *C. perfringens* expressing unconjugated SIV p27 (*Cp*-p27, blue), PTD-5-conjugated p27 (*Cp*-PTD-5-p27, red), 8K-conjugated p27 (*Cp*-8K-p27, green), or empty-vector *C. perfringens* (light gray). As negative controls, cells were left untreated (black) or exposed to purified p27 protein (dark gray). As a positive control, LPS was added to cells (dashed). Unfilled solid light curve represents isotype control. All histograms are pregated on $CD11c^+$ cells. *A*, Representative flow cytometric histograms from 2 to 5 independent experiments showing the level of expression of maturation markers. *B*, Average mean fluorescence intensity + standard error of the mean of maturation marker expression. Values of *p* were determined by Student's t-test against untreated cells. **p*<0.05; #*p*<0.10. *C,* Flow cytometric histograms show FITC-dextran internalization at 4°C (grey filled curve) or 37°C (unfilled curve). Average difference in mean fluorescence intensity (ΔMFI) is shown in the upper right hand corner of each histogram, with ΔMFI calculated by subtracting the 4°C MFI from the 37°C MFI.

3.5.3 Uptake of PTD-Conjugated p27 Delivered by *C. perfringens*

To determine whether conjugation to either PTD sequence enhanced uptake of p27, two cell lines were exposed to bacteria expressing equivalent levels of p27, and internalized protein per cell was quantified by Western blot. To examine the effect of PTD conjugation on internalization of p27 by both DCs and epithelial cells, the murine dendritic cell line DC2.4 and human colon epithelial cell line CaCo2 were exposed to *C. perfringens* expressing wild-type or PTD-conjugated p27. In both cell types, more p27 was detected in cells treated with PTD conjugate strains than wild type, with $p<0.10$ (Figure 9). In 6 of 10 experiments, there was consistently more p27 internalization by DC2.4 and CaCo2 cells following exposure to the 8K-p27 conjugate as compared to the PTD-5-p27 conjugate.

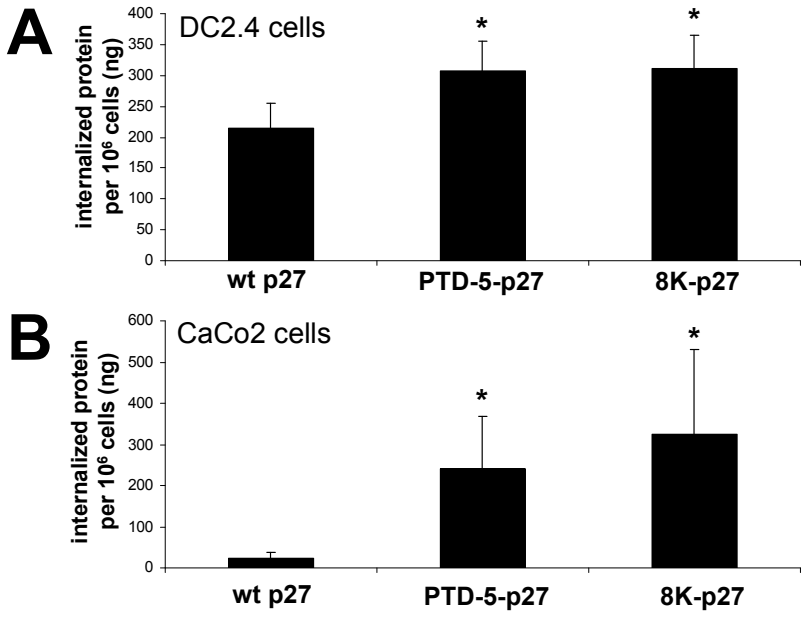


Figure 9. Internalization of *C. perfringens*-expressed SIV p27 conjugated to PTD sequences
DC2.4 (*A*) or CaCo2 (*B*) cell line cells were exposed to *C. perfringens* expressing unconjugated SIV p27 (wt-p27), PTD-5-conjugated p27 (PTD-5-p27), or 8K-conjugated p27 (8K-p27). Internalized protein was detected by quantitative Western blot and normalized to 10^6 cells. Graphs are representative of at least 3 independent experiments per cell line. Bars represent average internalized protein from 6 samples + standard error of the mean. Values of p were determined by Student's t-test against wt-p27-treated cells. *$p<0.1$.

Table 4. Viable cells (in millions) remaining after treatment with vaccine constructs in wells seeded with equal numbers of CaCo2 cells

	vaccine construct used for treatment		
	***Cp*-p27**	***Cp*-PTD-5-p27**	***Cp*-8K-p27**
trial 1	19.12	3.89	6.05
trial 2	20.49	3.44	3.34
trial 3	7.45	1.83	0.73

It is important to note that the viability of cells was also assessed after treatment with bacteria. Viability of DC2.4 cells was not altered. However, massive killing of CaCo2 cells was observed following treatment with PTD conjugates, while cells treated with the unconjugated construct in the same experiment maintained high viability (Table 4).

3.5.4 Functional Capacity of BMDCs Exposed to *C. perfringens* Expressing PTD-Conjugated p27

It was next determined whether the p27-specific immune stimulatory capacity of DCs was enhanced following exposure to PTD conjugates using a p27-specific IFN-γ ELISpot assay. Similar to *C. perfringens* expressing wild-type p27, a strong p27-specific response was stimulated by both PTD conjugate vaccines ($p<0.01$ compared with empty vector vaccine) (Figure 10 *A*). No significant difference in immune stimulation between wild-type and PTD-conjugated vaccine strains was observed. In addition, the percentage of p27 epitope presentation via MHC class I was not improved using *C. perfringens* expressing either PTD conjugate compared to *C. perfringens* expressing unconjugated p27 (Figure 10 *B*).

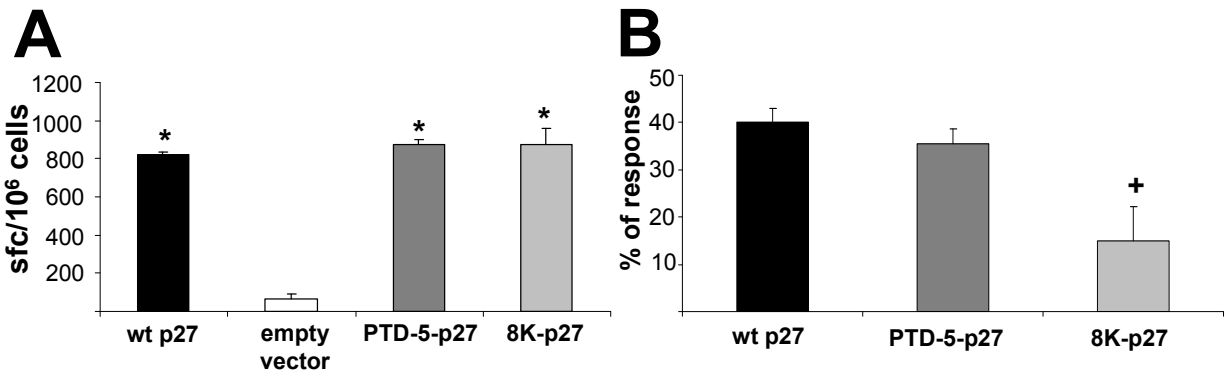


Figure 10. Effect of PTD-conjugation to p27 on BMDC-mediated IFN-γ stimulation
IFN-γ ELISpot assays were performed on p27-specific splenocytes with BMDCs exposed to *C. perfringens* carrying an empty expression plasmid (empty vector) or expressing unconjugated p27 (wt-p27), PTD-5-conjugated p27 (PTD-5-p27), or 8K-conjugated p27 (8K-p27). *A*, Averages of triplicate samples are shown with error bars indicating standard error of the mean. *$p<0.01$ via Student's t-test against empty vector. *B*, The percentage of total IFN-γ due to MHC class I epitope presentation as determined by ELISpot with antibodies directed against MHC class I or II molecules. +$p<0.05$ against both wt-p27 and PTD-5-p27 via Student's t-test.

3.5.5 Immunogenicity of *C. perfringens* Expressing PTD-Conjugated p27

In vivo immunogenicity of the oral *C. perfringens* vaccine expressing 8K-conjugated p27 was tested in small groups of mice. Spleen cells were assayed for p27-specific IFN-γ T cell immune response in via ELISpot. As a control, other groups of mice were fed with PBS or *C. perfringens* expressing unconjugated p27. As shown in Figure 11, conjugation of p27 to 8K did not improve the splenic response to p27 induced by *Cp*-p27. Both *C. perfringens*-based vaccines produced splenic p27-specific responses lower than the PBS control group and over 100 times lower than adenovirus carrying p27, a systemically delivered vaccine well-known to stimulate immunity in the spleen.

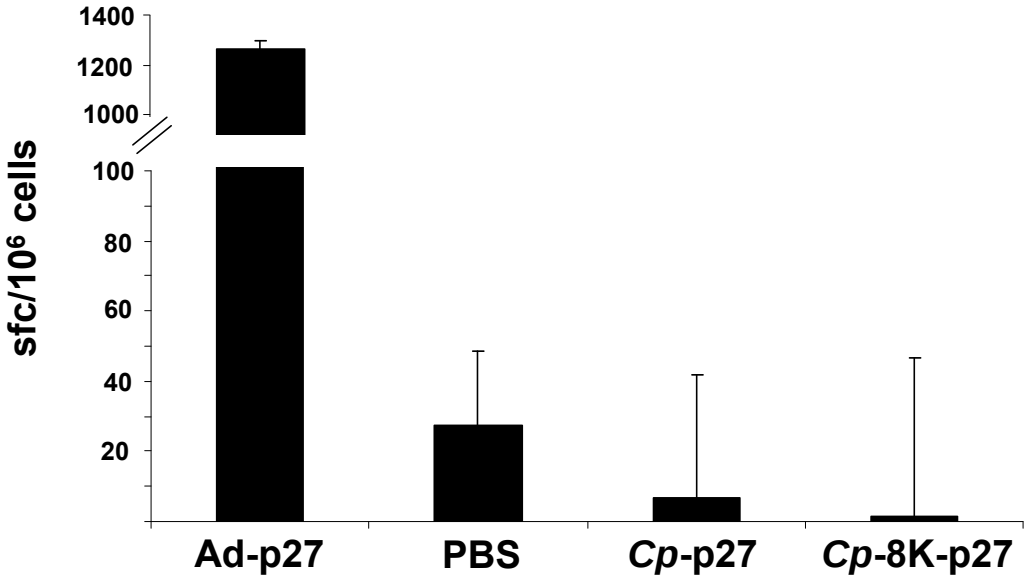


Figure 11. Effect of PTD conjugation to p27 on systemic immunogenicity *in vivo*
p27-specific IFN-γ ELISpots from spleen cells of mice inoculated intramuscularly with adenovirus expressing p27 (Ad-p27) or orally gavaged with PBS, *C. perfringens* expressing p27 (*Cp*-p27), or *C. perfringens* expressing 8K-conjugated p27 (*Cp*-8K-p27). Averages of at least 2 mice per group are shown with error bars representing standard error of the mean.

3.6 DISCUSSION

Since the dose of antigen delivered to intestinal immune tissue and the intracellular processing of antigen internalized by DCs have effects on the resultant immune response to the antigen, engineering antigen for ideal delivery and processing is important to achieve a maximal immune response. It was hypothesized that including a PTD sequence conjugated to vaccine-delivered p27 would both increase antigen delivery into DCs and enhance DC presentation of p27 antigens on MHC class I. These improvements were anticipated to prime stronger cellular immune responses *in vivo* than using p27 without PTD peptide.

The results presented here demonstrate that, when compared with *C. perfringens* expressing unconjugated p27, conjugation of PTD to p27 in the *C. perfringens* vaccine enhanced *in vitro* internalization of p27 by epithelial cells and DCs without enhancing or inhibiting the ability to induce maturation of DCs. DCs exposed to vaccine containing PTD-p27 conjugate protein stimulated similar levels of p27-specific IFN-γ as the unconjugated vaccine strain. This response appeared to be mediated by both MHC class I and MHC class II in the case of the PTD-5 conjugate but primarily by MHC class II in the case of the 8K conjugate. A pilot study of immunization using the 8K conjugate strain displayed no improved splenic cellular immunity when compared with the wild type *Cp*-p27 strain.

Despite the trend towards higher levels of DC maturation and p27-specific IFN-γ production in samples exposed to PTD-conjugated vaccines, further evaluation of the *in vivo* response to PTD-conjugated *Cp*-p27 vaccine strains is not being pursued in light of several important implications from the literature and critical evaluation of the data.

First, *in vivo* responses that were improved by including PTD-conjugation in other reported systems have only been observed when *in vitro* studies showed a minimum of 4-fold enhanced internalization into cells [345, 346]. The current study shows significantly improved internalization, but the increase is only 1- to 2-fold in DCs, the major APC in the gut.

Second, the overall percentage of response mediated by MHC class I antigen presentation was not enhanced by PTD-5-conjugation to p27. While MHC class I- and MHC class II-mediated IFN-γ ELISpot assays demonstrated a slightly stronger MHC-class I-restricted response with the PTD-5-conjugated vaccine construct, the increase was not statistically significant and was less than 2-fold higher when compared with the unconjugated construct (data not shown). The differences observed in these experiments are not expected to overtly alter biological function *in vivo*. Finally, the overall IFN-γ production in these assays was not enhanced in PTD-conjugated vaccine-treated cells when compared with the unconjugated vaccine.

Third, *in vivo* testing of the 8K-p27 fusion construct against the unconjugated *Cp*-p27 vaccine showed very low responses to p27 in the spleen of mice. While other experiments in the Gupta lab have demonstrated that mice immunized with the vaccine construct maintained low IFN-γ p27-specific responses in spleen but showed a robust response in the Peyer's patches, in light of the previous points the lack of enhanced splenic response may simply be due to the fact that the PTD-conjugate is not effective enough *in vivo*.

Fourth, recent literature indicates that delivery of certain PTD-conjugated proteins may be cytotoxic to cells, particularly when used at high concentrations [347, 348]. In *in vitro* experiments in the current study, massive killing of CaCo2 cells treated with PTD-conjugated vaccine constructs was observed, while cells treated with the unconjugated construct in the same

experiment maintained viability. Conjugation of β-galactosidase, antimicrobial peptides, and NEMO (NF-κB essential modulator)-binding domain to PTD-5 and 8K have not been reported to cause cytotoxic effects on other cell types, including epithelial cells from cervical (HeLa cells) and airway (A549 and HBE144 cells) mucosal sites [288, 291, 344, 349]. Several other PTD peptides attached to various cargoes have been observed to be safely delivered to CaCo2 cells without disrupting the integrity of the cells [350-352]. Therefore, it was unexpected that PTD conjugation to p27 would damage CaCo2 cells. However, Szeto *et al*. have demonstrated that related protein cargoes delivered to CaCo2 cells via identical PTD sequences differentially target the mitochondria [353]. In their study, protein targeted to the mitochondrial matrix induced mitochondrial swelling which leads to apoptosis, whereas protein targeted to the inner mitochondrial membrane did not cause cytotoxicity. Targeting of cationic peptides to the mitochondria may be mediated by their positive charge, but the reason why certain proteins delivered via PTD sequences enter the mitochondrial matrix is unclear.

Furthermore, few studies have successfully demonstrated enhanced immunity using PTD conjugates, and conflicting results about immune response induction via PTD-conjugated vaccination have been reported [354-357]. The most effective *in vivo* uses of PTD conjugates are those that aim to selectively destroy cells in the context of diseases like cancer and autoimmunity [349, 358, 359]. This suggests that the toxic side effects of many PTDs, including PTD-5 and 8K, may be best used in applications other than vaccination.

These findings raise concern that including a PTD sequence in the *Cp*-p27 vaccine would compromise the epithelial barrier of the mucosa, thus putting a vaccinee at risk for complications. Indeed, it has recently been reported that the very breakdown of the gut mucosa contributes to HIV pathogensis [360]. Furthermore, since the *in vitro* findings have not

demonstrated sufficiently higher internalization or p27-specific IFN-γ stimulation rates with PTD-conjugated p27 expressed by *C. perfringens* compared with wild-type *Cp*-p27, it is unlikely that a significant enhancement of immunity would be achieved through vaccination with these PTD-conjugated constructs. The observed benefits of slightly enhanced antigen internalization and maintenance of processing through the MHC class I pathway with the use of PTD conjugation to p27 in the *C. perfringens* vaccine are far outweighed by the harmful effects likely to be exerted upon gut epithelial cells upon oral inoculation. Thus, further use of PTD-conjugated *C. perfringens* expressing SIV p27 as an oral vaccine is not warranted.

4.0 MUCOSAL ADJUVANT OPTIMIZATION FOR USE WITH *CP*-P27

4.1 PREFACE

The study described in this chapter was performed in Dr. Phalguni Gupta's laboratory. Ruth Helmus and Poonam Poonam conducted the first two animal experiments together with the assistance of Lori Caruso and Dr. Yue Chen in vaccine administration and sample collection and processing. The final three experiments were conducted by Ruth Helmus with technical assistance by Poonam Poonam, Lori Caruso, Dr. Yue Chen, and Dr. Cheng-Li Shen. Flow cytometry was performed with the assistance of Luann Borowski, Kim Stojka, and Edwin Molina from the laboratory of Dr. Charles R. Rinaldo, Jr. LT(R192G) was graciously provided by Dr. John D. Clemens, and Dr. Ted M. Ross provided guidance for CpG ODN sequence selection. Statistical advising was provided by Dr. Patrick Tarwater, and Dr. Marsha P. Cole provided instruction and the use of GraphPad. Animals were cared for by the University of Pittsburgh Division of Laboratory Animal Resources. Portions of this work were presented as a poster abstract at the 2007 Keystone Symposia on HIV Vaccines (Optimization of anti-SIV gut mucosal vaccine response using *Clostridium perfringens*, adenovirus, and synergistic mucosal adjuvants. R. Helmus, P. Poonam, L. Caruso, Y. Chen, and P. Gupta.)

4.2 ABSTRACT

The use of mucosal adjuvants can improve immunogenicity of a mucosally-delivered vaccine as well as direct immunity towards a Th1/cellular response or Th2/humoral response. *Cp*-p27 is a *Clostridium perfringens*-based vaccine designed to deliver intact simian immunodeficiency virus (SIV) p27 to the inductive immune tissue of the gut. In this study, the three mucosal adjuvants (cholera toxin (CT), mutant *E. coli* heat-labile enterotoxin (LT(R192G)), and unmethylated cytosine-phosphate-guanine oligodinucleotides (CpG ODNs)) were evaluated for use with orally delivered *Cp*-p27 in mice to optimize gut cellular immunity. At optimal doses, all adjuvants improved IFN-γ ELISpot responses in small intestine Peyer's patches (PPs) as compared to unadjuvanted *Cp*-p27. The use of LT(R192G) or CpG ODNs generated better responses than CT. A combination of LT(R192G) and CpG ODNs provided higher immunity than either used alone in both PPs and the lamina propria gut effector tissue. However, the functionality of $CD8^+$ T cells was better when 25μg LT(R192G) was utilized alone. Overall, the use of 25μg LT(R192G) provided the best quality of cellular immunity without significantly compromising the strength of immunity.

4.3 INTRODUCTION

Two of the largest challenges in generating an effective vaccine against human immunodeficiency virus (HIV) are the development of mucosal immunity and inducing effective $CD8^+$ and $CD4^+$ T cell responses. Since HIV is largely transmitted via mucosal tissue and immune tissue in the gut is a primary viral target in early infection and remains a reservoir in chronic infection [39, 64, 74, 75, 78, 79, 312-314], induction of mucosal immunity is important for effective prevention of HIV infection and propagation. Stopping infection at the mucosa may ablate infection or lower the severity of resulting infection; indeed, once virus has established infection outside of mucosal tissue, it cannot be eliminated from the host [36]. Using the monkey model of mucosal exposure to simian immunodeficiency virus (SIV), Murphey-Corb *et al*. observed that monkeys who were SIV-negative following viral challenge displayed SIV-specific $CD8^+$ T cells in the small intestine [79]. Additional studies have shown that the presence of vaccine-induced SIV-specific gut $CD8^+$ T cells can slow the appearance of SIV in the blood [120, 121]. This demonstrates the capacity for gut mucosal immunity to slow the establishment of productive SIV infection. In addition, stimulation of immunity at one mucosal site can impart immunity in distal mucosal tissues [249-251]. Therefore, further improvements to vaccine strategies that induce gut immunity against SIV may be able to prevent persistent SIV infection.

Since mucosal tissue possesses a natural propensity for immune tolerance via the generation of Th3 or T-regulatory cells [252], an effective mucosal vaccine must overcome this tolerance to induce a Th1 response that includes $CD8^+$ T cells that produce multiple antiviral

cytokines and display cytotoxic capacity [40, 42, 136, 138-140]. An effective immune response would also include multifunctional $CD4^+$ T cells that concurrently express interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) [136, 137, 142, 212]. These types of $CD8^+$ and $CD4^+$ T cell responses have been associated with viral control following infection [40, 42, 136-141].

In mucosal tissue, an immune response is generated only under inflammatory conditions, such as those generated by pathogenic organisms that damage cells of the gut wall. Adjuvants are molecules which exert inflammatory effects by mimicking the presence of pathogenic threats. When used in mucosal vaccination, adjuvants can both overcome tolerance and direct the immune response towards either Th1 or Th2. The strength and type of immune response generated in response to mucosal vaccines is determined by the form and amount of adjuvant delivered with the vaccine antigen. To date, few adjuvants have effectively displayed safety in humans while retaining adjuvanticity

One classical mucosal adjuvant is cholera toxin (CT), which is derived from *Vibrio cholerae* and helps drive Th2 and possibly Th1 responses [265, 361]. This adjuvant is very adept at overcoming mucosal tolerance, leading to the generation of antigen-specific antibodies against numerous proteins when delivered orally. Whole CT causes diarrhea in humans, but safe mutants are being developed [263, 265, 362-364]. LT(R192G), an inactive, nontoxic mutant of the *Escherichia coli* heat labile toxin, stimulates Th1 and Th2 responses when delivered orally with protein [365]. This adjuvant has been found to be safe for use in humans while still retaining adjuvant activity [263, 366]. Cytosine-phosphate-guanine oligodinucleotides (CpG ODNs) are synthetic oligonucleotides containing unmethylated CpG motifs reminiscent of bacterial DNA; they are recognized by toll-like receptor 9, and ensuing signal transduction leads

to Th1 responses [278, 367]. Whereas CT and LT(R192G) likely stimulate the immune system through downstream effects of cAMP, CpG ODNs trigger immune responses by activating signal transduction through Ras and MyD88 pathways. Many studies have demonstrated that combining CpG ODNs with one of the bacterially-derived adjuvants can enhance resulting immune responses by stimulating immunity through both pathways [368-371].

Clostridium perfringens expressing SIV p27 (*Cp*-p27) can effectively deliver p27 to gut mucosal immune tissue when delivered orally. Inclusion of adjuvants in the *Cp*-p27 vaccine formulation is expected to enhance the immune response induced following inoculation. Therefore, it is important to determine the optimal dose of mucosal adjuvants for use with the *Cp*-p27 vaccine. It is hypothesized that combinations of optimal doses of strong mucosal adjuvants delivered with *Cp*-p27 would improve the strength of cellular immunity as determined by IFN-γ production via ELISpot and the quality of immune response by monitoring intracellular cytokine production and cytotoxicity via flow cytometric analysis.

4.4 MATERIALS AND METHODS

Animals

Female Balb/c mice were purchased from Charles Rivers Laboratories, Inc. and housed in a pathogen-free facility in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee and federal regulations. Animals were used at 6 to 8 weeks of age.

Clostridium perfringens *vaccine strain* Cp-*p27*

Construction of the *Clostridium perfringens* vaccine expressing SIV p27 (*Cp*-p27) has been described previously [306]. Culture and isolation of sporulated *Cp*-p27 was performed as described in Chapter 2. Isolated sporulating bacteria were sonicated, and the concentration of p27 was enumerated by desitometry of quantitative Western blots. Vaccine was then stored at -140°C until use.

Oral adjuvants

Whole, active cholera toxin was purchased from Sigma and stored at 4°C until use. HPLC-purified S-thiolated CpG oligodinucleotides (ODN) were purchased from Sigma-Genosys and stored at -20°C until use. CpG ODN sequences were: CpG-A=TCCATGACGTTCCTGACGTT; CpG-B=TGACTGTGAACGTTCGAGATGA [372, 373]. The isolation of LT(R192G) has been described previously [258]. LT(R192G) was provided by Dr. John D. Clements and was reconstituted to 1mg/mL in sterile water and stored at 4°C until use.

Vaccination

Animals were inoculated via gavage using an infant enteral feeding tube inserted down the esophagus into the stomach, where a total volume of 500μL containing *Cp*-p27 ± adjuvant(s) was delivered. Each *Cp*-p27 dose contained approximately 250μg p27 as determined by quantitative Western blot. Three inoculations per trial were conducted, with inoculations at 2 week intervals.

Serum sample collection and processing

Pre-immune serum samples were acquired through venopuncture of the lateral saphenous vein, and blood was collected into heparinized capillary tubes. At sacrifice, blood samples were collected via heart puncture, and blood was allowed to coagulate on ice for several hours before separation. For all samples, serum was separated from blood by centrifugation at room temperature at 750xg for 20 minutes. Serum samples were stored at -70°C.

Fecal sample collection and processing

Four to seven days before sacrifice, fecal matter was collected from each mouse and processed on ice. Approximately 50-150mg of fecal material was obtained from each mouse. Samples were weighed and fully resuspended in Complete Mini protease inhibitor cocktail (1 tablet/mL PBS containing 0.1% sodium azide; Roche) by adding 1mL per 100 or 200mg of fecal matter. Resuspended samples were vortexed and then centrifuged at 13000rpm for 10 minutes in a tabletop centrifuge. Supernatant was assayed immediately.

Tissue collection and cell isolation

Mice were sacrificed about 10 to 15 days after the final inoculation. The small intestine was aseptically removed and processed. Before cell isolation, the intestine was rinsed with 1mL sterile PBS. This intestinal wash was pelleted to remove solid matter using a tabletop centrifuge at 13000 rpm for 10 minutes, and the supernatant was stored at -70°C.

Dissection of intestinal tissue was performed on ice, and when tissues and cells were not being treated enzymatically, they were kept on ice. Fatty tissue was removed from small intestine tissue, and the lumen was thoroughly flushed with PBS. Peyer's patches (PPs) were carefully removed with fine scissors and then washed with agitation at 37°C for 20 minutes in 30mL pre-warmed EDTA-DTE solution (PBS containing 10% bovine growth serum (HyClone), 1mM EDTA, and 1mM dithioerythritol) in a 50mL conical tube placed lengthwise on an orbital shaker. PPs were next rinsed repeatedly with Hank's buffered saline solution and incubated at 37°C in 6-well plate wells in 5mL pre-warmed collagenase solution (RPMI 1640 containing 10% fetal calf serum and 1mg/mL collagenase D (Roche)) without agitation. PP tissues were then gently crushed, and released cells were passed through nylon mesh, pelleted for 5 minutes with centrifugation at 4°C at 1200 rpm, and washed.

To isolate lamina propria (LP) cells, following PPs removal the remaining intestinal tissue was cut open longitudinally and cut into 0.5-1cm pieces, working on ice. Pieces were placed in pre-warmed EDTA-DTE solution in a 125mL Erlenmeyer flask and stirred at 37°C for 30 minutes. The medium was poured off, and pieces were vortexed in fresh warm EDTA-DTE solution four times at room temperature to remove residual epithelial cells. Pieces were then placed in fresh warm EDTA-DTE solution in the flask and stirred at 37°C for an additional 15 minutes. Typically the medium was clear after this step. However, if medium was cloudy after

this step, the vortexing step was repeated and tissue was again placed in fresh warm EDTA-DTE solution and stirred at 37°C for an additional 15 minutes. This was repeated until medium was clear after the 15 minutes of stirring. Tissue pieces were then rinsed repeatedly with RPMI containing 2% bovine growth serum to remove EDTA. Pieces were then stirred at 37°C in prewarmed collagenase solution in the flask for 30 minutes, after which time samples were observed for cloudiness in the medium, indicating LP cell release. If medium was not cloudy, sample was returned to 37°C for an additional 15 minutes of stirring. If medium was cloudy, collagenase-treated tissue pieces were gently crushed, and released cells were passed through nylon mesh, pelleted with centrifugation at 4°C at 1200rpm for 5 minutes, and kept on ice in serum-containing medium. Remaining tissue pieces were returned to fresh collagenase solution for additional treatment, repeating the stirring at 37°C and cell isolation steps just described. When all tissue was digested (typically after a total of 2 or 3 collagenase treatments), cells were washed, the cell pellet was resuspended in 12mL ice-cold 40% isotonic Percoll in 1xPBS, and the cell suspension was distributed equally into 3 15mL conical tubes (i.e. 4mL per tube). Each 4mL cell suspension was then underlayed with 2mL ice-cold isotonic Percoll (9 parts Percoll to 1 part 10x HBSS (v/v)). Tubes were then centrifuged at 1700rpm with no brake at 4°C for 20 minutes. The resulting interface was harvested and diluted ≥10-times into fresh RPMI containing serum, and these LP lymphocytes were pelleted for 10 minutes with centrifugation at 4°C at 1500 rpm.

IFN-γ ELISpot

Detection of interferon-gamma (IFN-γ) from freshly isolated cells was performed using mouse IFN-γ ELISpot Kits from Mabtech activated and blocked as described in Chapter 2. PPs

cells were plated with $2x10^5$ cells per well in a volume of 200µL ELISpot media (RPMI-1640 with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 0.1mM non-essential amino acids, 2mM L-glutamine, 10mM HEPES, and 1mM sodium pyruvate) in ELISpot plates. Because of lower yields, LP cells were generally plated at $5x10^4$ or $1x10^5$ cells per well in 200µL. Samples were stimulated with two separate pools of SIVmac 239 gag 15-mer peptides overlapping by 11 amino acids (NIH AIDS Research and Reference Reagent Program) covering the majority of SIV p27 with peptides 5265 through 5298 of SIV gag with each peptide at a concentration of 5µg/mL. Each sample also contained a background control where a concentration of DMSO equivalent to that in the peptide pools was added to the well. As a positive control, each sample was also stimulated with 1mg/mL concanavalin A. All treatments for all samples were plated in triplicate, except when low LP yields made this impossible, in which case at least two wells per treatment per sample were plated. Cells were incubated at 37°C for 24h. Detection of IFN-γ spot-forming cells (sfc) was performed as described in Chapter 2. When dry, sfc on plates were counted on an automated ELISpot reader. Background sfc values from background control wells were removed as appropriate, and sfc were normalized to 10^6 cells.

SIV p27-specific ELISA

EIA/RIA Plates were coated overnight at room temperature with recombinant SIV p27 isolated from *E. coli*. After blocking plates, serial dilutions of serum or undiluted samples of fecal extracts or intestinal washes were placed in wells and incubated at room temperature for 2h (serum) or at 4°C overnight (fecal extracts and intestinal washes). Plates were washed, and AKP-conjugated α-mouse IgG_1 or IgG_{2a} antibody or biotin-conjugated α-mouse IgA (BD Biosciences) was incubated in appropriate wells for 1h at room temperature. For IgA detection,

plates were washed and a secondary AKP-conjugated streptavidin (Sigma) was then incubated in appropriate wells for 30 minutes at room temperature. Finally, 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma) was used to detect p27-specific antibody, and optical density was read on a plate reader at 405nm. Background values from negative control wells on each plate were subtracted.

Surface, intracellular cytokine, and CD107a staining

FITC-α-CD107a (clone 1D4B), R-PE-α-CD8 (53-6.7), APC-α-IL-2 (JES6-5H4), PE-Cy7-α-IFN-γ (XMG1.2), Biotin-α-TNF-α (MP6-XT3), and APC-Cy7-streptavidin were purchased from BD Pharmingen. PE-Cy5-α-CD3 (clone 145-2C11) was purchased from BioLegend. Isotype control antibodies were purchased from the same manufacturers according to the fluorescent conjugate used.

Freshly isolated cells (maximum 10^6 cells) were cultured for 5 hours at 37°C in 96-well plates in 200μL growth media (DMEM with 10% fetal calf serum, 1mM sodium pyruvate, 2mM L-glutamine, 0.025M 2-mercaptoethanol, and 1.25mM HEPES) containing 5μg/mL α-CD107a antibody, 3μM monensin, 5μg/mL brefeldin A, and 5μg/mL peptides spanning the entire p27 protein. As a background control, one well of cells of each sample was cultured without peptide. As a positive control, samples were cultured with 50ng/mL phorbol myristate acetate and 1μg/mL ionomycin instead of peptide.

After the culture period, plates were cooled to 4°C overnight. Cells were surface stained for CD3 and CD8, washed with FACS buffer (PBS with 0.1% bovine serum albumin and 0.1% sodium azide), fixed in 4% paraformaldehyde, and permeabilized with FACS buffer containing 0.2% saponin. Following permeabilization, cells were intracellularly stained for IL-2, IFN-γ,

and TNF-α, washed, fixed, and stored in the dark at 4°C in FACS buffer. All samples were fully analyzed by flow cytometry within 12 hours of staining.

Flow cytometry and analysis

Stained cells were analyzed using a BD Canto flow cytometer. FCS files were analyzed using FlowJo version 7.2.2 (Tree Star, Inc.). Cells in the lymphocyte gate were gated on $CD3^+CD8^+$ or $CD3^+CD8^-$ cells, and gates for individual cytokines and CD107a were established with control cells stained only for CD3 and CD8. Cells in the cytokine and CD107a gates were analyzed with Boolean gating to generate the percentage of cells expressing each combination of functional markers. Background expression values were subtracted from peptide-stimulated values for each sample. Graphical representation of functionality was achieved using SPICE software kindly provided by Dr. Mario Roederer of the NIH VRC. For SPICE analysis, individuals with no p27-specific response were excluded. A threshold value of the 75% confidence values of negative percentages for each T cell subset was used.

Statistics

Statistical analyses were performed using GraphPad Prism version 4. Unless otherwise noted, values of p were determined assuming a nonparametric distribution and employing a Kruskal-Wallis test followed by the Dunn procedure to compare groups. Antibody titer values were first transformed to $\log_{10}$ before analysis. Results were considered significant if $p<0.05$.

4.5 RESULTS

4.5.1 Single Adjuvant Screening

Since adjuvants have been observed to stimulate immunity differently depending upon the vaccine with which they are delivered, the optimal adjuvant(s) and their doses needed to be established for their use with the *Cp*-p27 vaccine. To screen a variety of adjuvants at different doses for their ability to aid in priming gut cellular immunity, small groups of mice (3 per group) were orally inoculated with equivalent doses of *Cp*-p27 and varying doses of cholera toxin (CT), mutant heat-labile toxin (LT(R192G)), or unmethylated cytosine-phosphate-guanine oligodinucleotides (CpG ODN). Control groups consisted of mice vaccinated orally with *Cp*-p27 without adjuvants or with PBS. Cells from small intestinal PPs were assayed for p27-specific IFN-γ production using an ELISpot assay. As shown in Figure 12, the highest immune responses were observed in groups of mice that received 5μg CT, 1μg LT(R192G), or 50μg of each CpG ODN. In contrast, the mice that received 1, 5, or 10μg CpG ODNs or 10 or 50μg CT did not induce any significant cellular responses compared to the PBS-only or *Cp*-p27-only control groups. LT(R192G) at 1, 5, 25, or 50μg produced cellular responses higher than the control groups, although this was not statistically significant. Serum (IgG_1 and IgG_{2a}) and fecal extract and intestinal wash IgA levels were low in all groups, with no difference observed between vaccinated mice and the PBS control group (data not shown).

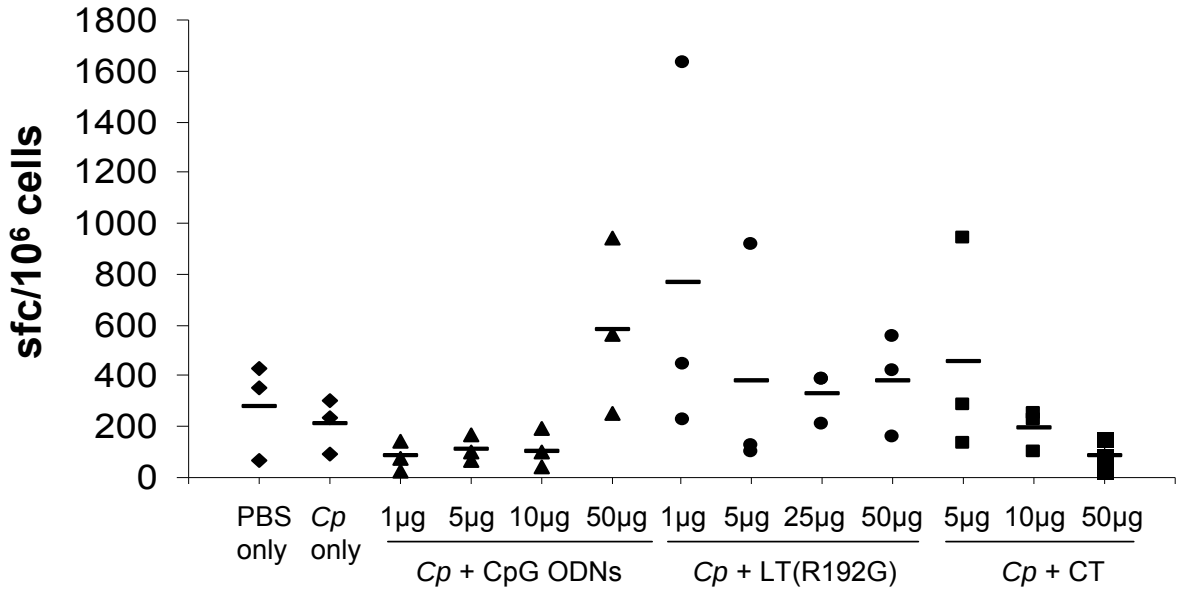


Figure 12. Adjuvant effects on p27-specific IFN-γ immune induction in Peyer's patches via oral *Cp*-p27 vaccination
Mice were immunized orally with *Cp*-p27 and various doses of adjuvants. Control animals received PBS or *Cp*-p27 without adjuvant. p27-specific IFN-γ ELISpot results from small intestinal PPs are shown. Results from individuals animals are represented by diamonds (control), triangles (CpG ODNs), circles (LT(R192G)), or squares (CT), and horizontal bars indicate the average response for each group.

4.5.2 Adjuvant Combinations

Since both CpG ODN and LT(R192G) primed greater cellular responses than CT when used with *Cp*-p27, efforts were concentrated on further study on CpG ODNs and LT(R192G) as adjuvants with *Cp*-p27. CpG ODNs and LT(R192G) in combination have been shown to stimulate stronger cellular immunity than when used alone [280, 368, 369]. In some of these studies, lower doses of adjuvants in combination were found to produce stronger cellular immune responses than higher doses of either adjuvant alone. In other studies, higher doses of one or both adjuvants were required in combination to induce strong immune responses. Thus, an experiment was initiated to determine the optimal dose of CpG ODNs for use with 1μg LT(R192G) to induce a cellular immune response following *Cp*-p27 vaccination.

Groups of mice were inoculated orally with *Cp*-p27 with various combinations of doses CpG ODNs with 1μg LT(R192G), and their cellular immune responses were compared with groups of mice that received each adjuvant dose alone or *Cp*-p27 alone. IFN-γ ELISpot assays of cells from PPs showed that 25μg CpG ODN provided better average cellular responses than 50 or 100μg CpG ODN (Figure 13). However, 50μg CpG ODN combined with LT(R192G) generated a better but statistically insignificant response than 25 or 100μg CpG ODN combined with LT(R192G). The highest response was in the group that received 1μg LT(R192G) and 50μg CpG ODNs. This was the only group that displayed a significantly higher cellular response compared with the group that received *Cp*-p27 without adjuvants. SIV p27-specific serum (IgG_1 and IgG_{2a}) and gut mucosal (IgA) antibody levels were also assessed, but antibody levels were low and no differences were observed between any of the groups (data not shown).

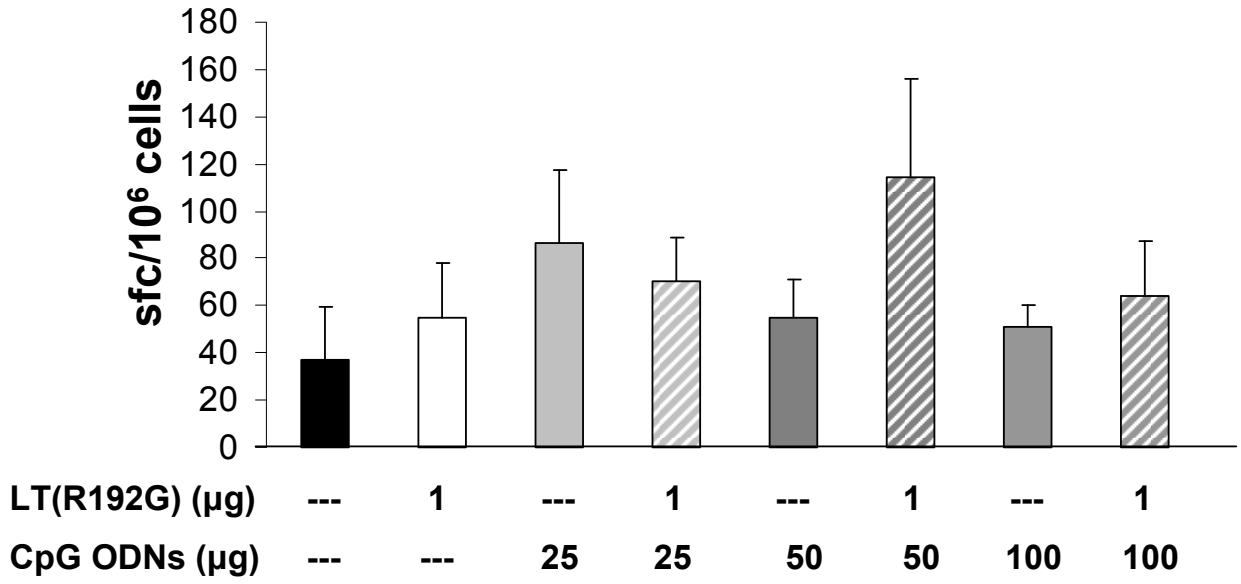


Figure 13. Effects of combinations of LT(R192G) and CpG ODNs on p27-specific cellular immunity to *Cp*-p27 in Peyer's patches
Mice were immunized orally with *Cp*-p27 with varying doses of CpG ODNs and/or 1μg LT(R192G) adjuvants. Control animals received *Cp*-p27 without adjuvant. Average p27-specific IFN-γ ELISpot results from small intestinal PPs of 4 mice per group are shown with error bars representing standard error of the mean.

4.5.3 Optimal LT(R192G) Dose

One unexpected result from the single adjuvant screening experiment was that the lowest dose of LT(R192G) produced stronger average responses than higher doses. LT(R192G) has been extensively characterized and is recommended to be used at 25μg per dose (J. D. Clements, personal communication). The single adjuvant screening experiment trial described currently (Figure 12) used a small number of mice in each group, and there was much variability within each group. For example, in the 1μg LT(R192G) group, one mouse displayed a cellular response 7-times that of the other two mice. To more clearly define which dose was optimal for use with *Cp*-p27, larger groups of mice (10 per group) were vaccinated with either 1μg or 25μg LT(R192G) and equivalent doses of *Cp*-p27. This experiment also included an assessment of both PPs and intestinal lamina propria (LP) cellular responses. As the effector tissue of the gut associated lymphoid tissue, the LP is the site where cells primed in the PPs and other inductive sites migrate and exert their effects. The response in the LP thus provides a more accurate representation of the level p27-specific cells that can act against an infection. After vaccination with *Cp*-p27, more cellular response was detected in the 25μg dose group than the 1μg group in both PPs and LP (Figure 14). This neared significance in PPs (p=0.0716 via Mann Whitney test) (Figure 14 *A*). As in the previous experiment, p27-specific serum IgG_1 and IgG_{2a} and intestinal wash IgA levels were very low in all groups and no differences were observed between groups (data not shown).

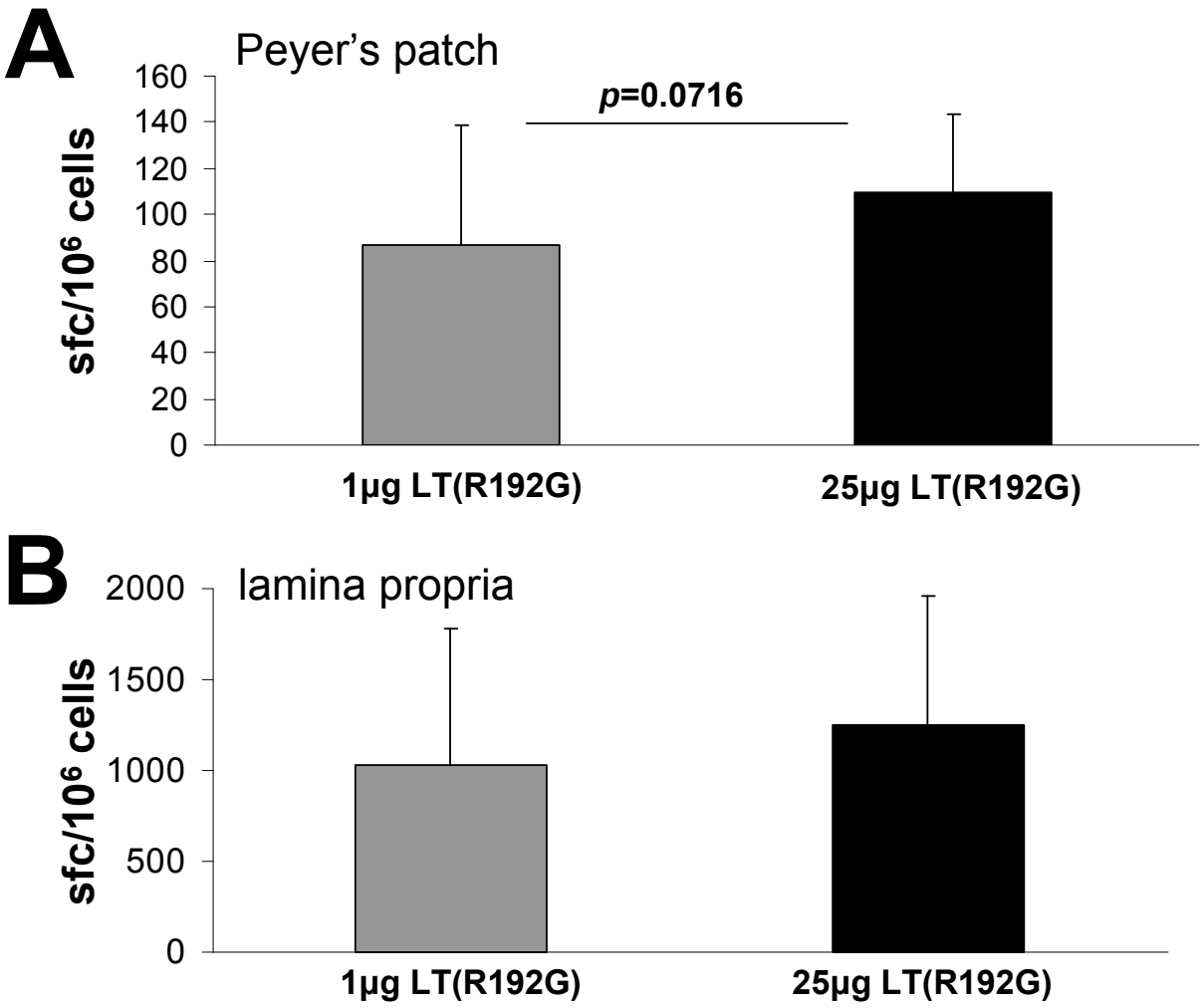


Figure 14. Optimal dose of LT(R192G) with *Cp*-p27 inducing p27-specific responses in gut inductive and effector tissues

Mice were immunized orally with *Cp*-p27 and 1µg or 25µg LT(R192G). Average p27-specific IFN-γ ELISpot results from small intestinal PPs of 10 animals (*A*) and lamina propria of 6 animals (*B*) are shown with error bars representing standard error of the mean. Value of *p* determined by the 1-tailed Mann Whitney test.

4.5.4 Inductive and Effector Responses of Optimal Adjuvant Combinations

An experiment was next conducted to confirm the enhanced response resulting from the use of both LT(R192G) and CpG ODNs using the 25µg dose of LT(R192G). The combination of 25µg LT(R192G) and CpG ODNs did not show a significantly higher level of IFN-γ ELISpot response in PPs than either adjuvant alone (Figure 15 *A*). In the LP, the average p27-specific IFN-γ ELISpot response was higher, although not significantly, in the LT(R192G) group than in the CpG ODN group, and the group that received both adjuvants displayed the strongest response (Figure 15 *B*).

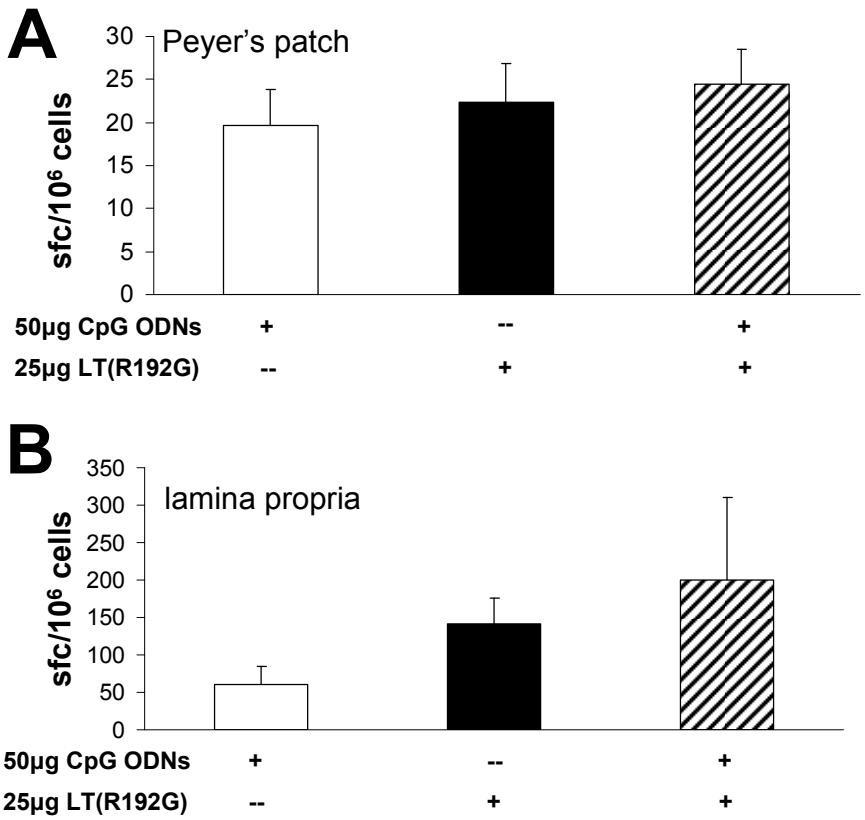


Figure 15. Effects of LT(R192G) and CpG ODNs on p27-specific cellular immunity to *Cp*-p27 in gut inductive and effector tissues
Mice were immunized orally with *Cp*-p27 with CpG ODNs and/or 25µg LT(R192G) adjuvants. Average p27-specific IFN-γ ELISpot results from small intestinal PPs of 10 animals (*A*) and lamina propria of 6 animals (*B*) are shown with error bars representing standard error of the mean.

A final vaccination trial was conducted to confirm these results and to determine the quality of the cellular response induced by each vaccine/adjuvant combination. The ability for $CD4^+$ and $CD8^+$ T cells to produce interleukin-2 (IL-2) and tumor necrosis factor-α (TNF-α) in addition to IFN-γ has been associated with better ability of these T cells to combat viral infections [40, 42, 136, 138-140]. The production of these cytokines was examined through intracellular staining followed by single-cell analysis in a flow cytometer. Therefore, PPs and LP cells from vaccinated mice were assayed with cell staining and flow cytometry in addition to IFN-γ ELISpot.

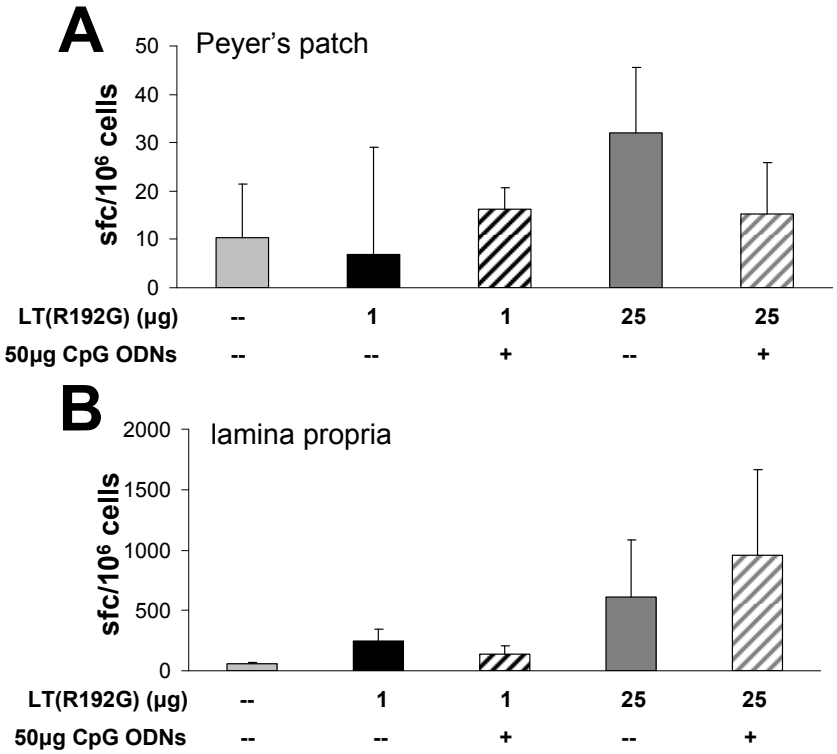


Figure 16. Effects of adjuvant type and dose on p27-specific cellular immunity to *Cp*-p27 in gut inductive and effector tissues
Mice were immunized orally with *Cp*-p27 with 1µg or 25µg LT(R192G) with our without CpG ODNs. Control animals received *Cp*-p27 without adjuvant. Average p27-specific IFN-γ ELISpot results from small intestinal PPs of 10 animals (*A*) and lamina propria of 6 animals (*B*) are shown with error bars representing standard error of the mean.

Like the data shown in Figure 15, the 25μg dose of LT(R192G) generated more p27-specific IFN-γ ELISpot response than the 1μg dose in both PPs and LP (Figure 16). The combination of CpG ODNs and 1μg LT(R192G) produced a greater average level of response in PPs than the LT(R192G) alone (Figure 16 *A*). The same result held true in the LP with the 25μg LT(R192G) dose (Figure 16 *B*). However, in PPs, CpG ODNs delivered with 25μg LT(R192G) resulted in a somewhat lower level of response than with just the LT(R192G) alone (Figure 16 *A*).

4.5.5 T Cell-Mediated Cytotoxicity Induced by Vaccination

As determined by CD107a surface staining, in $CD8^+$ T cells there was a trend towards recipients of LT(R192G) to have a larger percentage of cells displaying p27-specific cytotoxicity as compared with recipients of both LT(R192G) and CpG ODNs (Figure 17), though these differences were not statistically significant. Both PPs and LP $CD8^+$ T cells from the 25μg LT(R192G) group contained a much higher percentage of p27-specific CD107a-positive cells than all other vaccine groups (Figure 17).

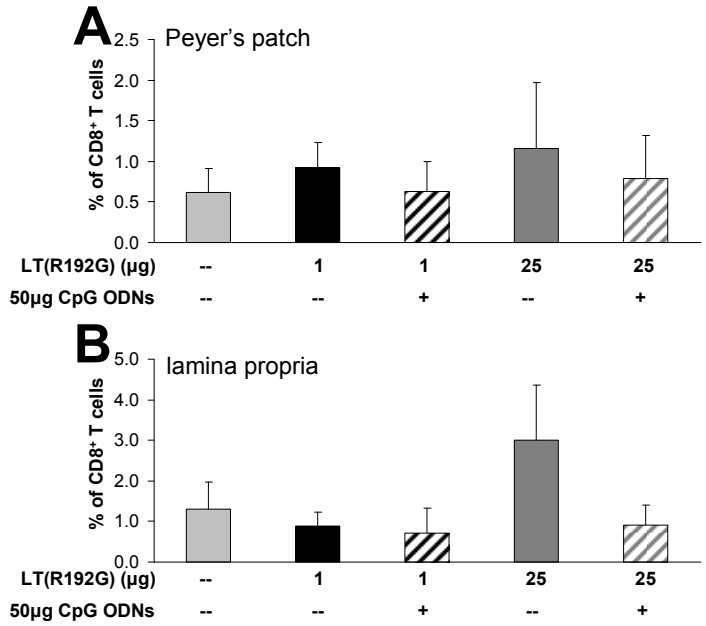


Figure 17. p27-specific degranulation in gut mucosal CD8$^+$ T cells following immunization with *Cp*-p27 and combinations of adjuvants
Cells from small intestinal Peyer's patches (*A*) and lamina propria (*B*) were surface stained for CD3 and CD8. SIV p27-specific surface expression of CD107a was detected on CD8$^+$CD3$^+$ cells via via flow cytometry. Bars represent the average values from 5-10 animals per group plus standard error of the mean. Light grey bars, no adjuvant; black bars, 1µg LT(R192G); black striped bars, 1µg LT(R192G) + CpG ODNs; dark grey bars, 25µg LT(R192G); dark grey striped bars, 25µg LT(R192G) + CpG ODNs.

4.5.6 Multi-Cytokine Analysis: Strength and Quality of Immune Responses

The percentages of CD3$^+$CD8$^+$ and CD3$^+$CD8$^-$ cells displaying any p27-specific cytokine response (IL-2, TNF-α and/or IFN-γ) were determined to evaluate the quality of response in each T cell subset. The percentage of cells displaying response to any cytokine was calculated. As the dose and number of adjuvants administered with *Cp*-p27 was increased, the response to any cytokine in LP CD8$^-$ T cells decreased (Figure 18). This was also the case in PPs CD8$^-$ T cells, with the exception that mice immunized using 25µg LT(R192G) and CpG ODNs displayed the best response (Figure 18). In PPs CD8$^+$ T cells, little difference in p27-specific cytokine responsiveness was observed between animals who received only LT(R192G) at either dose

(Figure 18). Use of CpG ODNs with either dose of LT(R192G) improved PPs $CD8^+$ T cell responsiveness, with recipients of 25μg LT(R192G) and CpG ODNs showing the largest percentage of p27-specific cells of all groups (Figure 18). $CD8^+$ T cells in the LP demonstrated greater percentages of p27-specific cytokine responsiveness when mice received 25μg LT(R192G) than 1μg or no adjuvant, and use of CpG ODN with LT(R192G) improved cytokine responsiveness (Figure 18). The highest $CD8^+$ T cell response in the LP was observed in the group that received 25μg LT(R192G) and 50μg CpG ODNs (Figure 18).

Percentages of p27-specific cells producing each cytokine varied depending on the dose and type of adjuvants administered. Compared with the no adjuvant control group, $CD8^-$ T cells from PPs displayed a significantly lower percentage of IFN-γ in mice immunized using 1μg LT(R192G), while immunization using 25μg LT(R192G) and CpG ODNs generated a significantly higher IFN-γ response, and a similar but statistically insignificant trend was also observed in PPs $CD8^+$ T cells (Figure 18). With either LT(R192G) dose, addition of CpG ODN slightly improved IFN-γ response in PPs $CD8^-$ T cells, whereas the opposite was true in LP $CD8^-$ T cells and very little difference was observed in LP $CD8^+$ T cells (Figure 18).

TNF-α levels were somewhat lower in both T cell subsets in PPs and LP in recipients of adjuvant than in mice who only received *Cp*-p27 (Figure 18). Use of CpG ODN with LT(R192G) improved the percentage of p27-specific TNF-α-producing cells compared with LT(R192G) only, although not significantly (Figure 18). In all cells from both tissues, IL-2-responsiveness was lower in adjuvant recipients than *Cp*-p27 only recipients, although slight improvements were observed in mice who received 25μg LT(R192G) in some cases (Figure 18). The combination of CpG ODN with LT(R192G) produced insignificantly lower percentages of IL-2 responsive cells in most cases (Figure 18).

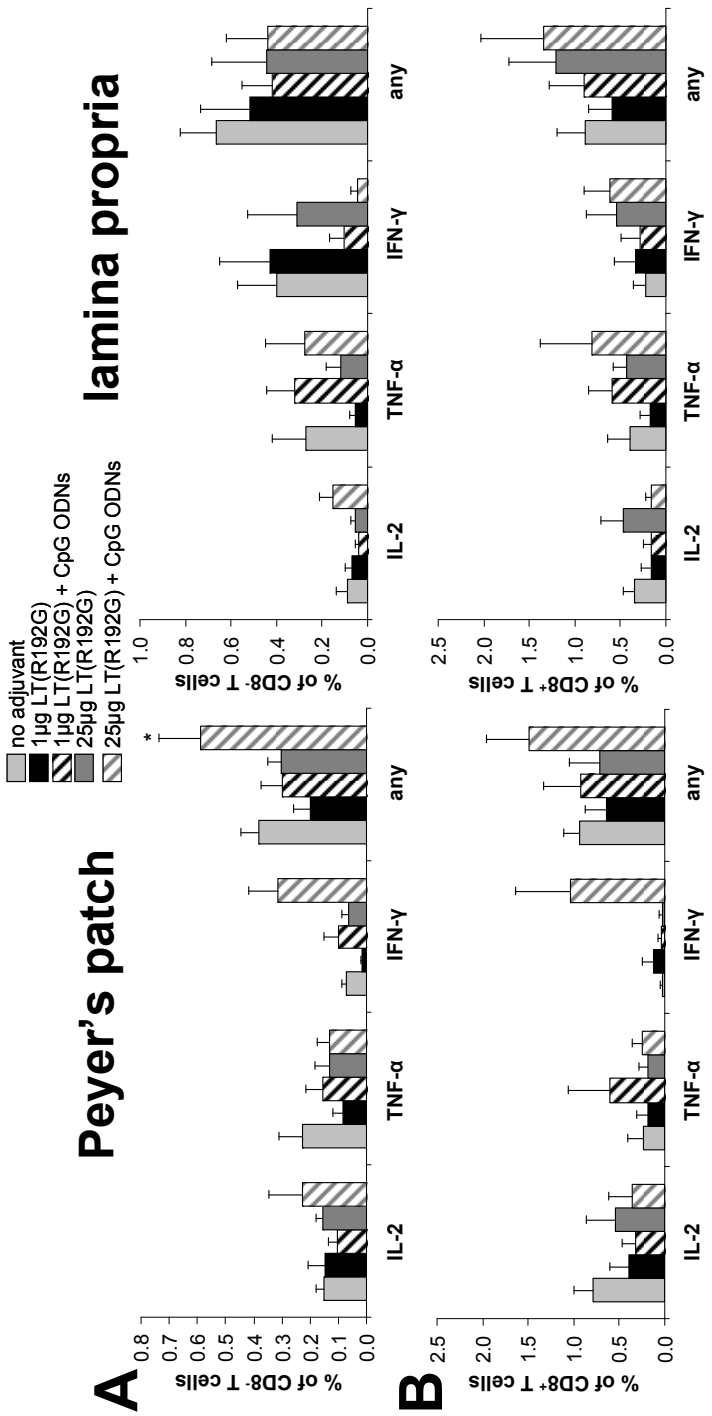


Figure 18. T cell p27-specific cytokine responsiveness in gut mucosal tissues following immunization with *Cp*-p27 and combinations of adjuvants

Cells from small intestinal Peyer's patches (*left*) and lamina propria (*right*) were surface stained for CD3 and CD8. SIV p27-specific IL-2, TNF-α, and IFN-γ production from $CD8^-$ (*A*) and $CD8^+$ (*B*) and $CD3^+$ cells was detected via intracellular staining and analysis via flow cytometry. Percentage of cells with any cytokine response was then determined ("any"). Bars represent the average values from 5-10 animals per group plus standard error of the mean. Light grey bars, no adjuvant; black bars, 1µg LT(R192G); black striped bars, 1µg LT(R192G) + CpG ODNs; dark grey bars, 25µg LT(R192G); dark grey striped bars, 25µg LT(R192G) + CpG ODNs. For "any" $CD8^-$ Peyer's patches cells, p=0.0597 via the Kruskal-Wallis test. *p<0.05 compared with 1µg LT(R192G) group.

The responses of each group were further evaluated for the percentage of cytokine response due to T cells with more than one function, as well as the level of multifunctionality (e.g. 2 or 3 functions), referred to as complexity. Overall, the percentage and complexity of multifunctionality in both $CD8^+$ and $CD8^-$ T cell subsets in PPs were not significantly affected by use of adjuvants with *Cp*-p27 inoculation. In the LP, any addition of adjuvant increased the percentage and/or complexity of multifunctionality compared with *Cp*-p27 inoculation (Figure 19). The exceptions to these trends occurred in the $CD8^-$ subset in the 1μg LT(R192G) group, which demonstrated percentages of multifunctional cells that were much higher in PPs and lower in LP compared with the *Cp*-p27-only group (Figure 19). The percentage of IL-2^+TNF-α^+ $CD8^-$ p27-specific PPs T cells in the 1μg LT(R192G) group was significantly higher than in the *Cp*-p27-only group (Figure 19). Although not significant, a similar trend was observed in the LP of mice who received 25μg LT(R192G), which displayed higher percentages of $CD8^-$ p27-specific response due to IL-2^+TNF-α^+ than mice who only received *Cp*-p27 (Figure 19). Recipients of 25μg LT(R192G) without CpG ODNs were the only group to display IL-2^+TNF-α^+IFN-γ^+ cells as part of their LP $CD8^+$ T cell response (Figure 19).

Immune response comparison between tissues within vaccine groups is also of interest. Inoculation with *Cp*-p27 generated responses that were slightly more multifunctional and complex in PPs than LP in both T cell subsets (Figure 19). In contrast, immunization using 25μg LT(R192G) with or without CpG ODNs stimulated higher percentages and complexity of multifunctionality in LP as compared to PPs in both T cell subsets (Figure 19).

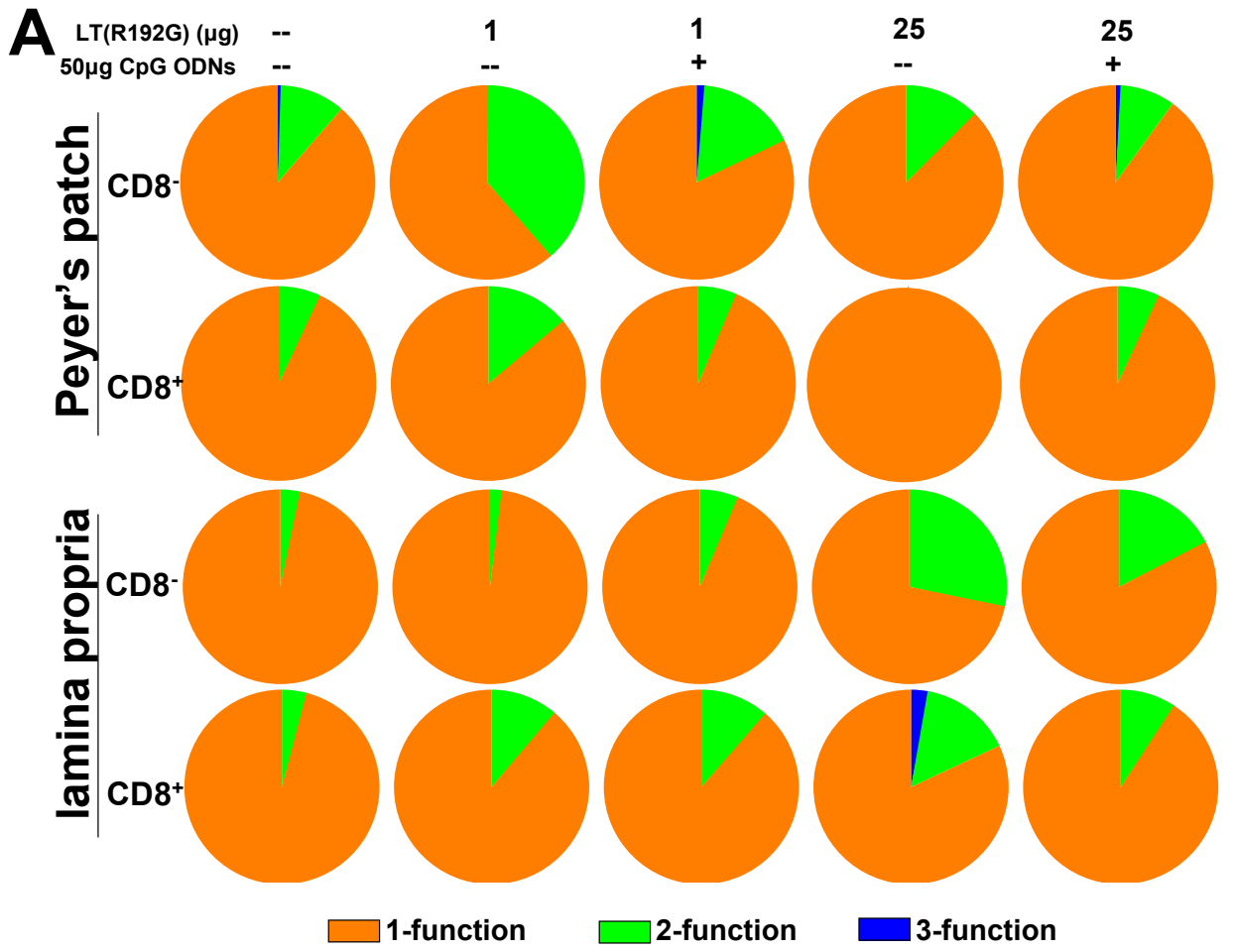
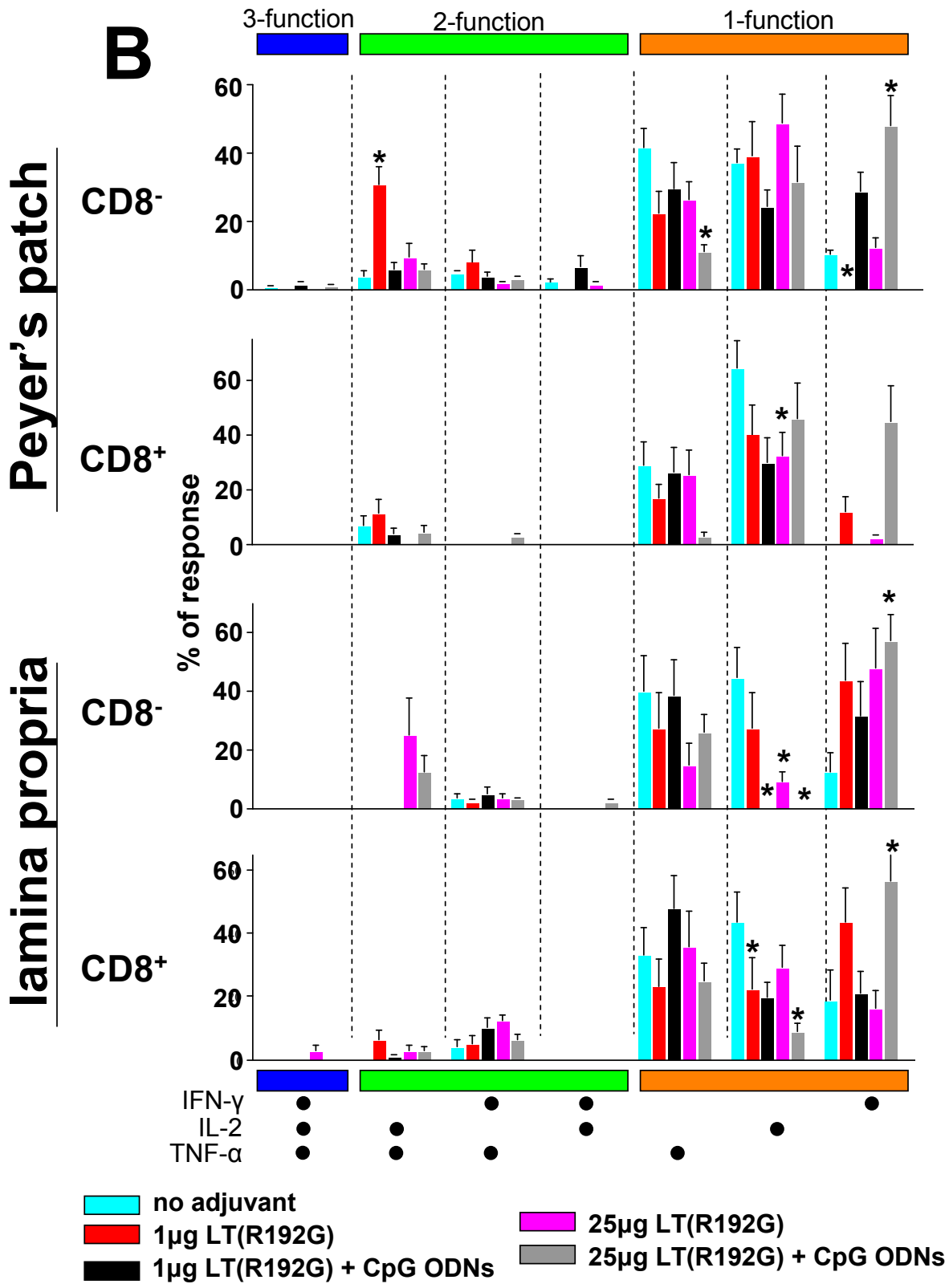


Figure 19. Multifunctional gut T cell cytokine responses in inductive and effector tissues resulting from vaccination

Data generated from Boolean-gated $CD8^-$ and $CD8^+$ $CD3^+$ cells stained for IL-2, TNF-α, and IFN-γ were analyzed for concurrent functionality using SPICE software. *A*, The average percentage of total response by Peyer's patch and lamina propria cells at each level of multifunctionality is represented by slices in pie charts. *B*, Bars representing average percent of total $CD8^-$ or $CD8^+$ p27-specific $CD3^+$ cell response in each tissue. *$p<0.05$ per Wilcoxen signed-rank test compared with the no adjuvant group.

(Figure 19 continued)



4.6 DISCUSSION

Mucosal vaccination typically requires the use of adjuvant to overcome tolerance and drive the induction of an appropriate immune response. Although *Cp*-p27 appears to possess inherent immunostimulatory capacity, likely due to the presence of its own unmethylated CpG DNA moieties and peptidoglycan, the coadministration of mucosal adjuvant with the bacteria was expected to alter the type of immunity induced by oral vaccination. Given the varying capacities of CT, LT(R192G), and CpG ODNs to drive the induction of $CD8^+$ and $CD4^+$ T cell responses, it was expected that including one or more of these adjuvants in the *Cp*-p27 vaccine milieu would affect the resultant immunity. Both 1μg and 25μg LT(R192G) was found to stimulate gut cellular responses in PPs and LP when delivered with *Cp*-p27. Although inclusion of CpG ODNs with LT(R192G) improved the strength of the response, the resultant cells displayed poorer quality of responses. The optimal adjuvant regimen to deliver with *Cp*-p27 to generate a strong, quality T cell response in gut effector tissue appears to be 25μg LT(R192G).

CT and LT and their mutants are related bacterial proteins that share many characteristics. However, their adjuvant capacities are not identical. Mucosal delivery of CT consistently results in the production of Th2-type cells in mouse models, particularly when delivered orally [262, 263]. On the other hand, LT generally induces the production of Th1-type cells in addition to Th2-type cells [263, 264]. These differences have been suggested to be due to the induction of 5-hydroxytryptamine by CT but not LT [263]. Other studies have demonstrated further mechanisms such as preferential induction of apoptosis in different T cells subsets by CT or LT [374-376]. Undoubtedly the reasons for different adjuvant characteristics

between CT and LT are complex and remain to be fully explained. The current study supports the concept that LT more potently stimulates cellular immunity (generally associated with Th1 responses), since mice who received LT demonstrated better antigen-specific IFN-γ responses than those who received CT.

Several mechanisms have been suggested for the adjuvant activity associated with orally administered CT and LT [263]. These include perturbation of the gut epithelial leading to increased permeabilization and therefore access of antigen to underlying cells; improved antigen presentation by cells such as dendritic cells (DCs); and altering B and T cell proliferation and immune maturation. The A subunit of both toxin proteins naturally possesses ADP-ribosylating activity, leading to an increase in cAMP after the B subunit of the toxin binds GM1-ganglioside on the host cell membrane and the toxin gains entry into the cytosol. The toxins prevent GTP hydrolysis by the α subunit of the GTP-binding protein family, thereby irreversibly activating adenylate cyclase and leading to watery diarrhea by activation of protein kinase A and the opening of membrane chloride channels. Other downstream effects of cAMP include activation of Raf/Ras and PI3-kinase, which may collectively result in the activation of transcription factors that can help promote T cell growth, including NF-κB [263, 377].

Uncoupling of the ADP-ribosylating activity from immunostimulatory activity has been demonstrated by mutating the enzyme active site or by preventing the dissociation of the A and B subunits of each toxin [258, 263, 364, 378-385]. Of these alterations of LT, the mutation of arginine to glycine at position 192 (LT(R192G)) has arguably maintained the highest level of adjuvant activity while displaying the lowest level of toxicity [258, 263]. Thus, in addition to the improved cellular immunity induced with the use of LT(R192G), on an entirely practical level

the use of LT(R192G) with the *Cp*-p27 vaccine is preferable to CT because it is more likely to applicable in humans.

A completely separate mechanism for immunostimulation is utilized by CpG ODNs [270, 272]. CpG ODNs interact with DCs by binding to intracellular Toll-like receptor 9, which recognizes the unmethylated cytosine-phosphate-guanine pattern in the context of certain flanking nucleotides. Signal transduction through MyD88-dependent and -independent pathways leads DC maturation and the production of cytokines that drive the formation of Th1-type responses. This tendency to drive cellular immune response formation helps to explain why the use of CpG ODNs as adjuvant to *Cp*-p27 inoculation generated the greatest PPs cellular responses in the current study. This response was observed with the high doses (≥25μg) of CpG ODNs but not low doses (<25μg). This is likely due to the fact that naked DNA, such as the CpG ODNs, is easily destroyed in the gastrointestinal system. Although the CpG ODNs utilized were generated with S-thiolation to ensure the highest level of stability, they remain targets for degradation. This will be important to consider for future development of this and other oral vaccines, since larger amounts of adjuvant involves greater overall price for the vaccine.

Because of the two distinct pathways through which CT/LT and CpG ODNs exert their adjuvant effects, it stands to reason that combining the adjuvants for simultaneous delivery could increase the strength of the resulting immune responses through additive effects. However, in practice this is not always the case. The results of the current study also demonstrated that only certain combinations of doses of LT(R192G) and CpG ODNs produced stronger responses than either adjuvant alone. It was observed that LT(R192G) can stimulate immunity that can be detected in LP, whereas the response formed in the presence of CpG ODNs was mainly detectable in PPs and seemed to lower the LP response when 1μg LT(R192G) was used.

Characterization of the nature of the immune response induced in mice using LT(R192G) with CpG ODNs as adjuvants has been examined using a variety of antigens delivered via many routes. McCluskie *et al*. observed that oral administration of LT(R192G) provided Th2-biased antibody response to antigen whereas administration of LT(R192G) and CpG ODNs displayed a more balanced Th1/Th2 response [368]. A similar trend was observed by Gerber *et a*l. after oral inoculation of mice with virus-like particles: immunogen delivered with CpG ODNs led to a predominantly Th1-type humoral response, while immunogen delivered with LT(R192G) provided more similar levels of Th1- and Th2-type antibodies [283]. Other studies have shown that this balanced LT-induced response is dependent upon the site of inoculation. Delivery of LT provided a Th1-bias when inoculation occurred intracolonically but not intragastrically [386]. Intranasal inoculation using LT or LT derivatives resulted in primarily Th2-type responses [282, 368, 369]. In addition, the dose of each adjuvant played a role in the type of immunity, with lower doses tending to bias responses more towards Th2-type responses and higher doses providing more Th1-type response, including more CTL activity. Furthermore, the Th1/Th2 response bias differed depending upon the protein antigen delivered with the adjuvant.

These studies exemplify the importance of determining the appropriate dose and adjuvants for use with each unique immunogen or vaccine. In the current study, the lower dose of LT(R192G) produced lower cellular responses than the higher dose in both PPs and LP. The quality of these responses differed as determined by intracellular cytokine staining, with both doses of LT(R192G) generating a more multifunctional response in $CD8^-$ cells than $CD8^+$ cells in PPs. The low dose (1μg) produced a more multifunctional response in $CD8^+$ cells than CD8- cells in LP, and the higher dose (25μg) displayed a more balanced $CD8^+/CD8^-$ response. This

suggests that LT(R192G) may stimulate both Th1- and Th2-type responses in PPs and LP, although further analysis beyond the scope of this project would be required to confirm this.

When either LT(R192G) dose was utilized with CpG ODNs or if no adjuvant was included, multifunctionality was reduced but T cells demonstrated more similar levels of multifunctionality in $CD8^-$ and $CD8^+$ populations in both gut tissues. IL-2 production of antigen-specific cells has been a hallmark of effective T cell activity [132], and it is interesting to note that more IL-2 production is observed in the LP $CD8^+$ cells of mice inoculated with only LT(R192G) than with both LT(R192G) and CpG ODNs. That the CD107a expression on $CD8^+$ T cells is also decreased in both PPs and LP when CpG ODNs are combined with LT(R192G) adds credence to the possibility that CpG ODNs are in fact negatively influencing the effector T cell immune response formation with this vaccine. Clearly, the strength and quality of cellular immune responses are affected by the dose and type of adjuvant used with *Cp*-p27, and overall the use of 25μg LT(R192G) provides the best quality T cell response without compromising the strength of response.

In conclusion, use of mucosal adjuvants at optimal doses improved gut cellular immune responses to *Cp*-p27 vaccination. The use of both LT(R192G) and CpG ODNs produced the strongest responses, but the best quality of response was generated by using only LT(R192G). For future studies directed at generating effective gut $CD8^+$ T cells using *Cp*-p27, vaccination should include 25μg LT(R192G) as an oral adjuvant.

5.0 MUCOSAL IMMUNE RESPONSE OF *CP*-P27 USING A PRIME-BOOST STRATEGY WITH ADENOVIRUS EXPRESSING P27

5.1 PREFACE

The study described in this chapter constitutes a manuscript in preparation. The experiments were performed in the laboratory of Dr. Phalguni Gupta by Ruth Helmus with the technical assistance of Poonam Poonam, Lori Caruso, Dr. Yue Chen, and Dr. Cheng-Li Shen in vaccine administration and sample collection and processing. Flow cytometry was performed with the assistance of Luann Borowski, Kim Stojka, and Edwin Molina from the laboratory of Dr. Charles R. Rinaldo, Jr. The adenovirus vaccine used in these experiments was generated in the University of Pittsburgh Vector Core by the laboratory of Dr. Andrea Gambotto in collaboration with Dr. Simon Barratt-Boyes [387]. LT(R192G) was graciously provided by Dr. John Clemens, and Dr. Ted M. Ross provided guidance for CpG ODN sequence selection. Statistical advising was provided by Dr. Patrick Tarwater, and Dr. Marsha P. Cole provided instruction and the use of GraphPad. Animals were cared for by the University of Pittsburgh Division of Laboratory Animal Resources. Portions of this work were presented as poster abstracts at AIDS Vaccine 2006 (A novel *Clostridium perfringens*-based SIV vaccine with adenovirus boosting induces strong systemic and gut mucosal immune responses. R. Helmus, P. Poonam, L. Caruso, Y. Chen and P. Gupta.) and the 2007 Keystone Symposia on HIV Vaccines (Optimization of anti-SIV gut mucosal vaccine response using *Clostridium perfringens*,

adenovirus, and synergistic mucosal adjuvants. R. Helmus, P. Poonam, L. Caruso, Y. Chen, and P. Gupta.)

5.2 ABSTRACT

Two major goals of HIV/SIV vaccination are to induce multifunctional cellular immunity and immunity in mucosal tissues such as the gut. An oral *Clostridium perfringens*-based vaccine (*Cp*-p27) that delivers SIV p27 to gut inductive immune tissue was evaluated for its ability to prime cellular immunity in the gut. Priming via oral vaccination with the *Cp*-p27 vaccine followed by boosting with a systemically delivered adenovirus expressing SIV p27 (Ad-p27) was performed to create a multifunctional gut immune response in the gut as well as systemic immune responses. Immunization with *Cp*-p27 alone generated multifunctional p27-specific cellular responses in small intestinal lamina propria (LP) but very little systemic response. In contrast, systemic inoculation with Ad-p27 generated systemic responses but a low cellular response with little multifunctionality in the LP. Priming with *Cp*-p27 and boosting with Ad-p27 resulted in the highest systemic and gut mucosal responses, as well as the highest degrees of p27-specific multifunctional $CD8^+$ T cells in the gut. These results indicate that priming of intestinal tissue with *Cp*-p27 can enhance the otherwise limited gut mucosal cellular response generated via systemic inoculation with Ad-p27.

5.3 INTRODUCTION

Growing evidence emphasizes the importance of the gut mucosa in HIV and SIV infection: $CD4^+$ T cells in the gut are rapidly infected and depleted soon after infection [64, 74, 75, 312-314]; $CD4^+$ T cell repopulation of the gut is prevented throughout infection [75-77]; cellular loss in the gut may promote bacterial translocation that contributes to generalized systemic immune activation [360]; and gut cells harbor virus throughout infection, thus serving as viral reservoirs [39, 78, 79]. In light of these findings, it is imperative to concentrate vaccine efforts on stimulating immune responses that prevent or curtail infection of the gut. Other mucosal tissues are important in the early steps of HIV infection, such as the rectum and vagina where the majority of HIV transmission occurs. Immunity induced in the gut-associated lymphoid tissue (GALT) may be able to afford immunity at these distal mucosal sites, also [250].

While inducing cellular immunity at mucosal sites is important in HIV/SIV vaccinology, the functionality of immune responses induced against HIV/SIV also affects the outcome of infection [40, 42, 136-140]. Functionality refers to a cell's ability to proliferate and carry out effector functions against a pathogen. For example, a correlation exists between control of HIV or SIV infection and the presence of $CD8^+$ T cells from peripheral blood mononuclear cells (PBMC) concurrently expressing surface CD107a as a marker of antigen-specific cytotoxicity and producing IFN-γ, TNF-α, IL-2, and/or MIP-1β [40, 42]. HIV-infected patients who slowly progress to AIDS disease display significantly higher percentages of HIV-specific PBMC $CD8^+$ T cells that demonstrate four or more of these functions than rapidly progressing patients [40]. PBMC-derived antigen-specific $CD8^+$ T cells concurrently producing IL-2 and IFN-γ are known

to be superior in their proliferative capacity and ability to eliminate antigen [132, 142-144, 148, 149]. While information about the functionality of gut cells during HIV/SIV infection is limited [80, 139, 140], it is expected that not just the level of response but also the quality of immunity, including multifunctional $CD8^+$ T cells, in this tissue will relate directly to the outcome of infection or effectiveness of vaccine-induced immune responses [388]. Thus, examining the functional cellular responses in mucosal tissues such as the gut may provide correlations between control of HIV/SIV infection and immunity, either natural or vaccine-induced.

To address the need for gut mucosal priming of multifunctional cellular responses, a recombinant *Clostridium perfringens* bacterial strain expressing SIV p27 (*Cp*-p27) is used for the development of an oral vaccine that induces cellular immunity in the gut. This orally delivered vaccine can deliver large amounts SIV p27 antigen to the terminal ileum of the small intestine, where gut inductive immune tissues known as Peyer's patches (PPs) are concentrated. Whereas strictly systemic administration of leading HIV/SIV vaccine candidates, such as adenovirus-vectored vaccines, can produce systemic immunity but have failed to produce strong mucosal immunity [216, 230], a number of studies have demonstrated that mucosal priming followed by systemic boosting is an effective vaccination strategy for producing immunity in both systemic and mucosal tissues [228, 241-246]. Thus, it is hypothesized that priming the gut mucosa with the oral *Cp*-p27 vaccine would enable the inductive (PPs) and effector (lamina propria) tissues of the gut to better respond to intramuscular immunization using adenovirus (Ad) serotype 5 carrying SIV p27 (Ad-p27). This report evaluates the strength and functionality of immune responses generated by these two vaccines using a prime-boost strategy in mice.

5.4 MATERIALS AND METHODS

Animals

Female Balb/c mice were purchased from Charles Rivers Laboratories, Inc. and housed in a pathogen-free facility in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee and federal regulations. Animals were used at 6 to 8 weeks of age.

Clostridium perfringens vaccine strain *Cp*-p27

Construction of the *Clostridium perfringens* vaccine expressing SIV p27 (*Cp*-p27) has been described previously [306]. Culturing of *Cp*-p27 was performed in the presence of 10μg/mL chloramphenicol at 37°C. Fresh 8h cultures of *Cp*-p27 grown in fluid thioglycolate broth (Difco) were used to inoculate modified Duncan-Strong medium [299] which was grown for 18h to induce sporulation. Sporulation of at least 90% of all bacteria in cultures was confirmed by phase-contrast light microscopy. Sporulated bacteria were isolated and washed with phosphate buffered saline (PBS) using centrifugation. Isolated sporulating bacteria were sonicated, and the concentration of p27 was enumerated by desitometry of quantitative Western blots. Vaccine was then stored at -140°C until use.

Adenovirus vaccine strain Ad-p27

E1/E3-deleted adenovirus serotype 5 expressing codon-optimized SIV *gag* p45 was constructed by the University of Pittsburgh Vector Core as described in Gao, *et al.* [387]. Determination of adenovirus particle concentration was performed by spectrophotometer analysis using a validated assay based on Adenovirus Reference Material obtained from the ATCC. Virus was stored at -70°C until thawing on ice for use.

Oral adjuvants

Whole, active cholera toxin (CT) was purchased from Sigma and stored at 4°C until use. 5μg CT was used for each vaccine dose. HPLC-purified S-thiolated cytosine-phosphate-guanine oligodinucleotides (CpG ODN) were purchased from Sigma-Genosys and stored at -20°C until use. CpG ODN sequences were: CpG-A=TCCATGACGTTCCTGACGTT; CpG-B=TGACTGTGAACGTTCGAGATGA [372, 373]. 50μg of each CpG ODN was used for each vaccine dose. The isolation of LT(R192G) has been described previously [258]. LT(R192G) was provided by Dr. John D. Clements and was reconstituted to 1mg/mL in sterile water and stored at 4°C until use. 1μg LT(R192G) was used for each vaccine dose.

Vaccination

Mice were inoculated with *Cp*-p27 via gavage and/or Ad-p27 intramuscularly. To gavage mice, an infant enteral feeding tube was inserted down the esophagus into the stomach. Here the *Cp*-p27 vaccine dose, consisting of bacteria expressing 250μg p27 plus adjuvants, was delivered in a total volume of 500μL in PBS. Control mice received 500μL PBS via gavage. 10^9 pfu of Ad-p27 were delivered in 50μL into the quadriceps muscle using a 26G needle. As a control, mice received 50μL PBS intramuscularly. Vaccines were administered at 3 week intervals.

Fecal sample collection and processing

Two to four days before sacrifice, fecal matter was collected from each mouse and processed on ice. Approximately 50-150mg of fecal material was obtained from each mouse.

Samples were weighed and fully resuspended in Complete Mini protease inhibitor cocktail (1 tablet/mL PBS containing 0.1% sodium azide; Roche) by adding 1mL per 100 or 200mg of fecal matter. Resuspended samples were vortexed and then centrifuged at 13000rpm for 10 minutes in a tabletop centrifuge. Supernatant was assayed immediately.

Tissue collection and cell isolation

Mice were sacrificed, and spleen, mesenteric lymph node (MLN), and small intestine were aseptically removed and processed. Spleens were gently crushed with glass stoppers to release splenocytes, which were passed through nylon mesh, pelleted with centrifugation at 4°C at 1200 rpm for 5 minutes, treated with 3-5mL red blood cell lysis buffer (Sigma) for 5 minutes with a gentle shake after 3 minutes, and washed with RPMI containing serum. Some cells were used fresh in subsequent assays, while others were stored in 1mL aliquots in 10% DMSO in FCS at -140°C until use. Fatty tissue on MLNs was carefully removed, and MLN tissues were gently crushed using glass stoppers to release cells, which were passed through nylon mesh and pelleted with centrifugation at 4°C at 1200 rpm for 5 minutes. Isolation of spleen and MLN cells was performed at room temperature with centrifugation using a centrifuge cooled to 4°C to preserve the integrity of isolated cells.

Dissection of intestinal tissue was performed on ice, and when tissue and cells were not being treated enzymatically, they were kept on ice. Before cell isolation, the intestine was rinsed with 1mL sterile PBS. This intestinal wash was pelleted to remove solid matter using a tabletop centrifuge at 13000 rpm for 10 minutes, and the supernatant was stored at -70°C. Fatty tissue was removed from small intestine tissue, and the lumen was thoroughly flushed with PBS. Peyer's patches and lamina propria cells were then isolated as described in Chapter 4.

Serum sample collection and processing

Pre-immune serum samples were acquired through venopuncture of the lateral saphenous vein, and blood was collected into heparinized capillary tubes. At sacrifice, blood samples were collected via heart puncture, and blood was allowed to coagulate on ice for several hours before separation. For all samples, serum was separated from blood by centrifugation at room temperature at 750xg for 20 minutes. Serum samples were stored at -70°C.

Thawing of splenocytes

Vials of cells were removed from -140°C storage, placed in a 37°C water bath for 2 minutes, and resuspended by dropwise addition of 1mL room temperature RPMI containing serum followed by addition of 8mL RPMI containing serum in 1mL aliquots, with mixing of cells after addition of each drop or aliquot. Cells were then pelleted for 5 minutes with centrifugation at 4°C at 1200 rpm.

IFN-γ ELISpot

Detection of interferon-gamma (IFN-γ) was performed using mouse IFN-γ ELISpot Kits from Mabtech. Plates were prepared as described in Chapter 2. Most cells assayed via ELISpot were freshly isolated. However, frozen-thawed spleen cells were used in the second experiment. Spleen, MLN, and PPs cells were plated with $2x10^5$ cells per well of the activated ELISpot plate in a volume of 200µL ELISpot media (RPMI-1640 with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 0.1mM non-essential amino acids, 2mM L-glutamine, 10mM HEPES, and 1mM sodium pyruvate). Because of lower yields, LP cells were generally plated at $5x10^4$ or

$1x10^5$ cells per well in 200μL. Samples were stimulated as with two separate pools of SIVmac 239 gag 15-mer peptides overlapping by 11 amino acids (NIH AIDS Research and Reference Reagent Program) covering the majority of SIV p27 with peptides 5265 through 5298 of SIV gag with each peptide at a concentration of 5μg/mL. Each sample also contained a background control where a concentration of DMSO equivalent to that in the peptide pools was added to the well. As a positive control, each sample was also stimulated with 1mg/mL concanavalin A. All treatments for all samples were plated in triplicate, except when low LP yields made this impossible, in which case at least two wells per treatment per sample were plated. Cells were incubated at 37°C for 24h. Detection of IFN-γ spot-forming cells (sfc) was performed as described in Chapter 2. When dry, sfc on plates were counted on an automated ELISpot reader. Background sfc values from background control wells were removed as appropriate, and sfc were normalized to 10^6 cells.

SIV p27-specific ELISA

EIA/RIA Plates were coated overnight at room temperature with recombinant SIV p27 isolated from *E. coli*. After blocking plates, serial dilutions of serum or undiluted samples of fecal extracts or intestinal washes were placed in wells and incubated at room temperature for 2h (serum) or at 4°C overnight (fecal extracts and intestinal washes). Plates were washed, and AKP-conjugated α-mouse IgG, IgG_1 or IgG_{2a} antibody or biotin-conjugated α-mouse IgA antibody (BD Biosciences) was incubated in appropriate wells for 1h at room temperature. For IgA detection, plates were washed and a secondary AKP-conjugated streptavidin (Sigma) was then incubated in appropriate wells for 30 minutes at room temperature. Finally, 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma) was used to detect p27-specific antibody, and

optical density was read on a plate reader at 405nm. Background values from negative control wells on each plate were subtracted.

Surface, intracellular cytokine, and CD107a staining

FITC-α-CD107a (clone 1D4B), R-PE-α-CD8 (53-6.7), APC-α-IL-2 (JES6-5H4), PE-Cy7-α-IFN-γ (XMG1.2), Biotin-α-TNF-α (MP6-XT3), and APC-Cy7-streptavidin were purchased from BD Pharmingen. PE-Cy5-α-CD3 (clone 145-2C11) was purchased from BioLegend. Isotype control antibodies were purchased from the same manufacturers according to the fluorescent conjugate used.

Freshly isolated cells (maximum 10^6 cells) were cultured for 5 hours at 37°C in 96-well plates in 200μL growth media (DMEM with 10% fetal calf serum, 1mM sodium pyruvate, 2mM L-glutamine, 0.025M 2-mercaptoethanol, and 1.25mM HEPES) containing 5μg/mL α-CD107a antibody, 3μM monensin, 5μg/mL brefeldin A, and 5μg/mL peptides spanning the entire p27 protein. As a background control, one well of cells of each sample was cultured without peptide. As a positive control, samples were cultured with 50ng/mL phorbol myristate acetate and 1μg/mL ionomycin.

After the culture period, plates were cooled to 4°C overnight. Cells were surface stained for CD3 and CD8, washed with FACS buffer (PBS with 0.1% bovine serum albumin and 0.1% sodium azide), fixed in 4% paraformaldehyde, and permeabilized with FACS buffer containing 0.2% saponin. Following permeabilization, cells were intracellularly stained for IL-2, IFN-γ, and TNF-α, washed, fixed, and stored in the dark at 4°C in FACS buffer. All samples were fully analyzed by flow cytometry within 12 hours of staining.

Flow cytometry and analysis

Stained cells were analyzed using a BD Canto flow cytometer. FCS files were analyzed using FlowJo version 7.2.2 (Tree Star, Inc.). Cells in the lymphocyte gate were gated on $CD3^+CD8^+$ or $CD3^+CD8^-$ cells, and gates for individual cytokines and CD107a were established with control cells stained only for CD3 and CD8. Cells in the cytokine and CD107a gates were analyzed with Boolean gating to generate the percentage of cells expressing each combination of functional markers. Background expression values were subtracted from peptide-stimulated values for each sample. Graphical representation of functionality was achieved using SPICE software kindly provided by Dr. Mario Roederer of the NIH VRC. For SPICE analysis, individuals with no p27-specific immune response were excluded. Threshold values were determined by calculating confidence values of negative percentages for each T cell subset. For PPs, the 90% value was used; for LP, the 75% value was used.

Statistics

Statistical analyses were performed using GraphPad Prism version 4. Values of p were determined assuming a nonparametric distribution and employing a Kruskal-Wallis test followed by the Dunn procedure to compare groups. Antibody titer values were first transformed to $\log_{10}$ before analysis. Results were considered significant if $p<0.05$.

5.5 RESULTS

5.5.1 Optimal Order of Mucosal and Systemic Inoculation in a Prime-Boost Vaccine Model

To determine whether the oral *C. perfringens* vaccine carrying SIV p27 (*Cp*-p27) performed better as a prime or a boost to a systemically delivered adenovirus serotype 5 encoding SIV *gag* vaccine (Ad-p27), groups of five mice were vaccinated three times with different regimens as described in schedule 1 in Figure 20 *A*, and resulting systemic and mucosal immunity was investigated. Group A was a control group, and the mice received PBS at each inoculation. Mice in group B received three oral doses of *Cp*-p27 at days 0, 21, and 42. Mice in groups C and D received one dose of Ad-p27 intramuscularly at day 0, and those in group D also received two subsequent oral *Cp*-p27 inoculations as a boost at days 21 and 42. Mice in group E received two oral inoculations of *Cp*-p27 at days 0 and 21 followed by an intramuscular boost with Ad-p27 at day 42. All *Cp*-p27 doses included CT and CpG ODNs as adjuvants. Eight to ten days after the final vaccination, mice were sacrificed. Peripheral blood serum, intestinal washes, and fecal samples were collected and examined for humoral immune responses. Cells isolated isolated from spleen, mesenteric lymph node (MLN), and small intestinal Peyer's patches (PPs) were examined for cellular immune responses.

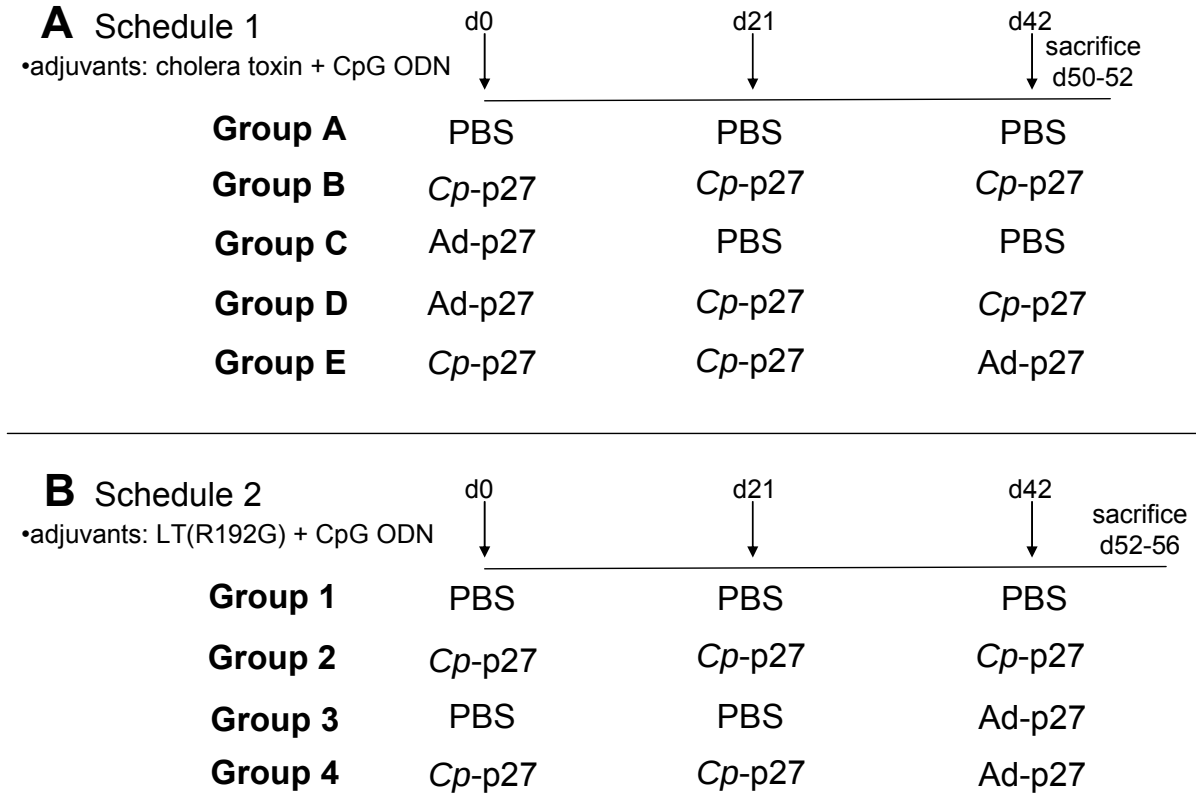


Figure 20. Vaccination schedules implementing *Cp*-p27 and Ad-p27
Female Balb/c mice 6 weeks of age were inoculated with orally delivered *Clostridium perfringens* carrying SIV p27 gene (*Cp*-p27) and/or intramuscularly delivered adenovirus serotype 5 carrying SIV *gag* gene (Ad-p27) at the indicated timepoints. Control inoculation with oral or intramuscularly delivered phosphate buffered saline was delivered as appropriate.

5.5.1.1 *Systemic Cellular Responses*

Animals in groups C, D, and E, who received Ad-p27, displayed cellular p27-specific IFN-γ production in spleen, while those from group B displayed a low response similar to the control group A. The strongest cellular response was observed in group E, which had received *Cp*-p27 followed by Ad-p27 boosting (Figure 21 *A*). The response in group E was statistically higher than group B and control group A ($p<0.001$ and $p<0.01$, respectively).

5.5.1.2 *Systemic Humoral Responses*

Serum antibodies specific for p27 were detected by ELISA (Figure 21 *B*). Mice from groups A and B displayed low antibody titers for both Th1-type (IgG_{2a}) and Th2-type (IgG_1) antibodies. The highest titers were observed in groups C and D. The titers in groups A and B were statistically lower than in group C (IgG_1) or groups C and D (IgG_{2a}) ($p<0.05$). Group E also displayed a higher IgG_{2a} titer than groups A or B, although this was not statistically significant. Antibody titers were probably lower in group E than in groups C and D because the mice in group E were sacrificed before antibody responses were matured, a process that normally takes approximately three weeks.

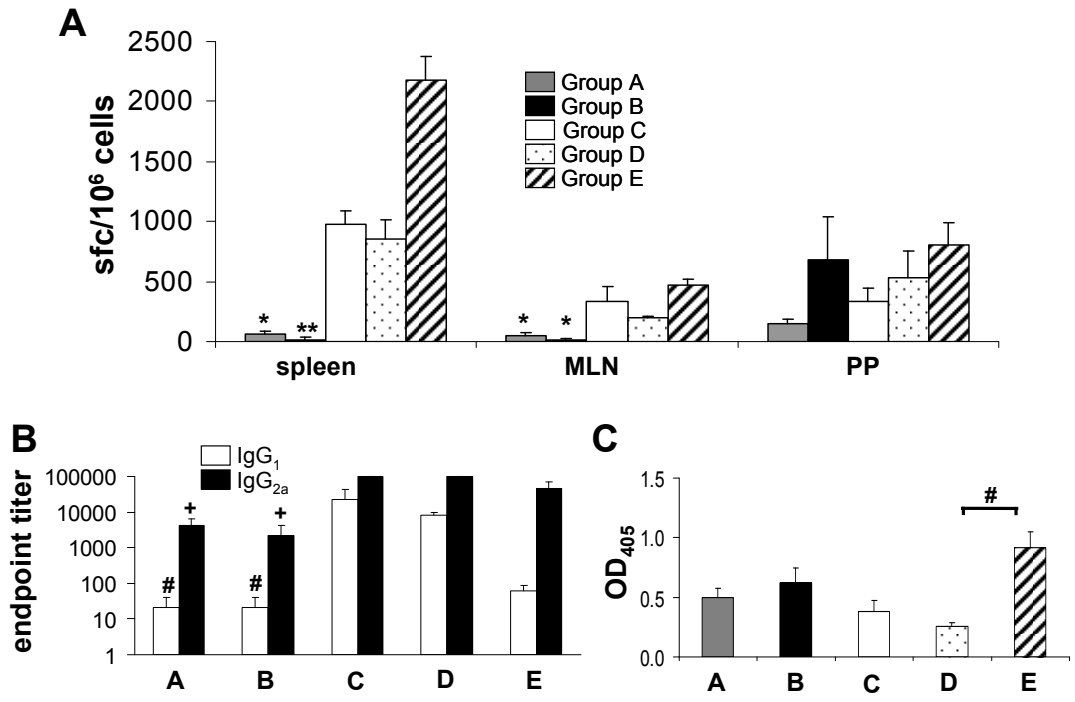


Figure 21. Immune responses stimulated by *Cp*-p27 and Ad-p27
Mice were immunized against SIV p27 with *Cp*-p27 and Ad-p27 vaccines as described in Figure 1. *A*, p27-specific IFN-γ ELISpot results from spleen, mesenteric lymph node (MLN), and small intestinal Peyer's patches (PPs). Grey bar, group A; black bar, group B; white bar, group C; speckled bar, group D; slashed bar, group E. Kruskal-Wallis test *p*-values: for spleen, $p=0.0003$; for MLN, $p=0.0005$. *$p<0.01$, **$p<0.001$ compared with group 5. Error bars represent standard error of the mean. *B*, Serum antibody titers detected by p27-specific ELISA. White bar, IgG_1; black bar, IgG_{2a}. For both isotypes, via the Kruskal-Wallis test of $\log_{10}$ transformed endpoint titer values, $p\leq0.001$. #$p<0.05$ compared with group D, +$p<0.05$ compared with both groups C and D. Error bars represent standard error of the mean. *C*, Intestinal wash OD_{405} values detected by p27-specific ELISA. Via Kruskal-Wallis test, $p=0.0107$. #$p<0.05$.

5.5.1.3 *Gut Mucosal Cellular Responses*

Mucosal tissues were also assayed for p27-specific IFN-γ production (Figure 21 *A*). Animals in group C group displayed minimal IFN-γ in PPs. This response was slightly heightened in group D. A p27-specific cellular immune response was observed in PPs from group B, and the highest response was in group E. These data indicate that priming with *Cp*-p27 stimulates a gut mucosal immune response that is improved by systemic Ad-p27 boosting.

Although a p27-specific IFN-γ was expected in the MLN, which drains the GALT (reviewed in [252] and [389]), no cellular response was detected in the MLN of mice in group B (Figure 21 *A*). Animals in group E, which received *Cp*-p27 followed by an Ad-p27 boost, displayed cellular responses in the MLN that were statistically higher than the PBS-only control group A as well as group B ($p<0.01$). Responses in MLN of group E were also higher than those in groups C and D, although not significantly.

5.5.1.4 *Gut Mucosal Humoral Responses*

SIV p27-specific IgA was detected in intestinal washes from groups B and E at higher levels than control group A. As shown in Figure 21 *C*, the highest response was observed in group E, which was statistically higher than group D ($p<0.05$) but not control group A. Low antibody responses were observed in groups C and D. Fecal samples did not contain detectable IgA in any group (data not shown).

5.5.2 Investigation of Vaccines' Contributions to Gut Immunity

These data suggest that orally delivered *Cp*-p27 is able to prime a gut mucosal immune response but not a systemic response. Furthermore, priming with *Cp*-p27 before Ad-p27 administration provided a stronger immune response than *Cp*-p27 boosting after Ad-p27 inoculation. In order to further explore the potential of *Cp*-p27 as a mucosal priming vaccine,

the strength and functional quality of cellular responses were measured in both the gut effector tissue, the lamina propria (LP), and the inductive PPs tissue after vaccination. In this second study, LT(R192G) and CpG ODN adjuvants designed for oral use [258, 372, 373] were employed with *Cp*-p27. This experiment was performed twice, with similar results.

In this experiment (see Figure 20 *B*, schedule 2), group 1 consisted of 5-6 control mice who received PBS only. Groups of ten mice were inoculated with oral *Cp*-p27 and/or intramuscular Ad-p27. Mice in group 2 received three inoculations of *Cp*-p27 at days 0, 21, and 42; mice in group 3 received one inoculation of Ad-p27 at day 42 (group 3); and mice in group 4 received two inoculations of *Cp*-p27 at days 0 and 21 followed by a boost of Ad-p27 at day 42. Mice were sacrificed 10 to 14 days after the last inoculation, which is the timepoint at which peak cellular responses to Ad-based vaccines are detected.

5.5.2.1 *Systemic Cellular Responses*

Spleen cells assayed by ELISpot for p27-specific IFN-γ production displayed no p27-specific response in mice immunized with *Cp*-p27 alone (group 2) or with PBS (group 1) (Figure 22 *A*). Mice who received Ad-p27 (group 3) displayed p27-specific splenic IFN-γ responses, significantly higher than group 2 ($p<0.05$). The cellular response was even was higher in group 4, with $p<0.01$ compared with group 2.

5.5.2.2 *Systemic Humoral Responses*

Group 2 displayed low titers of p27-specific IgG_1 and IgG_{2a} antibodies in the serum, similar to control group 1 (Figure 22 *B*). Groups 3 and 4 both had high titers of p27-specific IgG_1 and $IgG_{2a,}$ which were statistically higher than groups 1 and 2 ($p<0.001$). Within each group, the titers of Th1-type and Th2-type antibodies were similar, indicating no bias towards any one antibody isotype.

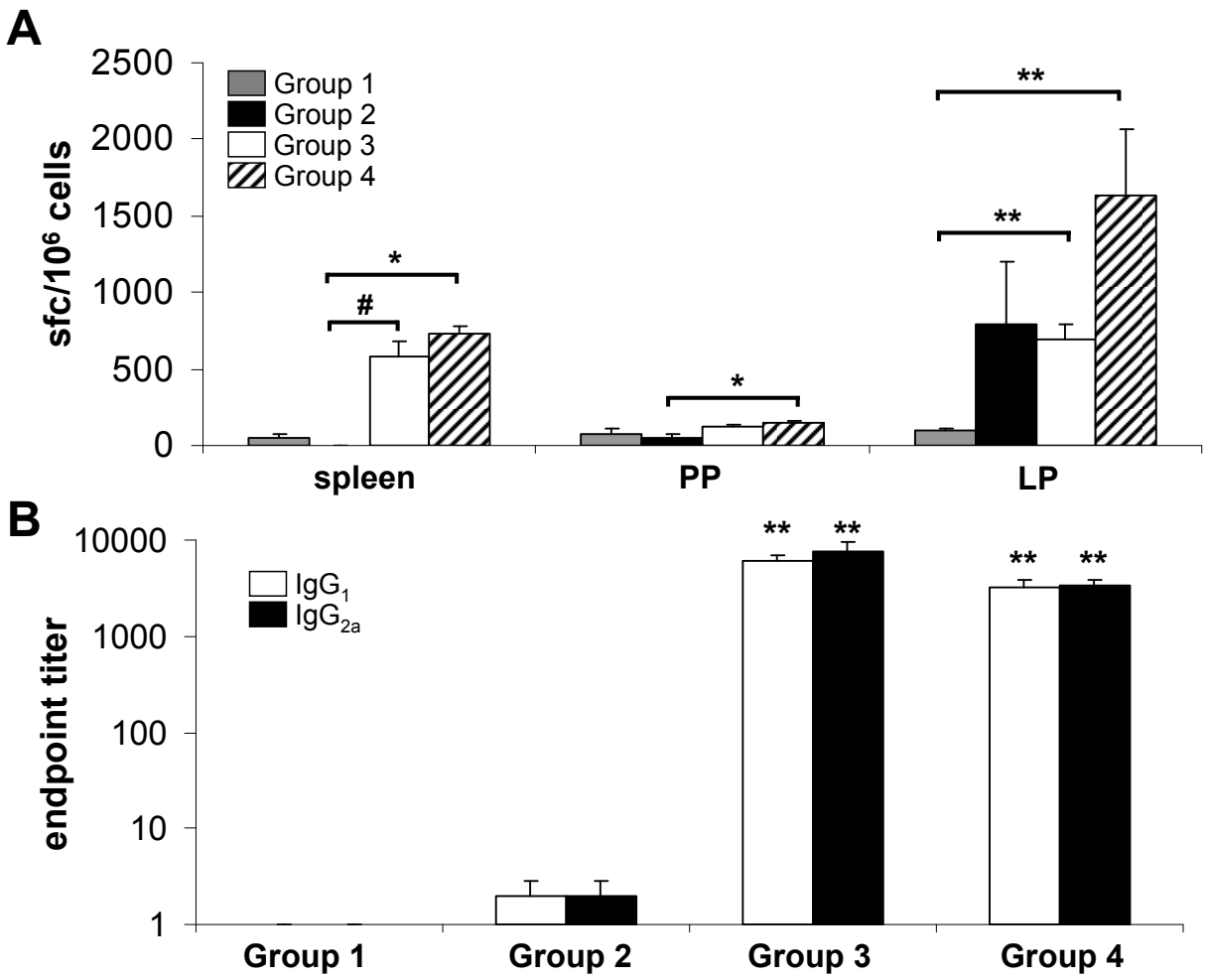


Figure 22. Systemic and gut mucosal immune responses generated from *Cp*-p27 and Ad-p27 vaccination
Mice were immunized against SIV p27 with *Cp*-p27 and Ad-p27 vaccines as described in Figure 1. *A,* p27-specific IFN-γ ELISpot results from spleen, small intestinal Peyer's patches (PPs) and small intestinal lamina propria (LP). Grey bar, group 1; black bar, group 2; white bar, group 3; slashed bar, group 4. Kruskal-Wallis test *p*-values: for spleen, $p=0.0011$; for PPs, $p=0.0056$; for LP, $p<0.0001$. #$p<0.05$, *$p<0.01$, **$p<0.001$. Error bars represent standard error of the mean. *B*, Serum antibody titers detected by p27-specific ELISA. White bar, IgG_1; black bar, IgG_{2a}. For both isotypes, via the Kruskal-Wallis test of $\log_{10}$ transformed endpoint titer values, $p<0.0001$. **$p<0.001$ compared with both group 1 and group 2. Error bars represent standard error of the mean.

5.5.2.3 *Gut Mucosal Cellular Responses*

Unlike the previous experiment using cholera toxin as an adjuvant with *Cp*-p27 (Figure 21), responses to *Cp*-p27 using LT(R192G) and CpG ODNs in animals of group 2 did not generate p27-specific IFN-γ ELISpot responses higher than then PBS control group 1 in PPs

(Figure 22 *A*). Cellular responses were observed in groups 3 and 4, with group 4 again showing the strongest p27-specific cellular response. As in the spleen, the cellular response in PPs was not significantly different between groups 3 and 4, but the response in group 4 was statistically higher than group 2 ($p<0.01$).

The LP showed a different hierarchy of p27-specific cellular response (Figure 22 *A*). In this effector tissue, all vaccinees displayed stronger responses than the control group 1. In the LP, group 2 demonstrated a slightly higher cellular response than group 3. The p27-specific IFN-γ response in group 4 was higher than any other group and very significantly higher than control group 1 ($p<0.001$). Whereas the magnitude of p27-specific IFN-γ production by splenocytes and LP lymphocytes was similar in group 3, in group 4 the LP response was nearly twice that in the spleen (Figure 22 *A*).

5.5.2.4 *Gut Mucosal Humoral Responses*

Intestinal p27-specific IgA and IgG was evaluated by ELISA, and all groups demonstrated low levels of antibody for both isotypes. There was no difference in OD levels in any vaccinated group compared with the PBS control group 1 (data not shown).

5.5.3 Function of p27-Specific T Cells

To evaluate other functions of the p27-specific gut mucosal cells, cells were stained for the cytotoxic degranulation marker CD107a, and intracellular cytokine staining was performed to detect IFN-γ, TNF-α, and IL-2 production. These four immune functions were then detected by flow cytometry. Cells were surface stained for CD3 and CD8 to differentiate $CD8^+$ and $CD8^-$ T cells. In PPs, $CD3^+CD8^-$ cells are 95-96% $CD4^+$; in LP, they are 88-90% $CD4^+$ (data not shown).

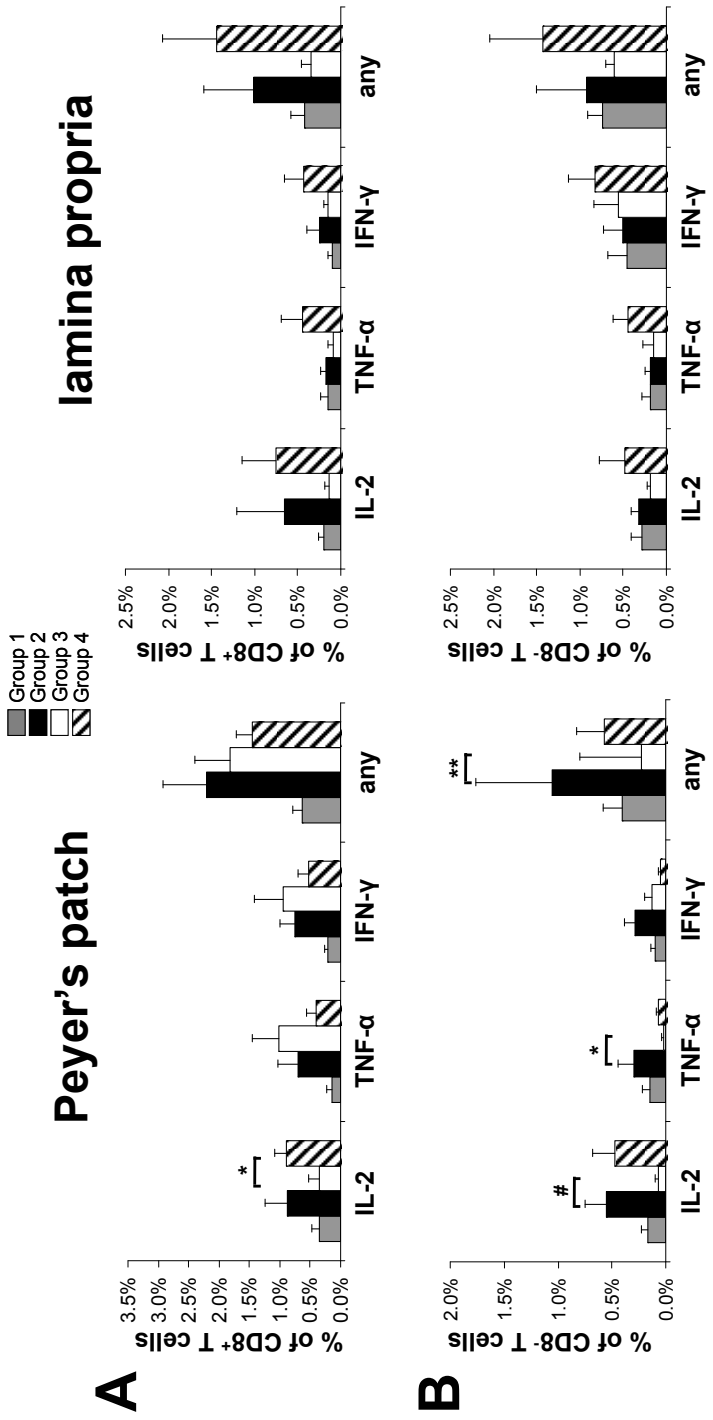


Figure 23. T cell p27-specific cytokine responsiveness in gut mucosal tissues following immunization with *Cp*-p27 and/or Ad-p27

Cells from small intestinal Peyer's patches (*left*) and lamina propria (*right*) were surface stained for CD3 and CD8. SIV p27-specific IL-2, TNF-α, and IFN-γ production from $CD8^+$ (*A*) and $CD8^-$ (*B*) $CD3^+$ cells was detected via intracellular staining and analysis via flow cytometry. Percentages of cells with each cytokine and any cytokine response were then determined. Grey bars, group 1; black bars, group 2; white bars, group 3; slashed bars, group 4. Error bars represent standard error of the mean. In all cases of statistical significance between groups, $p \leq 0.03$ via the Kruskal-Wallis test. #$p<0.05$, *$p<0.01$, **$p<0.001$.

5.5.3.1 *Individual Cytokine Levels Vary with Delivered Vaccine*

The percentage of cells producing each cytokine in the two gut tissues was calculated from flow cytometry results (Figure 23). Group 2 displayed slightly more production of each of IL-2, TNF-α, and IFN-γ in PPs $CD8^+$ and $CD8^-$ T cells compared with group 1. Group 3 demonstrated very little production of any cytokine except in PPs $CD8^+$ T cells, which had higher but statistically insignificant percentages of TNF-α and of IFN-γ producing cells than control group 1. IL-2 and TNF-α production in PPs $CD8^-$ T cells from group 3 were statistically lower than in group 2. The IL-2 production of PPs $CD8^+$ T cell in group 4 was statistically higher than in group 3. Group 4 demonstrated insignificantly higher percentages of IL-2, TNF-α, and IFN-γ producing PPs $CD8^+$ T cells compared with control group 1. In PPs $CD8^-$ T cells, group 4 had low levels of all cytokines except for IL-2. There was a slightly higher level of production of each cytokine by LP $CD8^+$ and $CD8^-$ T cells in group 4 compared with all other groups. Group 2 also showed a higher percentage of IL-2 producing cells than group 1, but this was not statistically significant.

5.5.3.2 *Multifunctional T Cells in Peyer's Patches*

All mice displayed production of at least one of the assayed cytokines in response to p27 in PPs T cells. The percentage of $CD8^+$ T cells showing any cytokine responsiveness to p27 was highest in groups 2 and 3 (Figure 23). The percentage of p27-specific cytokine-producing $CD8^-$ T cells was highest in groups 2 and 4, although no vaccine groups displayed significantly higher $CD8^-$ PPs T cell responses than the PBS control group. Group 2 displayed a statistically higher $CD8^-$ T cell response in PPs than group 3 ($p<0.001$).

The cells from each vaccinated group were then evaluated for multifunctionality (at least 2 different functions in the same cell) of cytokine production and degranulation. Within the

$CD8^+$ PPs T cell response, multifunctionality was observed significantly more often in group 4 compared with group 2 or group 3 (p=0.026 and p=0.013, respectively) (Figure 24). $CD8^-$ PPs T cells were also most multifunctional in group 4; mice from group 2 displayed some multifunctionality, and mice in group 3 demonstrated no multifunctionality within the $CD8^-$ T cell subset in PPs (Figure 24). The lack of multifunctional PPs $CD8^-$ cells in group 3 mice created statistically significant differences in complexity (i.e. 2- vs. 3- vs. 4-functions) of multifunctionality profiles when comparing group 3 with either group 2 or group 4 (p<0.001). These results demonstrate a difference in $CD8^-$ ($CD4^+$) and $CD8^+$ T cell responses in PPs dependent upon the vaccine vector used to deliver p27 antigen.

5.5.3.3 *Multifunctional T Cells in Gut Lamina Propria*

Most mice in all groups demonstrated a positive p27-specific response to at least one cytokine in the LP: 10/10 group 1 (100%); 13/13 group 2 (100%); 11/12 group 3 (92%); and 11/11 group 4 (100%). However, the percentage of p27-specific cells producing any cytokine in mice from group 3 was lower than that in PBS control group 1 mice in both $CD8^-$ and $CD8^+$ LP T cells (Figure 23). Of the p27 responsive mice, higher percentages of $CD8^-$ and $CD8^+$ T cells demonstrated a p27-specific cytokine response in animals vaccinated with *Cp*-p27 (groups 2 and 4) compared with only Ad-p27 (group 3) (Figure 23). The highest percentages were observed in group 4.

Although not statistically significant, a larger portion of the p27-specific response was due to multifunctional cells (cytokines and/or degranulation) in group 4 than in groups 2 and 3, particularly in the $CD8^+$ T cell subset (Figure 24). Of particular interest, the level of multifunctionality of LP $CD8^+$ T cells was greater in mice from group 4 (2-, 3-, and 4-function responses) than in group 3 mice (2-function responses only).

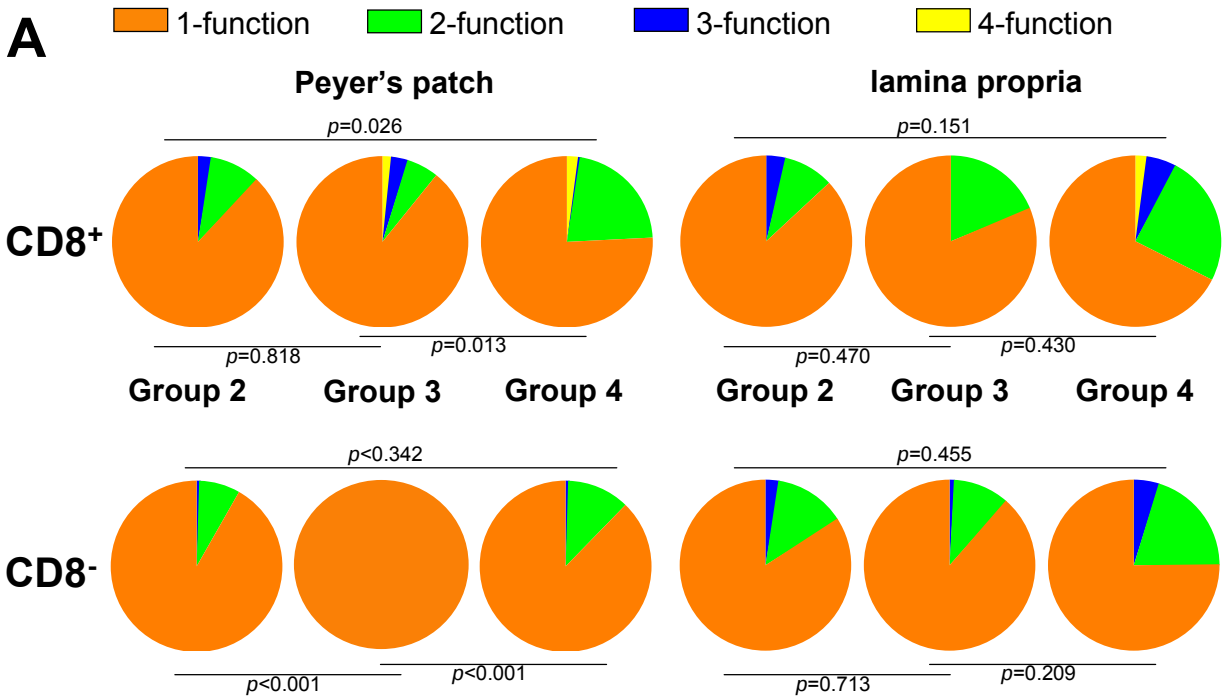
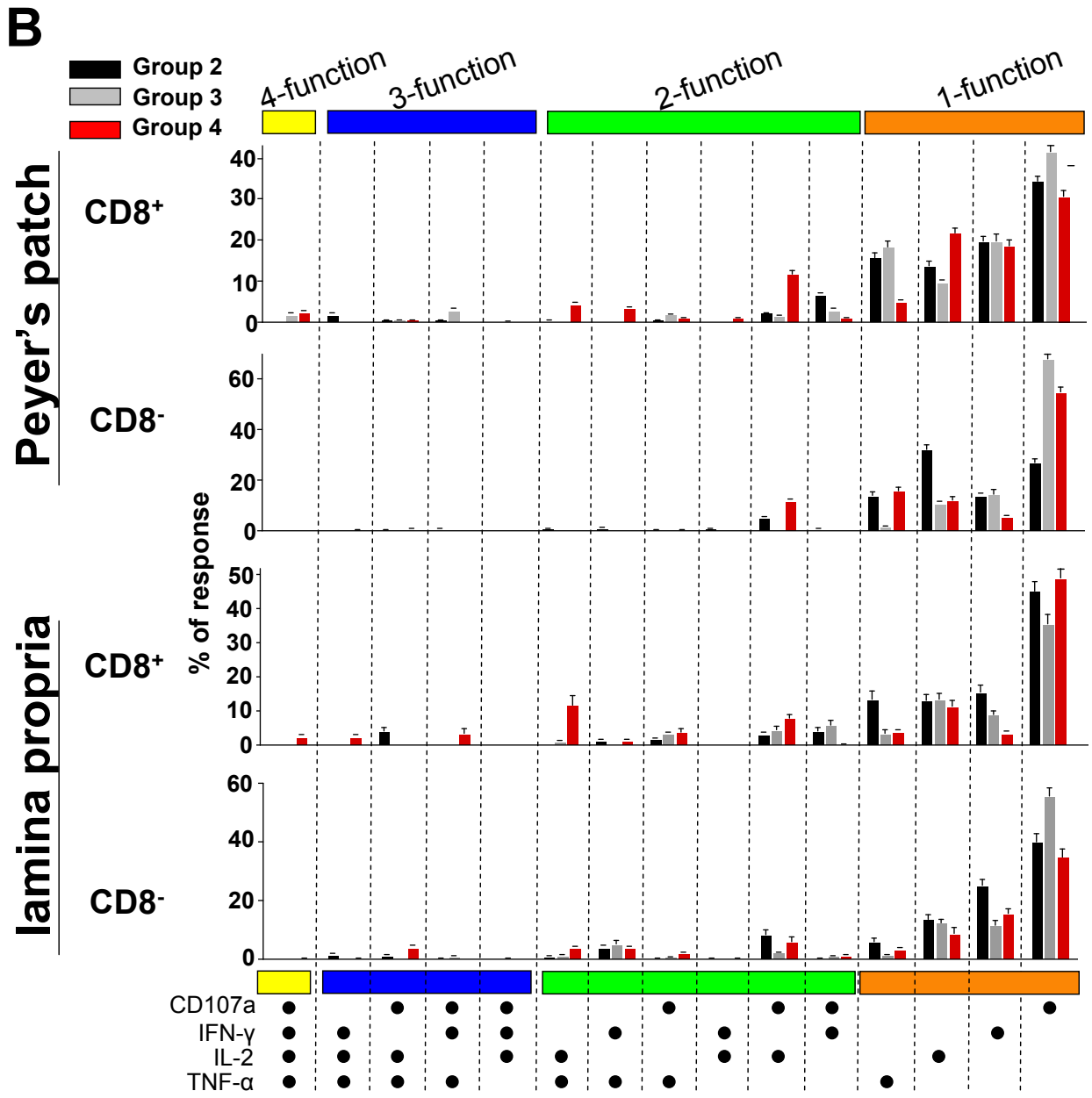


Figure 24. Multifunctional gut T cell responses in inductive and effector tissues resulting from vaccination
Data generated from Boolean-gated $CD8^+$ and $CD8^-$ cells from Peyer's patches and lamina propria stained for CD107a, IL-2, TNF-α, and IFN-γ were analyzed for concurrent functionality using SPICE software. *A*, The average percentage of total response at each level of multifunctionality is represented by slices in pie charts. Values of *p* were determined with SPICE software comparing distribution between pies grouped by slice color using 10000 permutations. *B*, The average percentage of total response for each combination of functions is shown with error bars representing standard error of the mean.

(Figure 24 continued)



5.6 DISCUSSION

Newly acquired HIV and SIV infections quickly target gut mucosal tissue, and the inability to restore the gut mucosal immune system following initial depletion of its $CD4^+$ T cells contributes to the pathology and persistence of HIV and SIV [64, 74, 75, 78, 79, 312-314]. Nevertheless, vaccine-induced gut $CD8^+$ T cell immunity has been shown to impede the dissemination of SIV in rhesus macaques following challenge [121], providing evidence in support of the hypothesis that containment of infection at mucosal sites can reduce the severity of HIV/SIV infection [34, 36]. The systemic and mucosal presence of both $CD8^+$ and $CD4^+$ multifunctional cellular responses against lentiviruses has also been associated with protection from or control of HIV and SIV infections [40, 42, 136-140].

One of the challenges of HIV/SIV vaccine design is the complexity of inducing immune responses in the gut. It is often difficult to deliver sufficient amounts of vaccine antigen to the relevant GALT inductive tissues, namely PPs. Both oral and rectal inoculation strategies are under investigation for this purpose, with varying levels of resultant mucosal and systemic responses [49, 121, 247, 248, 332, 390-397]. Some systemically delivered vaccines can induce a degree of mucosal response. Intramuscular immunization with recombinant Ad carrying HIV/SIV antigens has been shown to induce strong systemic cellular immunity, as well as limited cellular immunity in PPs [216, 230]. Under these circumstances, the gut mucosal immune response was several degrees of magnitude lower than the systemic immune responses. Data from a recent clinical trial using an Ad serotype 5-based vaccine demonstrated a lower than anticipated protective ability despite inducing strong systemic immunity against HIV (reviewed

in [207]); gut mucosal responses were not defined. In light of these findings and the importance of gut mucosal immunity against HIV and SIV, a strategy to improve the gut mucosal immunity generated from leading HIV/SIV vaccine candidates is desirable.

The current study attempted to generate multifunctional mucosal immune responses in the gut through immunization of mice with an oral *C. perfringens*-based vaccine against SIV p27. Despite low systemic humoral and cellular responses with this vaccine strategy, strong cellular responses in the inductive (PPs) and effector (LP) gut tissues were observed. $CD8^+$ T cells from the LP of *Cp*-p27-vaccinated mice contained cells concurrently displaying two, three, and four of the cytokine and cytotoxic immune functions associated with effective cellular immunity. Additionally, this study investigated whether the oral *Cp*-p27 vaccine used as a prime could improve gut mucosal responses to subsequent intramuscular inoculation of Ad-5 carrying SIV p27. These results indicate that mice that were first primed with *Cp*-p27 and then boosted with Ad-p27 generated stronger systemic and gut cellular responses to SIV p27 compared with unprimed Ad-p27 recipients. Multifunctionality of PPs and LP $CD8^+$ and $CD8^-$ T cell subsets was also increased in *Cp*-p27-primed mice that were then boosted with Ad-p27. Compared with mice who only received *Cp*-p27, the multifunctionality of $CD8^+$ T cells in LP was strikingly improved in mice boosted with Ad-p27.

Together, these observations suggest that different functional profiles are stimulated in inductive and effector gut lymphoid tissue depending on which vaccines mice receive. The observation that the percentage of responsive LP $CD8^+$ T cells in recipients of Ad-p27 only is much lower compared with mice who received *Cp*-p27 only and *Cp*-p27 boosted with Ad-p27 suggests that intramuscular Ad-p27 vaccination provides limited stimulation of LP $CD8^+$ T cell responses. Finally, the current study suggests that immunization with *Cp*-p27 stimulates

multiple cytokine and degranulation events in the lamina propria, enabling a subsequent inoculation of Ad-p27 to boost the memory T cell response in this effector tissue while also improving the functionality of T cells in inductive PPs tissue.

The ability of vaccine-induced multifunctional $CD8^+$ T cell immunity to predictably prevent HIV or SIV infection is not wholly understood. In rhesus macaques immunized with live-attenuated SHIV, animals who controlled viral replication against SIV challenge were found to have more multifunctional responses in PBMC-derived $CD8^+$ T cells than macaques who displayed high viral loads [42]. However, study of PBMC T cell immunity in an individual who acquired HIV after vaccination against HIV has suggested that infection can occur even in the face of multifunctional vaccine-induced responses similar to those seen in long-term non-progressor HIV patients [398]. Clearly there remains much to be learned regarding the relationship between immune responses and protection from and/or control of HIV/SIV infection. In light of the many reports that anti-HIV/SIV immunity in PBMC does not accurately reflect that in the gut tissues [74, 77, 399], it will be important to include these mucosal sites in future evaluations. Tasca *et al.* observed that uninfected SHIV-exposed rhesus macaques demonstrating resistance to infection following vaginal challenge displayed more jejunal gut lamina propria $CD8^+$ T cells with concurrent expression of IFN-γ and TNF-α than susceptible macaques [140]. Further studies into the quality of immune response, i.e. multifunctional $CD8^+$ T cells, in various gut tissues will provide insight into an understanding of the true definition of protective immunity.

In conclusion, oral vaccination of mice with *Cp*-p27 induces multifunctional cellular immunity in the LP, and boosting with intramuscular Ad-p27 enhances both LP and systemic immunity. Future studies with *Cp*-p27 will focus on the memory phenotype of induced T cells,

long-term immunity induced by vaccination, and the level of protection against challenge infection generated by *Cp*-p27 immunization. *Cp*-p27 should be considered as a mucosal prime for other vaccination strategies. Oral administration of vaccines is often preferable in the field to inoculation strategies requiring sterile needles, so including *Cp*-p27 in a vaccination regimen involving Ad or another injected vaccine would have the added benefit of reducing the number of needle-based inoculations. Inclusion of *Cp*-p27 could also lower the cost since culture of *Cp*-p27 does not require expensive techniques and reagents associated with vaccines generated through cell culture and other common vaccine production methods.

6.0 FINAL DISCUSSION AND CONCLUSIONS

6.1 EXPERIMENTAL FINDINGS OF CURRENT STUDY WITH *CP*-P27

6.1.1 Overview of Results

The experiments described in the preceding four chapters were aimed at addressing the hypothesis that the *Cp*-p27 vaccine, consisting of *Clostridium perfringens* expressing SIV p27 from the sporulation-induced *cpe* promoter, can deliver p27 to gut DCs and thereby prime mucosal and systemic humoral and cellular immunity against SIV. Since cellular immunity is important in control of SIV and HIV infection, the bulk of the experiments addressed the induction of T cell immune responses following exposure to *Cp*-p27 under *in vitro* and *in vivo* conditions. DCs of systemic and gut origin were observed to mature and stimulate p27-specific T cell responses following exposure to *Cp*-p27. Inclusion of PTD sequences conjugated to the p27 increased the uptake of antigen by DCs, but such conjugation did not improve p27-specific cellular immune responses. *Cp*-p27 delivered orally produced gut cellular immunity, which was improved by the coadministration of mucosal adjuvants. This response was greatest in strength with 25μg LT(R192G) plus 50μg CpG ODNs and optimal in functional quality with 25μg LT(R192G) alone. Systemic immune responses were not induced by *Cp*-p27 vaccination alone, but oral priming using *Cp*-p27 followed by an intramuscular Ad-p27 boost improved systemic cellular immune responses. Such a prime-boost strategy also improved cellular immunity in the gut effector lamina propria tissue compared with Ad-p27 or *Cp*-p27 alone. Altogether, this work

demonstrates that *Cp*-p27 can stimulate cellular immunity in the gut mucosal tissue after delivery to gut DCs, and that *Cp*-p27 can prime systemic immunity for an improved response to subsequent booster vaccination with Ad-p27.

6.1.2 Significance of Results

This is the first study demonstrating that the use of a non-invasive bacterial-based protein delivery system directed at the gut can induce strong, multifunctional cellular immunity in the gut. The safety, ease, and cost-effectiveness of such a delivery system make the *Cp*-p27 vaccine an attractive vector for further evaluation. This study did not evaluate *Cp*-p27-induced mucosal immunity generated in distal mucosal tissues. Studies in many mammals indicate that stimulation of immunity at gut mucosal can also generate immunity in vaginal and rectal tissue [48, 49, 238, 249-251, 311, 400-405]. Some evidence for this phenomenon exists in humans, although other findings suggest that primates do not readily display transfer of gut immunity to vaginal or rectal tissues [250, 400-404]. Regardless, the presence of cellular immunity at these sites of initial HIV transmission may not completely block early HIV infection, and cellular immunity at secondary infection sites, such as the gut, may be required to lower the severity of infection by controlling viral replication [36, 119]. Studies in the macaque model suggest that a vaccine-induced gut cellular response to SIV can slow the establishment of mucosally-acquired SIV infection and results in a lower viral set-point [120, 121], which often correlates with a longer period before developing AIDS as well as less transmission of virus [55, 56, 213, 214].

6.2 COMPARISON OF CP-P27 WITH OTHER VACCINE VECTORS AGAINST SIV AND HIV

6.2.1 Systemic Immunity

A number of other approaches to oral vaccination have been described in the literature. Oral inoculation using viral vectors including adenovirus, vaccinia virus, papilloma virus, and adeno-associated virus have all been studied in mice [228, 392, 406-408]. Orally delivered virus-based vaccines have been observed to result in between 80 and 600 antigen-specific ELISpot sfc/10^6 in spleen. In comparison, systemically delivered viral-based vaccines tend to provide IFN-γ ELISpot counts ranging from 500 to 1500 sfc/10^6 in mouse splenocytes.

Bacterial vectors such as *Lactococcus lactis*, *Salmonella enterica* serovar *Typhimurium* and *Listeria monocytogenes* are also under investigation as oral vaccine vectors and have been shown to induce systemic immunity in mice [236, 248, 331, 332, 334, 393, 395]. *Shigella flexneri* may also be useful as an oral vaccine vector but has thus far only been examined in the context of intranasal inoculation [237, 409, 410]. When delivered mucosally, many of the bacteria-based vaccines also result in respectable levels of antigen-specific systemic cellular immunity, detectable in the spleen at levels around 200-300 IFN-γ ELISpot sfc/10^6 cells or with high levels of T cell proliferation or cytotoxicity rates. The exception to these common observations comes from studies using *Listeria monocytogenes* engineered to express HIV gag, wherein mice immunized orally with a *L. monocytogenes*-based HIV vaccine demonstrated few or no HIV-specific $CD8^+$ T cells in the spleen [228, 332, 411]. Similarly, vaccination with *Cp*-p27 in the current study generated a low p27-specific cellular immune response in the spleen.

6.2.2 Gut Mucosal Immunity: Peyer's Patches and Mesenteric Lymph Nodes

In other studies [228, 332], cellular immune responses in the PPs were observed but not strong after oral inoculation using *L. monocytogenes*, similar to observations with *Cp*-p27 alone in the current study. *Salmonella*-based oral vaccination appears capable of stimulating antigen-specific PPs cellular immunity on the order of 250-400 sfc/10^6 cells, although these results are not conclusive since one report observed nearly identical antigen-specific response levels following inoculation with an empty vector *Salmonella* and another utilized a 6-day restimulation protocol before the assay in order to enhance the detectable response [409, 412]. Intramuscularly delivered Ad has been observed to generate 50 to 100 antigen-specific sfc/10^6 cells detected in an IFN-γ ELISpot assay of PPs cells, similar to the results obtained in the current study [216, 230].

The response generated by *Cp*-p27 was confined to gut tissue local to the site of p27 delivery and was not observed in the draining lymph node (MLN) or systemic tissue. Two possibilities may explain the lack of immunity detected in the MLN. First, the timepoints used for analysis may not represent timepoints when draining lymph nodes contain antigen-specific IFN-γ producing cells. This is supported by findings from Zhu *et al*. who observed that, following intrarectal administration of an adenovirus, the cellular response of the iliac lymph node draining the rectal mucosa was detectable at days 4 and 6 following inoculation but not at day 14, indicating that detectable response waned in the inductive tissue within two weeks [413]. Rayevskaya and Frankel [332] observed a similar phenomenon in which cellular PPs responses following oral inoculation with a *L. monocytogenes*-based HIV vaccine were highest at day 7 after the final inoculation, and the response was much lower by day 14. It is likely that the transient *Cp*-p27-induced response in the PPs represents the priming of cells in this inductive

tissue which then migrate and establish themselves in the LP effector site. Further characterization of the kinetics and memory phenotype of the responses observed following *Cp*-p27 immunization will make this clear.

An alternative explanation for the lack of *Cp*-p27-induced MLN cellular immune response takes into account the difference in trafficking of PPs and LP APCs, specifically DCs. LPDCs primarily travel to the MLN to present antigen, whereas PPDCs can relocate from the SED to T and B cell areas of the PP and may not frequently migrate to MLN [252, 266, 268]. Thus, even if the PPDCs exposed to *Cp*-p27-delivered p27 efficiently acquire antigen, the responses they stimulate may not be detectable in the MLN. Responses in the MLN and systemic tissue have been observed following oral vaccination with other bacterial-based vectors, and this may be because these other vectors deliver protein to cells in the LP through direct infection and subsequent active replication [247, 331-333]. For example, a *Salmonella*-based vaccine delivers vaccine antigen by entering macrophages in the gut LP [247, 331].

This explanation would also reconcile the differences in systemic immunity stimulatory capacity observed using different oral vectors noted above since the vectors use different methods of antigen delivery to gut tissue. *L. monocytogenes* infects both monocytes and DCs, the latter being most prevalent in the gut in PPs [229, 334, 389]. Likewise, *Cp*-p27-expressed protein is thought to be primarily taken up by DCs in PPs since protein is delivered to the ileum in particulate inclusion bodies which are preferentially transcytosed by the M cells that allow protein to access PPs. On the other hand, bacterial vectors such as *Shigella* and *Salmonella* can infect macrophages, which in the gut reside predominantly in the LP. Viral-based vectors also deliver protein by infecting cells in the LP. The handling of antigen by PPDCs and by macrophages or DCs from LP differs in both the pathways for epitope presentation on MHCs

and trafficking of cells to immune inductive sites [229]. The latter difference may play a major role in determining the systemic or mucosal homing of immune cells induced by these various vaccines. PPDCs are apt to prime immunity in PPs but do not necessarily travel to the MLN, and immune cells primed in PPs home to gut LP [414]. Gut LP macrophages may induce immunity when they travel to inductive sites in the MLN; cells primed in the MLN can seed the systemic immune system [229, 389].

In reports describing mouse cellular immune responses against HIV gag following oral inoculation, mice with cellular responses in PPs and MLN displayed protection from challenge with HIV gag-expressing vaccinia virus [332, 391-394]. Stronger responses were consistently observed in gut compared with spleen in these previously reported oral inoculation studies. In the current study, oral *Cp*-p27 vaccination followed by a systemic Ad-p27 boost also produced stronger immunity in the gut than in the spleen, whereas oral inoculation with the *Cp*-p27 vaccine alone induced almost no detectable response in the spleen. Although the previously described *L. monocytogenes*-based vaccine also did not induce strong systemic cellular immunity, upon boosting with an intramuscularly delivered Ad-vectored vaccine, the percentage of antigen-specific $CD8^+$ T cells in the spleen increased by at least 20-fold, a level at least 10-times that induced by the Ad vaccine alone [228, 332]. The strength of this response increased incrementally as more primes of the oral *L. monocytogenes* vaccine was administered before the Ad boost. A similar trend was observed in PPs, where low levels of HIV-specific $CD8^+$ T cells were detectable following *L. monocytogenes* vaccination, but response to an Ad boost was improved in a *Listeria* dose-number dependent manner. These *L. monocytogenes* studies and the current *Cp*-p27 study demonstrate that even when immunity induced by an oral vaccine is at undetectable levels, immunity exists that can be boosted with a separate vector.

Furthermore, the low levels of spleen, PPs, and MLN cellular immunity induced by the *L. monocytogenes* vaccine did not hamper its ability to effectively control a viral challenge [228, 332]. Although these CTL responses were rather low (approximately 20-25 lytic units/10^6 cells in each tissue) and did not persist at high levels in spleen, MLN, or PPs, the immunized mice were protected from challenge with gag-expressing vaccinia virus via either intraperitoneal or oral inoculation [332]. This may indicate that low levels of immunity in the gut mucosa are sufficient to prevent infection by a mucosally targeted pathogen and/or that stronger gut immunity may exist in other compartments, such as effector LP tissue, even when responses in inductive tissues (PPs and MLN) are low. These studies also raise the possibility that the prime-boost strategy with *Cp*-p27 and Ad-p27 could provide protection from challenge with p27-expressing vaccinia virus in mice.

6.2.3 Gut Mucosal Immunity: Lamina Propria

A few studies have reported the antigen-specific IFN-γ ELISpot response of gut LP cells following immunization. In one such study, an intrarectally delivered modified vaccinia Ankara-based vaccine provided similar levels of antigen-specific IFN-γ sfc (800-900 sfc/10^6 cells) in the small intestine LP as did *Cp*-p27 vaccine in the current study [396]. Other vaccine strategies have induced varying levels of cytolytic cellular responses in the LP. For example, Belyakov *et al.* generated about 20% specific lysis at a 12.5:1 effector:target ratio in LP lymphocytes via intrarectal inoculation of HIV peptides with CT; this response provided control of a subsequent intrarectal HIV envelope-expressing vaccinina virus challenge [49].

6.2.4 Functional Quality of Vaccine-Induced Gut Cellular Responses

Cellular immune responses in gut mucosal tissues of mice have also been assayed via intracellular cytokine staining for IFN-γ in a few reports [216, 396]. The data from these reports suggest that oral immunization stimulates stronger cellular immunity in the gut than the spleen, whereas intramuscular immunization creates the opposite result. Lin *et al.* reported that a high dose ($5x10^{11}$) of intramuscularly delivered Ad induced antigen-specific IFN-γ production in about 2% of PPs $CD8^+$ T cells [230]. In the current study, a lower dose ($1x10^9$) of Ad-p27 generated IFN-γ production in 0.95% $CD8^+$ T cells from PPs, and *Cp*-p27 induced an antigen-specific IFN-γ response in 0.75% of PPs $CD8^+$ T cells. The antigen-specific IFN-γ response of small intestinal LP $CD8^+$ T cell following Cp-p27 and/or Ad-p27 vaccination in the current study were also similar to responses observed in gut-associated tissue in other studies, including in the jejunal lamina propria of monkeys inoculated through various routes with a vaccine vectored by the poxvirus NYVAC [140, 391]. The current finding of about 1% of $CD8^+$ or $CD4^+$ T cells expressing IFN-γ in an antigen-specific manner following immunization with *Cp*-p27 or Ad-p27 is consistent with many other studies in animals and humans, including individuals who control HIV viral replication [138, 141, 147, 215, 415-417].

The combination of IFN-γ with other functions of $CD8^+$ T cells appears to associate with effective $CD8^+$ CTL responses and control of SIV/HIV infection [40, 132]. Higher percentages of vaccine-induced antigen-specific cells that express both IFN-γ and TNF-α have been observed in gut-associate tissue of monkeys who resisted infection than in susceptible vaccinated animals, similar to the current findings [140]. All published studies detailing vaccine-induced expression of more than two functions have been performed with systemic and not mucosal cells (examples in [215, 398, 415, 418]). In these studies the vector that was used affected the quality of the

cellular response. For example, heterologous prime-boost immunization of mice using a herpes simplex virus amplicon followed by an Ad-vectored vaccine provided more multifunctional $CD4^+$ and $CD8^+$ cells than homologous prime-boosting [215]. In the same study, multifunctional $CD8^+$ T cells expressing IFN-γ, IL-2 and/or TNF-α made up about 15% of the total antigen-specific response. Similar trends were observed in the current study in which multifunctionality was greater in mice primed with *Cp*-p27 and boosted with Ad-p27 than mice who were vaccinated with only one vector, with a maximum of about 15% (IFN-γ, TNF-α , IL-2) or 25% (CD107a, IFN-γ, TNF-α , IL-2) of the gut mucosal cellular response due to multifunctional cells.

6.3 CRITICAL EVALUATION OF *CP*-P27 AND SUGGESTIONS FOR FUTURE STUDY

6.3.1 Benefits and Drawbacks of *Cp*-p27

Oral vaccination with *Cp*-p27 is superior to many other vaccines in its ability to induce cellular immunity in the gut LP. However, one of the benefits many of these other vaccines have over *Cp*-p27 is the ability to induce systemic immunity. Both systemic and mucosal immunity are thought to be necessary for effective protection against HIV disease. As described above, there are different methods of protein delivery by *Cp*-p27 and systemic-inducing vaccines which likely determine the ability of these vaccines to induce systemic or mucosal immunity. Although the delivery mechanism of vectors such as attenuated *Salmonella*, *Shigella* and various viruses promotes both mucosal and systemic immunity, their ability to infect or colonize the gut may not be ideal for use in humans. Regions with the greatest threat of HIV tend to also have high rates of gastrointestinal (GI) disease, such as diarrhea, rendering the gut mucosa less resistant to control of attenuated bacteria. Complications might easily occur if a vaccine vectored by an

invasive or colonizing bacteria or virus were delivered to the gut of individuals already fighting gastrointestinal disease. The *C. perfringens* vectoring the *Cp*-p27 vaccine enters a non-replicative, dormant state in the small intestine and delivers vaccine protein to inductive sites without infecting or colonizing the mucosa. *Cp*-p27 thus has the advantage of not contributing to GI disease.

An additional benefit of the *C. perfringens* vaccine vector system is the ease with which different genes can be inserted into the expression plasmid and placed under control of the strong *C. perfringens cpe* gene. For example, a *C. perfringens* vaccine expressing HIV p24 has been constructed and is currently under investigation in animal studies. Also, p27 under control of the *cpe* promoter has been inserted into the chromosome of *C. perfringens*, eliminating the need for an expression plasmid [305]. The safety and adaptability of the *C. perfringens* vector would be of further value if the vector could also induce systemic immunity. Therefore, future study of the *C. perfringens* vaccine vector system should concentrate on achieving systemic immune responses through oral vaccination, increasing the safety of the *C. perfringens* vector, and broadening immunity by inducing responses against additional viral proteins and stimulating antibodies.

6.3.2 Systemic Immunity Via *C. perfringens*-Based Oral Vaccination

The BMDCs used in this study represent systemic DCs that are able to mediate systemic immune response formation [419, 420]. In addition, these DCs can stimulate gut immunity insomuch as BMDCs delivered intraperitoneally have been observed to travel to MLN where they stimulate T cells that home to the gut [420]. Perhaps this is how systemic antigen can gain access to the gut. That systemic Ad-p27 vaccination induced responses in MLN suggests that this may be the case in the current study. Thus, in order for *Cp*-p27 to generate systemic as well

as mucosal immunity, the vaccine vector may need to more efficiently deliver p27 to cells that can travel to the MLN, such as LP antigen presenting cells including epithelial cells. The attempt to improve p27 uptake by epithelial cells by incorporation of PTD sequences on the N-terminal of p27 proved to be an ineffective solution to this challenge, since PTD incorporation caused death of epithelial cells *in vitro*; therefore, alternative strategies should be pursued. While PTD-based improvement of the antigen appears to be less than ideal, other methods for enhancing and directing immunity can be explored. Some of these options include directly conjugating adjuvant to the antigen expressed by *C. perfringens*, or fusing an ubiquitination tag on the antigen to direct it for ubiquitination and proteasomal cleavage into MHC class I epitopes.

6.3.3 Safety of *Cp*-p27

Despite the fact that most *C. perfringens* rapidly undergoes sporulation in the intestine, rendering it nonreplicative and dormant, one of the safety concerns of utilizing *C. perfringens* as an oral vaccine vector is that *C. perfringens* carries a variety of toxin-encoding genes which can lead to disease. The *C. perfringens* type A from whence *Cp*-p27 was engineered encodes two major exotoxins, α-toxin (also known as phospholipase C) and θ-toxin (also known as perfringolysin O). Oral consumption of *C. perfringens* carrying these two genes is considered safe in normal humans, and it is only when such bacteria enter deep wounds that they are associated with disease, namely gas gangrene. However, there is the potential for *C. perfringens* delivered to the gut to enter tissue if the normal GI mucosal tissue is compromised. Given that preexisting GI diseases are often found in the regions in most desperate need of HIV vaccines, it is prudent to take as many precautions as possible. Because of this, *C. perfringens* strains with inactivation of both α-toxin and θ-toxin have been developed and have been shown to express SIV p27 and deliver antigen to the terminal ileum [304, 305].

Although there is no evidence for recombination of genetic material between food-acquired *C. perfringens* and gut resident *C. perfringens* or other bacteria in the gut, there is the potential for a resistance gene to be transferred if it were included in a vaccine. In the course of the development of the α- and θ-toxin-double knockout mutant, a *C. perfringens* strain expressing SIV p27 from a chromosomally-encoded gene [305]. This expression strategy has improved safety for human use in that it circumvents the need for an antibiotic resistance gene as is required in the plasmid expression strategy. To maintain the plasmid in recombinant *Cp*-p27, continuous culture with antibiotic is necessary, which is an added cost and may introduce traces of antibiotic into the final vaccine preparation. Unnecessary use of antibiotics in the absence of infection has fueled the emergence of mutli-drug resistance strains of many bacteria over the past several decades. In addition, genetic material encoded on the chromosome is less likely to transfer from a vaccine strain to naturally occuring strains of *C. perfringens* compared with plasmid-encoded genetic material. The plasmid-encoded version of *Cp*-p27 would be more likely to inadvertently introduce further antibiotic resistance genes or vaccine antigen genes into the human population. Thus, future research with *C. perfringens* expressing SIV p27 can be performed utilizing the new generation of *C. perfringens* with multiple safety mutations and lacking antibiotic resistance genes.

6.3.4 Broadening Immunity Using Additional Viral Protein Genes

The production of an immune response that is reactive against a variety of viral proteins is thought to aid in protection from disease progression. As virus mutates its proteins in an attempt to evade immunity, a broad immune response increases the likelihood of the immune system to successfully recognize viral protein epitopes and control virus. The incorporation of various viral proteins into a vaccine would thus be beneficial. Many different viral proteins

could be placed under control of the *cpe* protmoter in the *C. perfringens* vector, creating *C. perfringens* strains expressing other viral enzymes such as reverse transcriptase, accessory proteins required for replication such as tat, and structural proteins such as envelope. The inclusion of these and other proteins in vaccines have been useful in generating protective responses in the SIV macaque model [421-426].

The contribution of cellular responses against envelope proteins in providing protection against SIV or HIV disease progression is unclear. Some evidence suggests that envelope-specific $CD8^+$ T cell responses may not play a large role in protection of SIV-infected macaques from disease progression [114, 427]. Thus, vaccination that can also induce antibody responses against envelope proteins would likely be more beneficial. The incorporation of an envelope protein would be a special case for expression by *C. perfringens* as the bacterium lacks the capacity to correctly glycosylate this protein in a manner useful for successful antibody response formation against most portions of gp120. However, certain portions of the gp41 molecule do not require glycosylation-controlled structural constraints for production of neutralizing antibodies and are also quite conserved among all HIV types, thus making them attractive for use in *C. perfringens*. The membrane proximal region of gp41 contains targets for three broadly neutralizing antibodies [428-430]. The ELDKWA sequence comprising amino acids 662 through 667 of gp41 is the target of the broadly neutralizing antibody 2F5, which has been shown to effectively neutralize about 90% of all tested HIV isolates from around the world [428, 431]. Inoculation of peptides encoding the ELDKWA sequence and derivatives of it have been able to induce some HIV-neutralizing antibodies in animal models [432-434], and the expression of this sequence from the *cpe* promoter in *C. perfringens* would be a feasible approach for utilizing the bacterial vector to induce protective antibody production via vaccination.

6.3.5 Humoral Immunity Via *Cp*-p27 Vaccination

The *Cp*-p27 vaccine was detected to stimulate only very low levels of intestinal p27-specific IgA and IgG. Antibodies against p27 or the HIV capsid protein p24 are not known to neutralize SIV or HIV, respectively; however, it would be useful for *C. perfringens* expressing an envelope protein to induce a mucosal neutralizing antibody response to prevent infection. The expression from the *cpe* promoter encourages the expressed protein to accumulate in particulate inclusion bodies, which are more likely to be internalized and processed through pathways promoting Th1-type immune responses. If protein were expressed in a soluble form instead of in particulate inclusion bodies, a Th2-type immune response promoting antibody formation would be more likely to be induced. It may be possible to produce soluble protein in *C. perfringens* from the *cpe* promoter if sequences encoding secretion pathway-targeting motifs were included in the antigen gene placed under control of the *cpe* promoter.

Another way to induce a humoral response creating antibodies is through the use of adjuvants that promote Th2-type response formation. The adjuvants utilized in the majority of the current study, CpG ODNs and LT(R192G), are known to promote Th1-type immunity more than Th2-type responses. The use of CT has been recognized as promoting Th2-type immunity, particularly when the CT or CTB subunit is conjugated directly to the antigen being delivered. The results in Chapter 4 of the current study demonstrated lower cellular gut immunity induction through the use of CT versus CpG ODNs or LT(R192G). Although not tested in this study, the literature supports that this may be due to a preferential induction of Th2-type immunity that prevents cellular immune response formation and promotes antibody responses. This is consistent with the results of the current study in that cellular immunity decreased as the dose of CT increased. Thus, it seems possible that the inclusion of Th2-promoting adjuvants may aid in

the formation of antibody responses as a result of oral vaccination with a *C. perfringens*-based vaccine.

6.3.6 Additional Studies Suggested for *Cp*-p27

In addition to pursuing strategies to spread *Cp*-p27-induced immunity to tissues other than the gut, future studies should include investigations of the vaccine to induce long-term immunity and protection from challenge. The responses described in this study were determined about two weeks after the final vaccination. A vaccine trial not described in this study examined the response four weeks after two *Cp*-p27 vaccinations and determined that cellular immunity remained detectable in LP, although to a lower level than at two weeks. This is somewhat expected since IFN-γ production by T cells generally subsides as antigen is cleared. Future studies assessing long-term immunity should include evaluation of memory cell surface molecules and IL-2 production of antigen-specific cells [132, 206]. Testing the protective capacity of *Cp*-p27 against virus establishment and disease progression may involve utilizing the SIV monkey model of immunization and infection. In addition, challenge of mice with p27-encoding vaccinia virus can provide data about the protective effectiveness of *Cp*-p27 vaccine-induced immunity.

6.4 CONCLUDING REMARKS

Previous to the initiation of the study described in this manuscript, it was known that p27 could be delivered to the locale of terminal ileum PPs after oral administration of *Cp*-p27 and that *Cp*-p27-delivered p27 could enter DCs of gut and systemic origin [306]. The current study has shown that these DCs can mature and stimulate cellular responses. This was not

significantly improved by the incorporation of N-terminus PTD sequence fusion to p27. It is evident from this study that *Cp*-p27 can induce multifunctional cellular immunity in the gut at levels similar to those observed through other oral vaccination strategies; humoral immunity in the gut following *Cp*-p27 vaccination is evident but limited. The *Cp*-p27-induced response shows the ability to be improved with inclusion of adjuvants, which could be used to sway the quality of cellular immunity. Although the cellular response was limited to gut tissue and not draining LN and systemic tissues, *Cp*-p27 vaccination did not generate tolerance since subsequent systemic antigen delivery via intramuscular Ad-p27 vaccination resulted in cellular responses in the spleen. Additionally, there was a slight increase in systemic response when *Cp*-p27 was given before Ad-p27. *Cp*-p27 can thus prime gut immunity that can be enhanced by a systemic boost. Vaccination using the oral *Cp*-p27 and systemic Ad-p27 prime-boost strategy holds promise for showing effectiveness against p27-expressing vaccinia virus challenge since the level and functional quality of gut cellular immunity observed after this vaccination regimen was similar to that induced by other vaccine strategies that have provided protection against viral challenge. This evaluation of *Cp*-p27 contributes to the fields of HIV/SIV vaccine development and mucosal vaccinology by demonstrating an example of a mucosally delivered vaccine providing a prime for a systemic vaccine boost and by characterizing *Cp*-p27 as a novel vector to continue exploring for mucosal vaccine use.

In conclusion, *Cp*-p27 represents a novel vaccine vector that can stimulate gut mucosal cellular immunity displaying characteristics currently thought to correlate with protection from SIV disease progression. More study is required with *C. perfringens*-vectored vaccines to make them viable for use as vaccines that induce systemic and mucosal immunity through the production of both antigen-specific cellular responses and neutralizing antibodies. Many

questions still remain regarding basic immunology and the interplay between HIV/SIV and host cells. A continued focus on defining the mechanisms of immune response formation and the role of innate and mucosal immunity on protection from disease may provide a more accurate understanding of the rational goals for HIV prophylaxis. As correlates of protection are further defined, *Cp*-p27 may prove to be a basis for an inexpensive, easily administered vaccine for priming mucosal tissue and stimulating protective immunity.

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