INDUCTION OF STRONG CELLULAR IMMUNE RESPONSES IN THE GUT MUCOSA AGAINST HIV-1 USING A COMBINATION VACCINE OF RECOMBINANT CLOSTRIDIUM PERFRINGENS AND HIV-1 VIRUS LIKE PARTICLES

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Submitted to the Graduate Faculty of

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2009

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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INDUCTION OF STRONG CELLULAR IMMUNE RESPONSES IN THE GUT MUCOSA AGAINST HIV-1 USING A COMBINATION VACCINE OF RECOMBINANT CLOSTRIDIUM PERFRINGENS AND HIV-1 VIRUS LIKE PARTICLES

Poonam Poonam, PhD

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The gut mucosa is an important portal for HIV-1 transmission and infection. Therefore, a vaccine which can prevent virus transmission at mucosal surfaces would be an ideal HIV-1 vaccine candidate. *Clostridium perfringens* has been used as a vehicle to deliver SIV proteins in large quantity to the terminal ileum. A mucosal immunization strategy using *C. perfringens* should be able to induce potent mucosal immune responses against HIV-1. A recombinant *C. perfringens* expressing large amount of HIV-1 Gag protein (*Cp*-Gag) was constructed. Under *in vitro* conditions, *Cp*-Gag was found to induce bone marrow derived dendrite cell (BMDC) to mature and stimulate HIV-1 Gag specific T cell responses. Then *in vivo* experiments were performed in mice to demonstrate orally delivered *Cp*-Gag ability to prime gut mucosal T cell responses. Since oral tolerance is a major obstacle for orally delivered immunization approaches, a combination of mutated heat-labile enterotoxin of *E. coli* (mLT) and CpG containing oligodeoxynucleotides (CpG-ODN) were used as adjuvants for oral administration with *Cp*-Gag. Orally delivered *Cp*-Gag was tested for induction of HIV-1 Gag specific T cell responses in a prime-boost model with intranasal inoculation of HIV-1 virus like particles (VLP). HIV-1 specific cellular immune responses in both the effector (Lamina propria) and inductive sites

(Peyer's patches) of the gastrointestinal (GI) tract were significantly higher in mice immunized using *Cp*-Gag and VLPs in prime-boost approaches compared to mice immunized with either *Cp*-Gag or VLPs alone. Such cellular immune response was found to be mediated by both $CD8^+$ and $CD4^+$ T cells. These groups of mice also seemed to have HIV-1 specific multifunctional T cells in PPs and LP of the GI tract. In summary, mucosal immunization of mice with a *Cp*-Gag and VLPs in a prime-boost mode led to strong HIV-1 Gag specific cellular immune responses in both mucosal and systemic immune compartments. Such strong mucosal immune response could be very important to control HIV-1 infection at mucosal surfaces. The proposed vaccine strategy has great public health significance for developing a practical vaccine against HIV due to its safety, low production cost and easy administration.

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ACKNOWLEDGEMENTS

My acknowledgment can't begin without thanking my mentor Dr. Phalguni Gupta who I sincerely thank for taking me as his student and teaching me a lot. I cannot begin to describe how much his patience and mentoring have affected me both professionally and personally. Through his guidance, I have learned not only the fundamental values of research, but also how to succeed in a field as intricate as this. Thank you for all your encouragement and discouragement. Thank you for always being for me whenever I needed you. You have not only been my supervisor but a father figure to me. You have a very special place in my heart and always will be, no matter where I am.

I would also like to thank my committee members, Dr Ted Ross, Dr Todd Reinhart and Dr Russell Salter, for keeping me focused and providing helpful comments in my thesis research. Thank you very much for making the immense time commitment to be a part of my committee, for your assistance and guidance in this project and for being ready for a meeting no matter how short a notice I gave you and thank you a lot for your patience for reading my not so good drafts.

I absolutely could not have made it to this day without the help, support and friendship of my fellow lab members. A special thank to the clostridia vaccine group; Dr Yue Chen, Dr Ruth Helmus and Lori Caruso for their role in helping me finish this project.

Thank you to Yue Chen, for all her help and support. I don't have words to express my gratitude for all the things you have done for me. I depended on you numerous times to keep me on track and optimistic and you always were there for me!

To Ruth, I thank you for being my friend. I can't thank you for your constant encouragement, support and patience. You have been an asset to me throughout the several years we have worked together.

Thank you to Lori, for your help in my experiments and for always being sweet to me. My appreciation goes to Dr Chen Li for his help with immunization study and in processing mice cells.

I am extremely grateful to my other past and present lab members; Dr. Milka and Jackeline; thank you for your friendship, for always being with me in good and bad. Thank you for the wonderful time we had together. Thank you, Dr Ashwin for your support when things were not going right, you always encourage me and keep me focused. Thank you, Kathy, Deena, Varsha, Ayan and Payal for your support and encouragement. There exists an endless list of individuals to whom I am deeply indebted for aiding in the pursuit of my doctoral studies.

I would like to thank the chairperson of our department Dr Charles Rinaldo for sharing his knowledge and experiences in this field; and for his encouragement after my first departmental presentation. Thank you for helping and being with me when our freezer broke down and you helped in transferring all our samples into your freezer. It has been a privilege to be student in your department.

I would like to thank the department of Infectious Diseases and Microbiology and all the students, faculty and staff that made it such a pleasure to work and learn here. Thank you all!

Special thanks to my classmates for your constant support and friendship. I have learned so much from you. Thank you all for the wonderful time we have had together.

To my friends here – Jayanth Venkatachari, Nitin Bhardwaj, Nabanita Biswas, Jessica Radjio, Purushottam Narute, Jill Montogmery, Yue Jen Tung, I could always count on you to be there to give advice or to have good time together. You all are very special to me!

My friends outside this continent –Dr Sonika Tyagi, Dr Pallavi Singh, Dr Veenu Tripathi, Stafania Mazzitelli, Kristina Shih, Dr Sunil Verma and Dr Rajesh Singh. Thank you for being my friends!

Thank you to my love and my husband now, Amarendra Pegu, for being with me throughout the process of getting to graduate school and then getting through it. You have been forever patient with me, always! I would have never gotten this far if it was not you and your support. You have stayed up innumerable late nights correcting my drafts and grammar, working on my slides. You are always there to solve my problem and explain logic to me whenever I talk illogical things. The world may not understand me but you did. My best friend, my most trusted critic, and, the only person who can bring out the best in me. You truly are my soul-mate!

Last but not least, my family members for their support and encouragement always no matter what I chose to do. Thank you for your unconditional love and support. Mummy and Papa, thank you, for always being by my side. Thank you for your believe in me. I would also like to thank my siblings Arvind, Jyotindra, Robin, Reena, and to my youngest sister Priyanka for your selfless love and support.

1.0 INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) is a member of the genus *Lentivirus* from the *Retroviridae* family. Retroviruses are enveloped RNA viruses. Distinguishing features of this family of viruses are a life cycle which includes reverse transcription of the retroviral RNA genome into DNA, which is then integrated into the host genome. Simian immunodeficiency virus (SIV) is the nonhuman primate lentivirus most closely related to HIV-1. Therefore, SIV have proved a useful experimental model of HIV-1 pathogenesis. HIV-1 is the causative agent responsible for the current acquired immune deficiency syndrome (AIDS) pandemic, having spread all across the globe [2]. HIV-1 is primarily transmitted through heterosexual contact, although injection drug use, men who have sex with men (MSM) and mother-to-child transmission also constitute a considerable amount of virus transmission. Currently, there is no cure for AIDS, although therapies combining multiple expensive drugs have been used to lower viral load and ameliorate symptoms. Therefore, an effective HIV-1 vaccine to prevent infection is the most cost effective approach to contain the impact of HIV-1/AIDS on public health.

1.1 THE AIDS EPIDEMIC

Since the discovery of HIV-1 over 25 years ago, the global AIDS epidemic has become one of the greatest threats to human health and development. In 2007 approximately 33 million people

were living with HIV-1 (Figure 1). 2.7 million new HIV-1 infections and 2 million deaths due to AIDS occur each year [3].

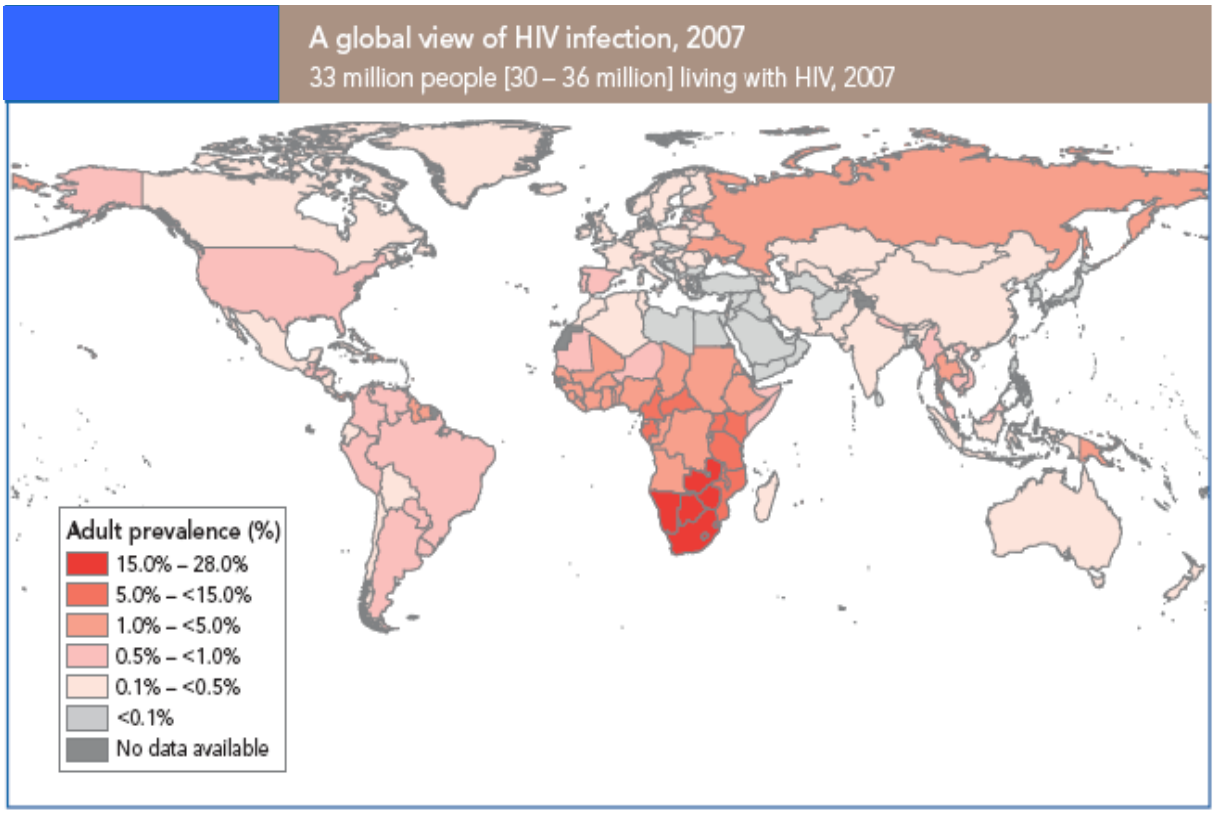

Figure 1. The global HIV-1 epidemic in 2007.

From the "Report on the global HIV-1/AIDS epidemic 2008" published by UNAIDS. Adapted from publicly available data from UNAIDS. <www.unaids.org>. Reproduced by kind permission of UNAIDS. © 2008 Report on the global AIDS epidemic.

Highly active antiretroviral therapy extends the lives of infected individuals. However, much of the HIV-1 epidemic is due to infections in countries where access to health care and medicines is limited [4]. The most heavily affected region is sub-Saharan Africa, where in a few countries more than one in five adults is infected with HIV-1. At the end of 2007, there were nine countries in Africa where more than one tenth of the adult population aged 15-49 was found to be infected with HIV-1. In three countries, within the same continent, at least one adult in five

is living with the virus. Strikingly, 23.9% of adults in Botswana and 18.1% in South Africa are infected with HIV-1. Currently, there are approximately 22 million Africans living with HIV-1/AIDS. The latest UNAIDS estimates indicate that 35,000 people in North Africa and the Middle East acquired an HIV-1 infection in 2007, bringing the total number of people living with HIV-1/AIDS in the Middle East and North Africa to an estimated 380,000 [3].

The epidemic of AIDS in Asia appears to be of more recent origin, and many Asian countries lack adequate systems for monitoring the spread of HIV-1. Half of the world's population lives in Asia, so even small differences in the infection rates can result in large increases in the number of people infected. In 2007, about five million people were living with HIV-1 in Asia including 2.4 million in India. The AIDS epidemic is rapidly increasing in Eastern Europe and Central Asia too, where the number of people living with HIV-1 increased 150% between 2001 and 2007 [3]. Therefore, it is necessary to concentrate efforts towards designing a safe, effective and affordable vaccine for HIV-1 to control the growing epidemic worldwide.

1.2 HIV-1 BIOLOGY

1.2.1 HIV-1 Virion Structure

The HIV-1 virion is composed of two 9.2 kb RNA strands enveloped by a lipid bilayer derived from the membrane of the host cell (Figure 2). The genomes of HIV-1 contain three prototypic genes flanked by non-coding long terminal repeats (LTR) sequences. The *gag* gene encodes the structural proteins matrix, capsid, and nucleocapsid. The *pol* gene, encodes the viral enzymes reverse transcriptase (RT), protease (Pro), and integrase (In). The *en*v gene, encodes the outer

glycoprotein that mediates viral entry of permissive cell types. Additionally, there are six open reading frames (ORF) composed of two genes that are essential for replication, *tat* and *rev* [5, 6], and the four accessory genes HIV-1 replication, *vif, vpr, vpu* and *nef* [6].

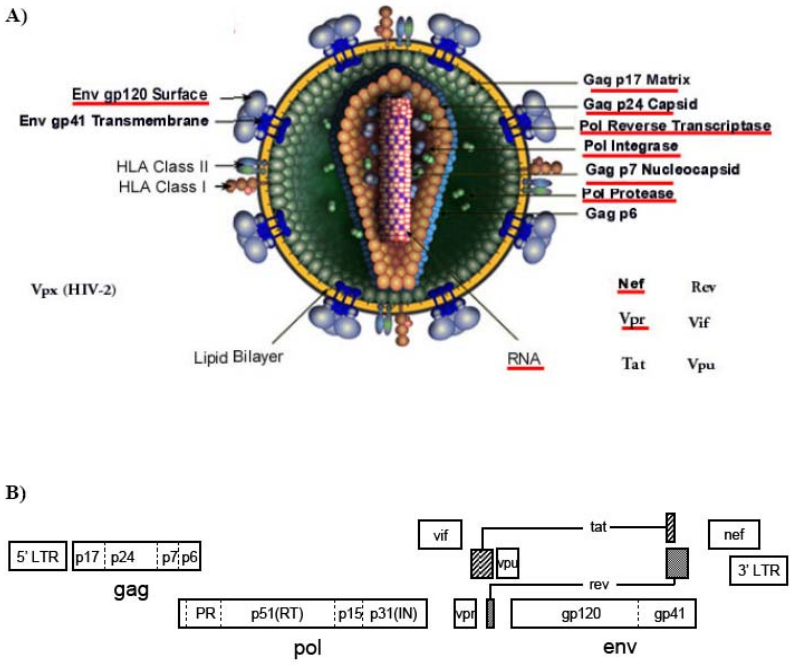

Figure 2. HIV-1 virion structure and genomic organization.

A) Mature HIV-1 virion structure. Image was reproduced from a publicly available source from the Los Alamos National Laboratory HIV-1 Database, Los Alamos, New Mexico. www.HIV-1.lanl.gov. B) HIV-1 genomic structure.

1.2.2 HIV-1 Lifecycle

HIV-1 enters a cell by binding of viral surface protein gp120 to cellular glycoprotein CD4, mainly expressed on $CD4^+$ T cells, monocytes and macrophages. This initial binding facilitates further binding of gp120 to two main co-receptor molecules, the chemokine receptors CCR5 (R5 tropic virus) and CXCR4 (X4 tropic virus) on the host cell [7, 8]. This binding of co-receptors

promotes a conformational change of gp120 which induces fusion of gp41 on the virus membrane to the target host cell membrane followed by subsequent release of the viral core into the cell cytoplasm [9]. Following release of the viral core, the HIV-1 RNA genome is reverse transcribed into DNA by virion reverse transcriptase and a host of additional viral and cellular proteins [9]. The viral DNA is then integrated into the host cell genome. Transcription of the viral genome occurs in the nucleus of the host cell mediated by the viral LTR and several host and viral cell transcription factors. Following the synthesis of viral proteins and shuttling of full-length copies of the viral RNA genome to the cytoplasm, the virion is assembled at the plasma membrane where budding occurs [7]. Generally, the structural polyproteins Gag-Pol and Env accumulate at the host cell plasma membrane and interact with the genomic RNA to package the genome into virions. Complete virions then bud from the plasma membrane. Final maturation of the virion occurs after budding from the cell and involves cleavage of the Gag polyprotein into a proline-rich protein (p6), a nucleic acid binding protein (p7), matrix (p17), and capsid (HIV-1 p24) [10].

1.2.3 Natural History of HIV-1 infection

There are four major modes of HIV-1 transmission: 1) sexual intercourse, 2) blood transfusion, 3) sharing contaminated needles from intravenous drug use and 4) mother-to-child during the prenatal period [11]. Many immunological and viral hallmarks are observed during the course of infection of HIV-1 [12, 13]. The majority of HIV-1 infected individuals follow three basic stages of disease progression: (i) primary or acute infection, (ii) asymptomatic (chronic) infection and (iii) symptomatic infection, better known as AIDS (Figure 3).

Primary or acute infection by HIV-1 is mainly characterized by flu-like symptoms that become present within 1 to 4 weeks after infection. These symptoms include sore throat, fever, muscle ache, swollen lymph nodes and rash [14]. Primary HIV infection is associated with a burst of HIV viremia and with abrupt and rapid decline of $CD4^+$ T cell number in the peripheral blood. The decrease in circulating $CD4^+$ T cells during primary infection is probably due both to HIV-1 mediated cell killing and to re-trafficking of cells to the lymphoid tissues and other organs [15]. During primary infection, HIV-1 transmission to a new host occurs and the virus targets $CCR5^+$ $CD4^+$ effector memory T cells [16, 17]. These target cells are present predominantly at mucosal sites of the gastrointestinal (GI) tract. As a consequence, a massive depletion of $CCR5^+$ $CD4^+$ cells occurs at these mucosal sites within the first few weeks of acute infection [16, 18]. Mucosal depletion is soon followed by a state of chronic activation resulting in 1) increased numbers of activated and memory T cells, 2) increased production of proinflammatory cytokines and 3) increased turnover of immune cells [16]. During the acute phase, viremia load can be as high as 10^6 to 10^7 copies/ml in plasma [14, 19]. Soon after infection, a 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 [20-22]. The $CD8^+$ T cell response is also high during acute infection and its ability to control viremia during the acute phase is usually the best predictor of a long-term asymptomatic period [14]. Following acute stage, an infected individual enters a clinically asymptomatic phase (chronic disease), which can persist for an average of 10 years in humans [23]. The asymptomatic stage varies from individual to individual. Low levels of virus replication and no apparent illness or clinical symptoms are present. Moreover, there is a decrease in viremia which could be due to an immune response mediated by $CD8^+$ T cell killing

of virus infected cells, substrate exhaustion due to the massive loss of target cells or a combination of both. During this time until the symptomatic phase begins (8 to 10 years in a typical progressor), there is a persistent low level of virus replication in the lymph nodes and peripheral blood, high viral genetic diversity and an increased viral replicative fitness. Low level virus replication is probably maintained by an antiviral $CD8^+$ T cell response. Although this phase is clinically asymptomatic, the delicate balance of the host immune system and viral replication dynamics are at constant war; cell turnover levels are high, leading to a persistent state of activation resulting in chronic progressive immune dysfunction [24]. Although a robust immune response is mounted soon after infection with the infiltration of $CD8^+$ T cells and production of proinflammatory cytokines and chemokines, it is generally too late and the infection is already well established. HIV specific neutralizing antibodies also arise during chronic infection and may help in limiting viremia [25].

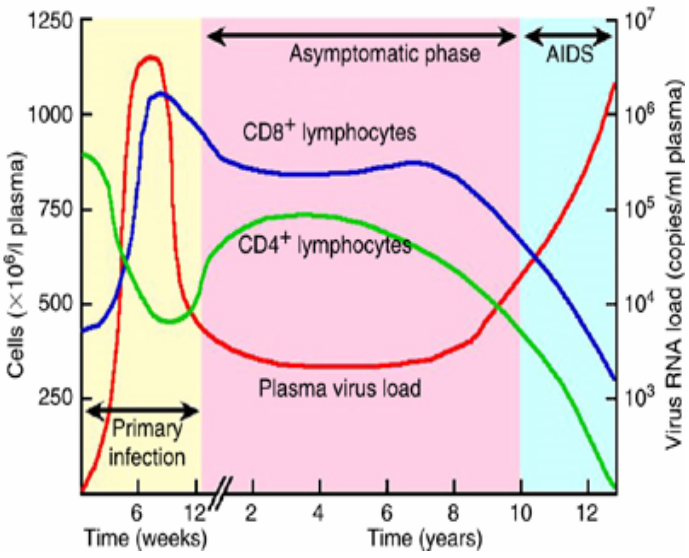

Figure 3: Schematic of typical course of HIV-1 infection showing changes in CD4 and CD8 T cell counts in peripheral blood and plasma viral load.

Figure from Munier et al., Immunol Cell Biol volume 85, 6-15, 2007 with permission from Nature publishing group.

The symptomatic phase is characterized by increased viral load, a decline in $CD4^+$ T cells to less than 200 cells/ul bloods and a deteriorated $CD8^+$ T cell response. During symptomatic phase $CD4^+$ T cell level constantly decline and reach a level in which the individual becomes susceptible to AIDS-defining opportunistic infections [e.g. *Pneumocystis carinii* pneumonia (PCP)] and neoplasms [24, 26, 27]. Because HIV-1 infection immediately diminishes the capacity for immune response formation, a vaccine that provides immunity before HIV-1 exposure and infection is desirable.

There is a small percentage of individuals including: (i) high risk commercial sex workers (likely repeat exposure to HIV-1 virus) that do not become infected [28-30] (ii) infected individuals who do not exhibit evidence of disease progression over extended period of time with slow or no decline in CD4+T cells, termed long term non-progressors (LTNP) [31, 32], and (iii) 'elite controllers' that can control virus without any treatment [33, 34]. Therefore, study of the HIV-1 specific immune responses in these individuals who are resistant to viral infection or control viral replication might shed light on the appropriate immune responses that need to be induced by a protective HIV-1 vaccine.

1.3 HIV-1 AND THE MUCOSA

The most common mode of HIV-1 transmission is through sexual contact with HIV-1-infected individuals. HIV-1 can enter the body during vaginal or anal sex and in some cases via oral route during oral sex, or through the surface tissues of the genitals [35]. The mucosa has large areas vulnerable to infection by pathogenic microorganisms with viral infection and replication observed in both urogenital and intestinal mucosa in humans and macaques [36, 37].

Several studies using immunohistochemistry of gastrointestinal (GI) tissues from chronically HIV-1-infected individuals, confirmed that the GI tract was significantly depleted of $CD4^+$ T cells [38-40]. These studies have shown that intestinal lamina propria (LP) could be a site of rapid CD4 lymphocyte destruction during HIV infection. Similar studies performed in rhesus macaques have also shown that within days of infection, there is a similar dramatic depletion of GI tract $CD4^+$ T cells in SIV infected animals [37, 41].

Studying the pathogenesis of $CD4^+$ T-cell depletion in the human GI tract during acute HIV-1 infection is challenging and not well defined as to why mucosal tissues are the primary targets for HIV-1, but it is likely that the nature of GI tract resident cells and the ability for virus or virally-infected cells to enter the GI mucosa contribute to infection of the GI tract. A recent study has shown that lymphocytes, including natural killer (NK) cells and $CD4^+$ and $CD8^+$ T cells, can bind to HIV-1 gp120 via the α4β7 gut homing molecule, suggesting a method by which virus is preferentially delivered to the GI tract following transmission [42]. Because envelope proteins remain associated with the host cell membrane following fusion, infected cells as well as free virus may bind to α4β7 and thus be transported to the GI tract [42, 43]. Many studies have examined depletion of the specific target of HIV-1: $CD4^+$ T cells that express the HIV-1 co-receptor CCR5 in the gut mucosal tissues. Phenotypic analysis of $CD4^+$ T cells in the GI mucosa of healthy individuals has demonstrated that the majority of T cells in mucosal tissues expresses CCR5 and is very permissive to *in vitro* infections. Such analyses have clearly demonstrated almost complete depletion of $CCR5^+$ $CD4^+$ T cells from mucosal sites of HIV-1-infected humans and SIV-infected rhesus macaque [44-46]. Preferential targeting of $CCR5^+$ $CD4^+$ T cells is consistent with reports showing more extensive depletion from the lamina propria (LP) as compared with inductive sites such as Peyer's patches (PP). The preferential loss

of $CCR5^{+}CD4^{+}$ T cells from the GI tract clearly suggests a "virus-centric" mechanism [38, 46]. Flow cytometric sorting of precisely defined T-cell subsets followed by quantitative polymerase chain reaction for viral DNA have shown that $CD4^{+}$ T cells in the GI tract are 10-fold more frequently infected by the virus than are those in the peripheral blood [47, 48]. It has also been shown that about 60% of GI $CD4^{+}$ T cells are infected by the virus *in vivo* [49].

The mechanisms underlying this phenomenon are not well defined but they have been explored extensively. In one PCR based study using the SIV model, it was demonstrated that at peak viremia (10 days post infection) 30 to 60% of memory T cells throughout the body (including the GI tract) are infected by SIV and suggested that direct viral cytopathicity is responsible for $CD4^{+}$ T-cell depletion in the intestines [47, 48]. Contemporaneously, another study suggested that the effects of direct viral infection alone could not account for the magnitude of GI $CD4^{+}$ T-cell loss, since only 7% of GI $CD4^{+}$ T cells were found to contain HIV-1 RNA [50]. These researchers based their findings on in situ hybridization as opposed to PCR-based techniques, which may account for the observed differences. Nevertheless, they found that SIV infection triggers Fas-Fas ligand-mediated apoptosis in lamina propria $CD4^{+}$ T cells and concluded that direct viral cytopathicity and indirect effects such as apoptosis of infected and uninfected $CD4^{+}$ T cells are both involved in the pathogenesis of mucosal $CD4^{+}$ T-cell depletion [48]. Therefore a mucosal HIV-1 vaccine is needed that can elicit a robust immune response in the GI tract to prevent the massive depletion of $CD4^{+}$ T cells in this mucosal tissue.

1.4 THE IMMUNE RESPONSE TO HIV-1 INFECTION

Both humoral and cellular components of the immune system generate a response against HIV-1 infection. Although initially these responses are able to control the viral load during the acute phase of the infection, there is no complete clearance of the virus. HIV-1 is able to persist by integrating into the chromosomes of resting T cells and thereby creating a latent reservoir, which marks the chronic phase of infection. In addition, due to the ability of HIV-1 to easily develop mutations that confer resistance against the initial immune response, HIV-1 is slowly able to escape immune control and ultimately progress to AIDS.

1.4.1 $CD8^+$ T cells in HIV-1 infection

It is known that $CD8^+$ T cells recognize peptides presented within the binding cleft of MHC class I (MHC-I) molecules. Most cells present peptides derived only from endogenous proteins on MHC-I molecules and, by doing so, become targets of the effector function of $CD8^+$ T cells. HIV-1-specific cytotoxic T lymphocytes (CTLs) recognize cells displaying viral antigen on MHC-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 $CD8^+$ CTLs to Fas on target cells engages the caspase cascade, leading to apoptosis [51, 52]. Second, and most commonly, CTLs degranulate, releasing perforin and granzyme proteins from intracellular lytic granules into the space between the CTL and its target [53]. Monomeric perforin inserts into the target cell plasma membrane and can polymerize to form pores through which granzyme can use to enter the target cell. Granzymes are serine

proteases which can cleave cellular proteins to initiate the caspase cascade and therefore lead to death of the target cells via apoptotic pathways.

The responses of HIV-1 specific $CD8^+$ T cells or cytotoxic T lymphocytes (CTL) appear to play an important role in the control of viral replication during natural history of HIV-1 infection. Early studies showed that the HIV-1 specific $CD8^+$ T cell responses emerge during acute infection coincident with initial control of primary viremia [54-56]. Potent cellular immune responses have also been reported in long-term non-progressors [57], and in individuals with specific HLA alleles and the breadth of HIV-1 Gag-specific T lymphocyte responses have been correlated with control of viral replication in HIV-1-infected individuals [58, 59]. These data indicate the potential importance of cellular immune responses in immune control of HIV-1. Depletion of $CD8^+$ T cells in SIV-infected macaques, a non-human primate model of HIV-1 infection, results in increased viral load and more rapid disease progression [60]. Strong vaccine-induced CTL responses in monkeys can control viral load and maintain $CD4^+$ T cells following challenge with SIV [61]. Therefore $CD8^+$ T cells play a key role in both systemic and mucosal immune responses to HIV-1.

Mucosal $CD8^+$ T cell responses are key to the control of viremia at the mucosal site of infection and limiting the extent of the viral spread in the body [62]. During the first few days of infection, there are only a few virus-infected cells in the mucosal tissues of vagina or rectum [63-65]. The presence of CTLs at these sites during this period should be able to prevent the spread of infection to other tissue compartments. On the other hand, the lack of CTL responses in these sites will lead to increased viral replication and subsequent spread of virus to other tissue compartments including secondary lymphoid organs like lymph nodes and the GI tract. The presence of antigen specific $CD8^+$ T cells in the colon has been associated with lower viremia in

monkeys [66, 67]. Also vaccine induced SIV specific $CD8^+$ T cells in the GI tract of monkeys abrogated or led to a milder productive SIV infection on mucosal challenge of these vaccinated monkeys [68, 69]. Therefore an HIV-1 vaccine must induce strong CTL responses at mucosal sites to be effective in protecting against mucosal HIV-1 infections.

1.4.2 $CD4^+$ T cells in HIV-1 infection

The $CD4^+$ T cells play a key role in the generation of immunity by providing appropriate 'help' for the development of humoral and cell mediated immune responses. The $CD4^+$ T cells are important for their help in the maintenance of $CD8^+$ T cell functions during chronic infections [70, 71]. For humans, loss of $CD4^+$ T cells during HIV-1 infection often precedes or is associated with $CD8^+$ T cell dysfunction and AIDS progression [72]. $CD4^+$ T cells also appear to play an important role in the optimal priming of $CD8^+$ T cells during acute infections [73, 74]. The common finding of these recent reports is that secondary expansion of memory $CD8^+$ T cells following re-stimulation is dramatically reduced if the $CD8^+$ T cells were originally primed in the absence of $CD4^+$ T cells [75].

The massive depletion of $CD4^+$ T cells during acute HIV-1 infection occurs predominantly in the $CCR5^+$ memory population, which is the major population found in the GI tract [18]. Similar depletion of $CD4^+$ memory T cells has also been found in SIV-infected monkeys [47, 50, 76]. The depletion of these $CD4^+$ memory T cells is a result of induction of cell death by both direct infection and bystander cell killing of these cells [77]. It has been reported that at the peak of viremia, more than 80% of $CD4^+$ memory T cells can die, of which only a fraction of cells are infected [47]. It has also been suggested that the level of depletion of GI tract $CD4^+$ memory T cells correlates with the time to the development of AIDS [78].

1.4.3 Multifunctional T cells and HIV-1 infection

Virus-specific $CD8^+$ T cells contribute to viral control by directly killing of virus-infected cells, secreting antiviral factors, and chemotactic factors that recruit other cells of the immune system [79]. Although virus-specific $CD8^+$ T cells are often measured by a limited number of parameters, such as IFN-γ and/or IL-2 secretion, the functional profiles of T cells are certainly more diverse. For example the correlation between IFN-γ production and health of HIV-1-infected individuals is weak and IFN-γ levels in vaccinated individuals do not correlate with protection [80-82]. 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 is a growth factor that is secreted by T cells to promote the proliferation and differentiation of antigen specific T cells in an attempt to respond to pathogenic infections swiftly and efficiently. Typically IL-2 is considered one of the $CD4^+$ T cell cytokines but $CD8^+$ T cells are also quite capable of producing IL-2. Unlike $CD4^+$ T cells though, $CD8^+$ T-cell production of IL-2 is typically much more restricted. The selective impairment of IL-2 secretion following TCR stimulation is a critical determinant of immune dysfunction during HIV-1 infection, resulting in the inability of HIV-1-specific $CD4^+$ T cells to mediate protective immunity upon restimulation [83-85]. IL-2 secretion thus serves as a reliable marker of functional HIV-1-specific $CD4^+$ T cells.

TNF-α is mainly produced by monocytes and macrophages but can also be secreted by T cells, natural killer cells, basophils, eosinophils, dendritic cells, neutrophils, and mast cells [86]. TNF-α synthesis can be stimulated by viral infections and help in the controlling viral replication. In the case of T cells, engagement of the TCR triggers TNF-α release, which

amplifies the Th1 response by inducing the synthesis of IL-12 and IL-18 [87]. These factors are important in up regulating IFN-γ production. TNF-α can alternatively kill virally infected target cells by binding its cognate receptor on their cell surface. This phenomenon is due to the fact that the TNF receptor contains an intracellular 'death domain' which triggers an apoptosis signaling cascade [87, 88].

The cytotoxic capacity of $CD8^+$ T cells is imperative in HIV-1 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 [81, 89]. CD107a can be detected on the surface of $CD8^+$ T cells although these molecules are not normally found on the surface of $CD8^+$ T cells but are found in the cytotoxic granule membrane [90]. Therefore only after the degranulation of activated $CD8^+$ T cells, CD107a and b molecules are made accessible on the cell surface for direct labeling by specific antibodies. Therefore CD107a has been associated with the cytotoxic capacity of $CD8^+$ T cells. The chemokines macrophage inflammatory protein [MIP-1α or MIP-1β] can also be found in cytotoxic granules, the latter type of which is upregulated rapidly upon activation [91].

Flow cytometry has revolutionized the HIV-1 field by allowing researchers, to measure multiple parameters and T cell effector functions at the single cell level [92]. This has been especially powerful in the measuring of phenotype T cells including $CD8^+$ and $CD4^+$ T cells and their classification as naïve, effector, central memory, or effector memory [93-95]. These can be further combined with the functional correlates (IFNγ, TNFα, IL-2, CD107a and b, perforin, granzyme A and B) which have been termed as 'functional signatures' [96, 97]. Understanding these functional patterns of antigen-specific responses is likely to lead researchers to the crucial missing pieces of viral control in acute and chronic infections. Multiparametric flow cytometry

has been used to examined five or more functions of $CD8^+$ T cells (surface CD107a mobilization for degranulation and IFN-γ, IL-2, MIP 1β, and TNF-α production) in HIV-1-infected progressors and non-progressors and found that non-progressors preferentially maintain HIV-1-specific $CD8^+$ T cells that have all five functions on a cell-by-cell basis [98].

The association between maintenance of this multifunctional phenotype of effector $CD8^+$ T cells and non-progression suggests that multifunctional $CD8^+$ T cells are an important component of a protective immune response. Growing evidence supports the concept that evaluation of the multifunctionality of T cells displaying more than three of the functions of the cytokine production, IFN-γ, TNF-α , IL-2, and degranulation via CD107a/b surface expression are more often detectable in individuals with low viral loads and healthy levels of $CD4^+$ T cells [98-105]. However, whether a vaccine can induce sustained levels of multifunctional T cells and whether such responses are associated with protection against viral challenge are unknown. But definitely understanding these functional patterns of antigen-specific responses is likely to lead researchers to the crucial missing pieces of viral control in acute and chronic infection.

1.4.4 Antibody responses in HIV-1 infection

Virus neutralizing antibody titers represent key immune correlates of protection for most licensed viral vaccines, and thus early studies focused on developing immunogens derived from HIV-1 envelope subunits. However these immunogens failed to generate protective immunity and were largely unsuccessful in clinical trials [106, 107]. Despite these early setbacks, neutralizing antibodies (NAbs) remain a major focus for HIV-1 vaccine development. A number of rare human NAbs have been isolated from HIV-1–infected patients that indeed do broadly

neutralize diverse HIV-1 strains, such as NAbs 2F5 and 4E10 against the gp41 membrane proximal region and NAb 1b12 reactive with the gp120 CD4-binding site [108-111].

HIV-1 envelope constructs made in the laboratory express the binding sites of these antibodies (i.e., they are antigenic), but when these HIV-1 envelopes are injected into animals or human subjects, they do not induce broadly neutralizing antibodies [108, 110, 111]. It is poorly understood why HIV-1 infected individuals fail to induce broadly neutralizing antibodies with specificities like the rare human NAbs. The causes of poor envelope immunogenicity might be due to a combination of various factors. The HIV-1 envelope is heavily glycosylated, with up to 40% of envelope mass carbohydrate creating an envelope glycan shield [110]. Also the HIV-1 envelope is quite flexible and confers a considerable energy barrier to B cells that would recognize broadly neutralizing epitopes. Therefore the construction of vaccine vectors that elicit broad neutralizing antibodies against HIV-1 remains a challenge and is a potential roadblock in the development of a successful HIV-1 vaccine.

1.5 HIV-1 VACCINES

Developing a safe and effective preventive HIV-1 vaccine is a critical priority in the overall plan to contain the global AIDS epidemic. However, progress on the development of a vaccine has been slow since the AIDS epidemic was first recognized in 1981. An effective HIV-1 vaccine should be able to stimulate both types of immune responses: a humoral response, in which B cells produce neutralizing antibodies against the viral surface glycoprotein to target free virus [112] and a cellular response, in which T cells lead to the elimination of already infected cells. These two types of immune responses have been implicated in control of HIV-1 infection. 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 and these $CD8^+$ T cells develop into cytotoxic T lymphocytes (CTLs) which play an important in the elimination of virus infected cells and therefore are important in virus clearance [113]. CTLs at sites of mucosal transmission are important for preventing viral spread [114, 115]. A Th2-type environment, dominated by IL-4, promotes B cell response maturation and antibody production. Much of the cytokine production driving Th1- or Th-2 responses occurs in activated $CD4^+$ T cells, although other cells also play vital roles. Therefore, $CD4^+$ T cells are essential for an immune response since they provide co stimulatory signals and produce cytokines that lead to activation of viral specific B and T cells [116].

1.5.1 Current HIV-1 vaccine candidates

The development of a safe, globally effective, easy to administer and affordable HIV-1 vaccine is the best hope for controlling the AIDS pandemic. There has been much intense research going on in this field and significant progress has been made. [117]. Multiple vaccine concepts and vaccination strategies have been tested, including DNA vaccines, subunit vaccines, live vectored recombinant vaccines and various prime-boost vaccine combinations. More than 35 vaccine candidates have been tested in Phase I/II clinical trials, involving more than 10,000 volunteers [117, 118], and two Phase III trials have been completed, themselves involving more than 7500 volunteers [119].

In a recent setback to the HIV-1 vaccine field, an adenovirus type 5 (Ad5) based vaccine developed by Merck and NIH failed to provide protection against HIV-1 infection in the

randomized STEP study [120]. The vaccine was made up of a recombinant replication-incompetent adenovirus type 5 (Ad5) that expresses the relatively well-conserved HIV-1 *gag, pol*, and *nef* genes from subtype B strains of HIV-1 and was aimed to prevent infection or reduce early viral burden though the induction of T-cell immunity alone. The results show the vaccine failed to achieve either objective, and suggest an increase of HIV-1 infection rate in male participants on the vaccine (4.6%) compared with controls (3.1%, p=0.07) [121]. Although the reasons for failure in this vaccine trial are unknown, it is suspected that lack of humoral antibody and mucosal immune response, and preexisting immune response to Ad5 may have contributed to such lack of efficacy of vaccine to prevent HIV-1 transmission. Therefore, future candidate HIV-1 vaccines probably must induce immunity in both mucosal and peripheral blood compartments, and also yield protective humoral responses in addition to inducing cellular responses. It has also been speculated that the use of more than one vector in heterologous prime boost approaches might be better in inducing protective immune responses than repeated administration of the same vector, as was the case in the STEP trail.

The heterologous prime-boost vaccination approach has been shown to be an efficient way to induce T cell responses in animals and in humans. Sequential immunization with two different replicating recombinant viral vectors was found to induce a strong $CD8^+$ T cell response against malaria [122]. Further studies in mice have shown that the heterologous prime-boost strategy with two different vectors encoding the same antigen is more efficient in inducing cellular immune responses than the use of a single vector [123, 124]. Studies in nonhuman primates have revealed that the prime-boost approach induces strong cellular immune responses and can lead to protection against diseases such as malaria or SIV in primates [61, 125].

We have still not been successful in making an effective HIV-1 vaccine, due to multiple hurdles and challenges. The development of a safe and effective vaccine is hampered by the high genetic variability of the virus [117, 126], the lack of knowledge of immune correlates of protection [127, 128], the difficulty of generating broadly neutralizing antibodies [112], the absence of relevant and predictive animal models and the complexities related to the preparation and difficulty in conducting multiple large-scale clinical trials, especially in developing countries [117, 129, 130]. There are several ongoing efforts in developing novel HIV-1 vaccines, which use new approaches. More intense and elaborated research on mucosal immunology has shown the path for these newer vaccine approaches.

1.5.2 Mucosal HIV-1 Vaccines

In general mucosal vaccines using oral or nasal routes have the potential advantages of being painless, easy to administer on a large scale and easy to store, and deliver than current systemic vaccine technologies [131-133]. Since most of the HIV-1 infections are transmitted through rectal and vaginal mucosal contact, it is imperative for a successful vaccine to induce immunity at the mucosal surface. 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. [131].

The development of a mucosal HIV-1 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 [134, 135]. Antigen delivery has been tested using attenuated live

virus, killed virus, recombinant virus, DNA, dendritic cells and peptides. Oral immunization strategies have been shown to induce good immune responses in the GI tract [136, 137], whereas nasal immunization strategies have been reported to induce robust immune responses in the respiratory and genitourinary tract [138]. Therefore a mucosal immunization strategy using both oral and nasal routes should be able to induce potent immune responses at most of the mucosal surfaces. The mucosal immune responses can also be optimized by the choice of delivery system for the vaccine components. Several bacterial and viral vaccine vectors are currently being considered as HIV-1 vaccine candidates and some of these are known to generate strong mucosal immune responses, depending on how they are administered.

Scientists are also looking at how substances called adjuvants delivered along with the vaccine candidate can be used to improve the mucosal immune responses induced. Adjuvants are already used with several licensed vaccines for other diseases to boost the level of immune responses and their duration. Several research groups are looking at novel substances that can specifically increase the production of antibodies and immune cells at mucosal surfaces.

Live-attenuated virus has been shown to be very effective at eliciting protective immune responses against HIV-1 in non-human primate models [139, 140]. However, live-attenuated virus vaccines for HIV-1 has limit in human due to safety issues. The most significant safety issue is the potential of live-attenuated HIV-1 to revert to a pathogenic form after inoculation, as well as the possibility to recombine with wild-type virus in an infected host.

An alternative approach to live-attenuated virus vaccines for HIV-1 is the use of virus-like particle (VLP) vaccines. VLPs, or pseudovirions, are self-assembling, non-replicating, non-pathogenic, genomeless particles that are similar in size and conformation to intact virions. These immunogens can be administered as purified particles or as DNA plasmids expressing the

viral proteins to form VLPs *in vivo* [139]. Several successful VLP vaccines have been developed and tested in human trials attesting to the efficacy of HIV-1 VLPs as a promising HIV-1 vaccine candidate [141, 142]. Studies on HBV and HPV VLP vaccines have revealed a number of characteristics in the context of the induction of immunity. HBV VLP vaccines are effective in priming cellular and humoral responses and have been successfully used for hepatitis B vaccination [143, 144]. Intranasal delivery of VLPs seems more efficient and requires lower doses of antigen than oral delivery [141]. VLPs are easy to make and highly immunogenic. Therefore VLPs can be used as a potent mucosal HIV-1 vaccine candidate.

Systemically delivered viral vectors can induce mucosal immune response formation against HIV-1 or SIV, most notably in the gut, rectal, and genital mucosa [145, 146]. However, the strength of these responses is generally poor. For example, Ad-vectored vaccines have been shown to induce low levels of mucosal immune responses after systemic inoculation, which are approximately 10 times less than the immune responses induced at systemic sites [145, 146]. Mucosal immune responses have also been seen in individuals inoculated intramuscularly with a pox-based vector [147]. But overall it is widely accepted that direct delivery of antigens to mucosal site achieves more effective mucosal immunity [114, 131, 148]. However there are very few mucosal vaccines against any infectious disease and the mucosal vaccines already available provide protection via induction of antibody responses. There are no current mucosal vaccines that are known to induce strong protective cellular immune responses. Therefore understanding biology of the mucosal immune system to develop better mucosal vaccines that can induce both humoral and cellular immunity is important.

1.6 THE BIOLOGY OF MUCOSA

The major obstacle in mucosal vaccine development is the challenge of induction of immunity in the mucosa. While 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.

1.6.1 Anatomy of the mucosal immune tissue

The gut-associated lymphoid tissue (GALT) is the largest component of the lymphoid organ system and is divided into inductive and effector sites. Inductive sites are comprised of organized lymphoid tissues such as the Peyer's patches and the draining mesenteric lymph nodes, which are distributed throughout the wall of the small and large intestines. In contrast, the effector sites consist of lymphocytes scattered in a less organized fashion throughout the epithelium and lamina propria of the gut mucosa [131, 132] (Figure 4).

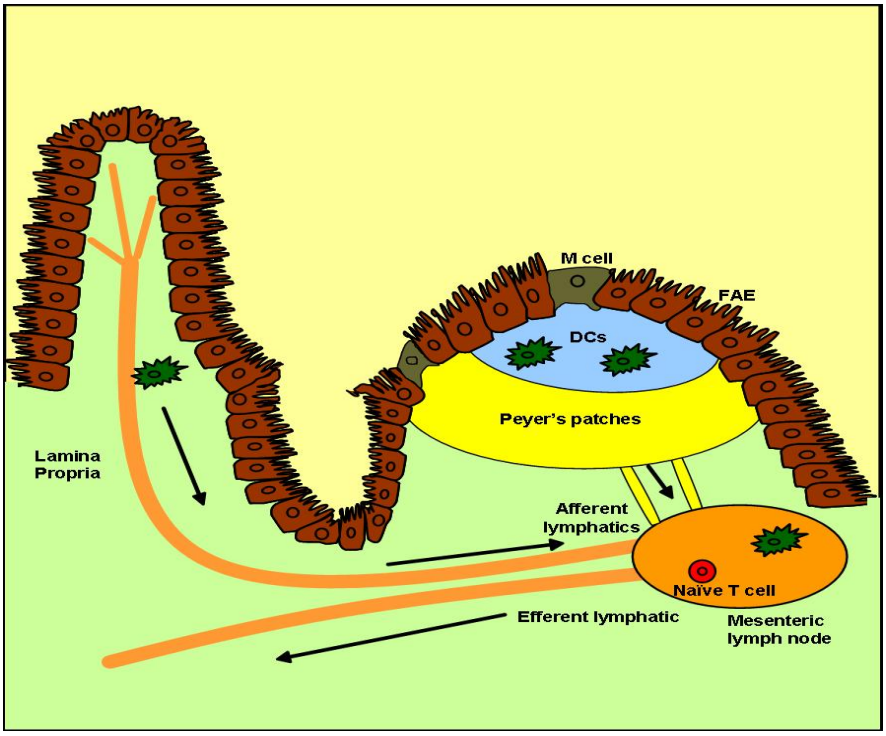

Figure 4. The gastrointestinal immune system.

Adapted from "The biology of oral tolerance and issues related to oral vaccine design". Poonam P. Curr Pharm Des. 2007 vol 13, 2001-7

1.6.1.1 Peyer's Patches

The Peyer's patches (PPs) are highly specialized lymphoid follicles in the small intestinal wall that contain naïve B cells, follicular dendritic cells (FDCs), and T cells [149]. PPs are separated from the lumen by a monostratified epithelium called follicle-associated epithelium (FAE), and a subepithelial dome (SED) containing numerous DCs. The FAE is characterized by the presence of microfold (M) cell. M cells are specialized in the capture and translocation of microorganisms and particulate antigens from the lumen to the underlying SED. In the SED, antigens can be captured and processed by antigen-presenting cells (APCs), such as DCs [150]. Following antigen uptake, these DCs present the antigen to specific $CD4^+$ T cells, either in the thymus dependent areas (TDA) of the PPs or in the T cell rich areas of the draining lymph node following migration to it. Primed T cells are induced to express the $\alpha_4\beta_7$ integrin and the CCR9

chemokine receptor molecules that lead to the recirculation of these differentiated T cells into the bloodstream and ultimately the migration of these cells into the intestinal tissues [132]. This mechanism is a possible explanation of the anatomic compartmentalization of the mucosal immune response and the mechanism by which antigen delivery to mucosal surfaces can trigger immunity at local and distal sites.

1.6.1.2 Mesenteric Lymph Nodes

The mesenteric lymph nodes (MLNs) are highly organized lymphoid structures and the largest lymph nodes in the body. MLNs are a part of the intestinal immune system and these are defined as draining lymph nodes that are connected to PPs, lamina propria, and to the peripheral immune system through afferent or efferent lymphatic vessels. The lymphocytes that are primed in the PPs exit through the afferent lymphatic vessels to the MLNs. Then, they migrate into the bloodstream through the thoracic duct and accumulate in the mucosa [132]. Accumulation of lymphocytes in the MLNs requires both L-selectin and $\alpha_4\beta_7$ integrin adhesion molecules, which normally direct lymphocytes to enter peripheral and mucosal tissues, respectively [150]. As a result of these unique anatomical features, the MLNs might be at crossroads between the peripheral and mucosal recirculation pathways.

1.6.1.3 Lamina Propria

The lamina propria (LP) of the GI tract is a meshwork of connective tissues underlying the GI epithelium that contains a broad spectrum of myeloid and lymphoid cells. The intestinal LP appears to contain DCs that are exposed to intestinal luminal antigens. These DCs present antigen locally or they immigrate to distant sites where they may prime T cells against intestinal derived antigens [151]. LP is also important for intestinal immune responses since orally

delivered antigens are presented by resident DCs in the LP, and the MLNs appear to contain mostly DCs that have migrated from the LP and not from PPs [152-154]. LP associated DCs have been shown to either uptake intestinal antigens that appear in the LP or directly sample luminal antigens by extending dendrites through the epithelium [155, 156].

1.6.2 The Mucosal Immune System

The mucosal immune system is an important first line of defense against infection. The mucosal surface is a major natural route of HIV-1 entry and the GALT is a major site of early HIV-1 replication [37, 41, 157]. The GALT comprises inductive immune tissue that collect antigen from the mucosal surface and generate immune responses against pathogens present in the GI tract. 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.

The various sites of the mucosal immune system are interconnected and show a lot of similarities in overall mucosa structure. Therefore, generation of a protective immune response in one mucosal area is able to afford protection at other mucosal sites [158, 159]. Therefore, mucosal antigen delivery can induce mucosal immunity at local and distal sites as well as systemic immune responses [147, 158, 159]. For example oral immunization against typhoid using *Salmonella typhi* vector has been shown to induce immune responses in the small intestine, ascending colon, mammary and salivary glands, although it is relatively inefficient at evoking response in the distal segments of the large intestines, tonsils or the female genital tract mucosa [159, 160]. On the other hand, intranasal immunization in humans results in strong immune

responses in the upper airway mucosa and regional secretions (saliva, nasal secretions) but weak immune responses in the gut [113, 159].

1.6.3 HIV-1 and the mucosa

The most common way that HIV-1 can be transmitted is through sexual contact with HIV-1-infected individuals. HIV-1 can enter the body during vaginal or anal sex and in some cases through oral sex, through the surface tissues (mucosa) of the genitals [35]. The mucosal surfaces have enormous surface areas that are vulnerable to infection by pathogenic microorganism. Infection and replication of virus have been observed in both urogenital and intestinal mucosa in humans and macaques [36, 37]. Several studies have shown that there is a similar dramatic depletion of GI tract $CD4^+$ T cells in HIV-1 infections [38-40]. Therefore, because of the significance of GI tract T cells in HIV-1 infection, the development of a strong cellular immune response in the intestine should be a major function of a mucosal vaccine against HIV-1.

1.6.4 Challenges in development of mucosal vaccines

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 are wholly dependent upon the production of humoral, not cellular responses [159]. This underscores the difficulty that the scientific community has experienced in developing mucosally delivered vaccines that induce antigen-specific $CD8^+$ T cell responses. Ongoing studies of novel mucosal

vaccine vectors may provide ways to induce cellular responses in addition to humoral responses at both mucosal and systemic sites.

1.6.4.1 Mucosal tolerance and the use of adjuvants

Oral vaccines represent an attractive approach for immunization against various mucosal infections due to their importance in eliciting protective immune responses both at mucosal and systemic sites; and also due to their ease of administration. However, despite many attractive features, developing an oral vaccine has often been difficult. A major obstacle in the development of orally delivered vaccines has been oral tolerance and therefore only a few oral vaccines have been approved for human use; such as vaccines against polio virus [161], *Salmonela typhi* [162], *V. cholera* [162] and rotavirus [163]. These vaccines are derived from live attenuated pathogens and have a major drawback of potential reversion of the attenuated strain to the wild type pathogenic strain. Therefore there is a need for the development of oral vaccines other than the live attenuated oral vaccines, which can be used as a mucosal vaccine against various mucosal infections.

Adjuvants have been used in oral immunization to overcome tolerance and direct the immune response either towards a Th1 or Th2 type response. The best known mucosal adjuvants are the secreted enterotoxin of *V. cholerae* and *E. coli* - cholera toxin (CT) and the *E. coli* heat labile enterotoxin (LT) respectively [164, 165]. These two enterotoxins are commonly used as adjuvants in small animal models. Both enterotoxins have almost similar immunological and physiochemical functions. Mutated form of LT (mLT) has been used as an adjuvant and was shown to induce both Th1 and Th2 immune responses, and when used in SIV vaccine formulations, it led to enhanced SIV-specific CTL levels [164, 166]. Furthermore, the safety of mLT adjuvant has been demonstrated in mice, nonhuman primates and humans [167].

The toll-like receptor-9 (TLR-9) ligand - synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotide motifs (CpG ODNs) is another promising mucosal adjuvant. CpG ODNs directly stimulate B cells and DCs, thereby promoting the production of Th1 and pro-inflammatory cytokines and the maturation/activation of professional antigen-presenting cells. CpG ODNs are effective as a mucosal adjuvant with intranasal delivered antigen [168, 169] and has shown to enhance adaptive Th1 humoral and cellular immune responses in mice and primates [170].

Apart from the natural adjuvants, synthetic components have also been used widely as adjuvants. Liposomes, which are phospholipid vesicles, have been evaluated both as adjuvants and as a delivery system for antigens and adjuvants and have been successfully used in numerous mucosal vaccination studies [171, 172]. Immune stimulating complexes (ISCOMS) have also been used to as adjuvants to enhance immune responses and present another attractive way to design oral vaccine vectors. ISCOMS are about 40 nm small particles and are made of Quil A (a potent adjuvant), cholesterol and phospholipids which can entrap multiple antigens [173]. ISCOMs have been used for parenteral immunization as well as for mucosal delivery of antigens [174]. Antigens from viral, bacterial and parasitic sources have been used with ISCOMS and they were able to boost both humoral and cellular responses against these antigens [173].

1.7 *CLOSTRIDIUM PERFRINGENS* VECTOR AS A MUCOSAL VACCINE CANDIDATE

Antigen delivery has been tested using attenuated live virus, killed virus, recombinant virus, DNA, dendritic cells and peptides. Recently the potential for recombinant bacteria to be used as vectors has been investigated. HIV-1 or SIV vaccine vectors have been designed using *Salmonella typhimurium, Shigella flexneri,* and *Listeria monocytogenes* [175-177]. A non-cytotoxic (cytopathic gene deleted) *Clostridium perfringens*, a gram positive, anaerobic bacterium, has been used in our laboratory to deliver a large amount of SIV proteins to the mucosal surface of the small intestine, especially to the GALT [1]. *C. perfringens* type A is a normal flora bacterium of the intestines of humans and other animals [178]. A small percentage (<1 %) of *C. perfringens* isolates produce an enterotoxin (CPE) that causes a mild food poisoning and some mild nonfood borne GI diseases. When foods contaminated with *cpe*-positive vegetative *C. perfringens* are ingested, conditions in the upper GI tract induce *C. perfringens* sporulation, which results in the expression of CPE protein that causes diarrhea [179]. Since mucosal surfaces are naturally exposed to *C. perfringens* in the lumen, therefore *C. perfringens* derived vectors can be potent vehicles to deliver immunogens to the lymphoid tissues of the gut and may stimulate strong mucosal immune responses without much safety concerns [1].

The unique characteristics of the biology of *C. perfringens* make it an attractive model for the development of a mucosal vaccine against HIV-1. These unique features include the presence of the *cpe* gene, which has a very strong promoter that leads to the expression high levels of cpe protein (15% of total bacterial protein) and induction of this promoter occurs only during the sporulating phase in the *C. perfringens* life cycle [180]. Therefore the *cpe* promoter

can be used to drive HIV-1 antigen production by making a recombinant *C. perfringens* construct in which the *cpe* gene will be replaced with that of the desired viral gene leaving the *cpe* promoter region intact. Removing the *cpe* gene from the bacteria will also make it nonpathogenic with respect to GI diseases. Furthermore, since the cpe protein is present in the form of inclusion bodies in the cytoplasm of the sporulating cell, it will be able to survive the harsh digestive environment of the intestine. Therefore the desired viral protein should also be protected and thus will be available for inducing immune responses [181].

These unique features make *C. perfringens* a better oral vaccine delivery model than other oral vaccine models involving attenuated gastrointestinal microbes, such as *Listeria monocytogenes* [175], *Salmonella typhimurium* [176] and *Shigella flexneri* [177], none of which has a strong promoter. Furthermore, although these microbes are attenuated, safety of using these microbes as vaccines is of great concern, because of their potential reversion into wild types *in vivo*. *C. perfringens*, on the other hand, because of the deletion of the *cpe* gene, is least likely to revert to wild type *in vivo*.

1.7.1 Safety concerns of recombinant *C. perfringens* expressing HIV-1 proteins

Despite the fact that most *C. perfringens* bacteria rapidly undergo sporulation in the intestine, rendering it non replicative 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. Although the *C. perfringens* type A that has been used in developing earlier vaccine vectors was devoid of the Cpe enterotoxin, it still carries two other exotoxins, α-toxin (also known as phospholipase C) and θ-toxin (also known as perfringolysin O). Oral inoculation 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 GI tract to cross the mucosal tissue lining and enter the underlying tissue, which is an anaerobic environment suitable for *C. perfringens* growth and thus toxin production. There is no evidence that this has ever occurred in humans, but given the preexisting GI diseases often found in regions in most desperate need of HIV-1 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 which has been shown to express SIV p27 and deliver viral antigen to the terminal ileum [182, 183].

Since it has been shown that inactivating the *cpe* gene renders *C. perfringens* avirulent in the intestine [1], a vaccine against HIV-1 using *cpe* gene-deleted *C. perfringens* should be safe for use in humans. Furthermore, since the type A *C. perfringens* (used in this study) present in normal intestine flora are *cpe*-negative and reside predominantly in the large intestine there is no chance for the wild type *cpe* gene and other toxin genes to be reintroduced in our vaccine construct through recombination *in vivo* since the vast majority of recombinant *C. perfringens* should be lysed in the ileum before passage to the large intestine.

Although there is no evidence for recombination of genetic material between food-acquired *C. perfringens* and GI tract resident *C. perfringens* or other bacteria in the gut, there is the potential for a resistance gene to be transferred if included in a vaccine. In the course of the development of the α and knock out mutants, a *C. perfringens* strain expressing SIV Gag was created that lacks the antibiotic resistance gene [182]. Thus, future research with *C. perfringens* expressing HIV-1 proteins can be performed utilizing this new generation of *C. perfringens* with multiple safety mutations and lacking antibiotic resistance genes.

Therefore in this dissertation, the development of a novel heterologous prime boost vaccination strategy using recombinant *C. perfringens* expressing HIV-1 proteins and HIV-1 VLPs is reported. It has been demonstrated that these vectors induce robust systemic as well as mucosal antigen specific cellular immune responses. Therefore these results present an alternative vaccination approach for generation of cellular immune responses against HIV-1 at mucosal sites of infection and merit further study of recombinant *C. perfringens* and VLPs as mucosal HIV-1 vaccine candidates.

2.0 SPECIFIC AIMS

The mucosal sites such as the gastrointestinal (GI) tract are major targets for early HIV-1 infection [18, 39]. Strong immune responses against HIV-1 at these sites may protect early infection. Thus an effective vaccine against HIV-1 should induce strong immune at the mucosal sites to prevent early HIV-1 infection. Mucosal vaccines represent an attractive approach for immunization against HIV-1 infections due to their importance in eliciting protective immune responses both at mucosal and systemic sites. Therefore a vaccine that can prevent virus transmission at mucosal surfaces by inducing strong mucosal immune responses at the site of infection would be an effective HIV-1 vaccine candidate**.** Oral immunization strategies have been shown to induce immune responses in the GI tract [137, 138], whereas nasal immunization strategies have been reported to induce immune responses in the respiratory and genitourinary tracts [136, 138]. Therefore, using these routes for immunization with HIV-1 vaccine candidates should enable the development of protective immune responses against HIV-1 at its major sites of primary infection.

A vaccine containing the recombinant *Clostridium perfringens* expressing HIV-1 Gag has been designed to stimulate immunity in the GI tract with the expectation that the resulting immunity may be transferred to other mucosal sites such as vaginal and rectal tissue. It has been shown that recombinant clostridia are potent vehicles for delivering SIV-Gag to the lymphoid tissues of the GI tract [1]. Therefore I propose to use recombinant noncytotoxic (cytopathic gene

deleted) *C. perfringens* as a novel oral vaccine vehicle to deliver HIV-1 proteins in large quantities to the terminal ileum in order to induce strong mucosal immune responses against HIV-1. Orally administered HIV-1 antigens via *C. perfringens* will survive the low pH, bile salts and proteolytic enzymes of the stomach and GI tract and will be exposed to M cells in PPs which will induce strong mucosal immunity against the virus. Recombinant *C. perfringens* expressing HIV-1 proteins alone may not be enough to give strong, durable cellular and humoral immune responses. Therefore a prime/boost vaccine approach may elicit better and more effective immune responses [184]. Priming or boosting with another HIV-1 vaccine construct like virus like particles (VLPs) may elicit more effective mucosal immune responses. VLPs are non-infectious virus particles and have become a promising candidate in the quest for a safe and effective vaccine against HIV-1/SIV [185, 186]. Therefore, combinations of intranasal delivered VLP and orally delivered recombinant *Clostridium perfringens* expressing HIV-1 proteins in different prime-boost vaccination approaches may elicit stronger mucosal immune responses than either vector alone since two independent mucosal compartments will be targeted by each vector which will lead to a better coverage of the mucosal immune inductive sites. **Thus I hypothesize that the oral immunization of recombinant *Clostridium perfringens* expressing HIV-1 proteins and intranasal administration of HIV-1 VLP in a prime boost strategy will elicit strong mucosal immune responses against HIV-1.** This hypothesis was addressed by the specific aims listed below.

1. **To construct and characterize the vaccine vectors; a recombinant *C. perfringens* expressing HIV-1 proteins and HIV-1 Virus like particles.** Recombinant *C. perfringens* expressing HIV-1 proteins were constructed and expression of these proteins *in vitro* was

2. **To determine the ability of recombinant *C. perfringens* expressing HIV-1 proteins to induce DC maturation and its ability to prime HIV-1 specific T cell responses *in vitro*.** Bone marrow derived dendritic cells were exposed to recombinant *C. perfringens* expressing HIV-1 proteins and expression of maturation markers on cell surface was monitored by flow cytometry. The antigen presentation function of BMDCs was assessed through ELISPOT assays by measuring HIV-1 Gag specific gamma interferon (IFN-γ) producing T cell responses following restimulation by vaccine-exposed BMDCs.

3. **To measure the mucosal HIV-1 specific cellular immune responses in mice after immunization with recombinant *C. perfringens* expressing HIV-1 proteins alone or along with HIV-1 VLPs in a prime boost strategy.** Mice were immunized with recombinant *C. perfringens* expressing HIV-1 Gag along with optimized adjuvants either alone or in different prime-boost combinations with HIV-1 VLPs. HIV-1 Gag specific IFN-γ secreting T cells in the spleen, mesenteric lymph nodes, lamina propria(LP) and Peyer's patches (PPs) of the immunized mice were detected by ELISPOT assays. Characterization of HIV Gag specific T cell responses was further evaluated by using $CD4^+$ or $CD8^+$ T cell depletion in all mucosal and systemic samples by ELISPOT assay. The functionality of HIV-1 Gag specific GI tract T cells responses in inductive and effector tissues was characterized with multi-color flow cytometry. In addition, HIV-1 Gag specific humoral responses were assayed with ELISA.

3.0 CONSTRUCTION AND CHARACTERIZATION OF RECOMBINANT *C. PERFRINGENS* EXPRESSING HIV-1 GENES AND HIV-1 VIRUS LIKE PARTICLES

3.1 PREFACE

The study described in this chapter was done by Poonam Poonam with technical help from Dr. Yue Chen and Sean McBurney. The construction of recombinants expressing HIV-1 *Gag* genes was done according to the methods previously described by Chen et al [1]. The PJRC200 plasmid was a kind gift of Dr Bruce McClane of the University of Pittsburgh

3.2 ABSTRACT

An effective HIV-1 vaccine is necessary for preventing HIV-1 infection. Since most HIV-1 infections are acquired via mucosal surfaces, an effective preventive vaccine against HIV-1 should induce mucosal immunity as well as systemic immunity to prevent systemic infection from the mucosal surfaces. A noncytotoxic (enterotoxin gene deleted) *Clostridium perfringens*, a gram positive anaerobic bacterium, has been used in this study as a novel oral vaccine vehicle to deliver HIV-1 proteins in large quantities to the terminal ileum to induce strong mucosal and systemic immune responses. A recombinant shuttle plasmid (pJRC200) carrying HIV-1 genes was constructed by inserting the HIV-1-p24 or HIV-1-p55 genes in the place of the cytotoxic

region of the *cpe* gene which is under the control of the *cpe* promoter. When this recombinant plasmid was transformed into *C. perfringen* entertoxin (cpe) negative *C. perfringens*, large amounts of the viral protein were produced during sporulation of the *C. perfringens*, as determined by a semi-quantitative western blot analysis. These recombinant clostridia were then used in the vaccine approaches described in subsequent chapters. Also HIV-1 virus like particles (VLPs) were constructed and used as an intra nasal vaccine vector. The VLPs were obtained by transfection of COS cell lines with plasmid DNA encoding HIV-1 Ada strain *gag/env* genes. Supernatant was collected and then the VLPs were purified from these supernatants by ultracentrifugation. A western blot was performed to verify the expression of HIV-1 proteins in the VLPs. These result shows high expression of whole Gag or p24 proteins in sporulating media and amount of proteins produced are directly related to high % of spores of *Clostridium perfringens.*

3.3 INTRODUCTION

An effective vaccine against HIV-1/SIV must induce mucosal immunity to prevent early HIV-1 infection. Because the gut is the major site of $CD4^+$ T cell depletion and HIV-1 replication [157] and the mucosal layer of the gastrointestinal (GI) tract is naturally exposed to bacteria in the lumen, a bacterial vector provides an attractive vehicle. A number of efforts have been made to develop a vaccine that elicits a mucosal immune response against HIV-1. *Clostridium perfringens* has also been considered as a vehicle for antigen delivery to the GALT [1]. 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. A unique property of *C. perfringens* is the presence of a very highly active *cpe* promoter. The *cpe* promoter is activated only during sporulation [187, 188]. The bacterial protein expressed from the *cpe* promoter remains untouched by proteases and bile salts of the intestinal lumen presumably because expressed proteins are present in the cytoplasmic inclusion body. 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. Oral ingestion of *C. perfringens* not carrying the *cpe* gene may be considered safe for humans [189]. Therefore recombinant *C. perfringens* is now being developed as a vaccine vector against HIV-1. In addition to its ability to deliver intact protein to the mucosal surfaces of the GI tract, *C. perfringens* has the benefits of being an inexpensive vaccine vector that can be delivered orally without the requirement of needles. In this report we have shown construction of recombinant clostridia expressing high level of HIV-1 Gag protein and produced HIV-1 VLP.

3.4 MATERIALS AND METHODS

3.4.1 Construction of recombinant *C. perfringens* carrying HIV-1 Gag and envelope genes

The construction of recombinant *C. perfringens* carrying HIV-1 Gag (p24 or p55) and Envelope (gp120 or gp140) were constructed in similar manner as described previously for SIV-p27 [1]. Briefly, the construction of recombinant *C. perfringens* expressing these genes have been achieved using the pJRC200 plasmid, which contains *cpe* gene cassette including its promoter,

open reading frame and downstream sequence [190]. For construction of recombinant plasmid containing HIV-1 *Gag* genes, the entire HIV-1-p24 or HIV-1-p55 ORF was amplified by PCR from a molecular clone of HIV-1 pNL4-3 with the primers p24 forward (TTCGAACCCATCGTGCAGAACATCCAG) and p24 reverse (CCTAAGGACAGCACGCGGGCCTTGTG) or p55 forward (TTCGAAATGGGCGCCCGCGCCAGCGTG) and p55 reverse (CCTAAGGATTGTGACGAGGGGTCGCTGCC), designed to contain either BstB1 or Bsu36I restriction site at the 5' end of each primer (underlined). The resultant PCR products were then digested with these two enzymes and inserted into the plasmid pJRC200 [191] which was also digested with BstB1 and Bsu36I to remove most of the cpe ORF to get the recombinant pJRC200 plasmids containing the p24 or p55 genes (cp24 or cp55). Similarly pJRC200 plasmids carrying HIV-1 envelope gene gp120 (cp120) and gp140 (cp140) (a gene segment encoding the transmembrane domain of gp41 in addition to the full length gp120 protein), were constructed. HIV-1 gp120 and gp140 genes were amplified from the full length clone of the HIV-1 isolate ADA. Primers pair for amplifying these genes were as follows: HIV-1-gp120 and gp140 F (GTTCGAAATGAAAGTGAAGGGGATCAGGAAGAA) and HIV-1-gp120 R (AGCTAAGCCTCTTTTTTCTCTCTGCACCAC) and HIV-1-gp140 R (AGCTAAGCATCCCTGCCTAACTCTATTTACTATAGAAAGTACAG). Then these plasmids were individually electroporated into a *cpe* negative strain of *C. perfringens* (C. perfringens strain ATCC 3624).

3.4.2 Bacterial growth conditions

A vegetative culture of *C. perfringens* (non toxic C. perfringens strain ATCC 3624) was grown overnight at 37°C in FTG media (fluid thioglycolate broth) (Difco). A Modified Duncan-Strong medium (MDS) was used to obtain sporulating cultures of *C. perfringens* [192]. All media were supplemented with 10–20 ug of chloramphenicol per milliliter for growing transformants containing pJRC200- based plasmid constructs. The percentage of sporulating *C. perfringens* cells formed in MDS was determined by observation under a phase-contrast microscope.

3.4.3 Transformation of *C. perfringens* with pJRC200- based plasmid constructs

Electroporation was performed to introduce cp24/cp55/cp120/cp140 or the control pJRC200 into *C. perfringens*. Vegetative cultures of clostridia were grown at 37°C to late log phase in TGY broth (3% Trypticase, 2% glucose, 1% yeast extract, and 0.1% cysteine), washed twice with SMP buffer (270 mM sucrose, 1 mM $MgCl_2$, and 7 mM Na_2HPO_4, pH 7.3), and then resuspended in 1 ml of SMP buffer. Then 400 microliters of those washed cells and plasmid DNA (5 ug) in 0.4 cm cuvette (Bio-Rad) were placed in a Bio-Rad Gene pulser set for 2.5 kV, 200Ω and 25 μFD. After pulsing, 3 ml of warm (37°C) TGY broth was added and the mixture was incubated for 3 hours at 37°C. An aliquot of this mixture was placed on BHI agar (brain heart infusion agar) (Difco) plate containing 10 ug/ml of chloramphenicol. Those plates were incubated for 24 hours at 37°C in an anaerobic atmosphere, created using a BBL GasPak jar.

3.4.4 Western blot analysis

The expression of HIV-1 Gag or Env proteins was confirmed in sporulating cultures of the transformed bacteria by a semi quantitative western blot. Recombinant *C. perfringens* were harvested, washed, and sonicated in PBS after culture in FTG or MDS media. Cells were then lysed and separated on a 15% SDS-PAGE gel. Protein was transferred to nitrocellulose membrane and incubated with anti-HIV-1 monoclonal antibody (from NIH AIDS Research and Reference Reagent Program) for an hour at room temperature. After washing, the membrance was incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Nordic Immunological Laboratories) for an hour. Protein bands were detected with SuperSignal West Pico Chemiluminescent Solution (Pierce). Blots were analyzed via densitometry using Quantity One software (Bio-Rad).

3.4.5 Vaccine constructs

The construction and purification of HIV VLPs has been developed in the Dr Ted Ross's laboratory and described elsewhere [193]. Briefly, HIV-1 VLPs were obtained by transiently transfecting the monkey fibroblast cell line, COS, with a plasmid expressing Gag, Gag–Pol, VLP as described previously [193]. The culture supernatants of the transfected COS cells were collected and subjected to ultracentrifugation (100,000 g through 20% glycerol, w/v) for 4 hours at 4°C. The subsequent pellets were resuspended in PBS and overlaid onto 20–60% sucrose gradients (11 steps, 4% increments). Further ultracentrifugation was performed for 17 h at 100,000 g at 4°C and eleven fractions (20–60%, 1 ml, weight per volume) were collected top to bottom from the gradient. The particles were precipitated with equal volumes of 20%

trichloroacetic acid (TCA) and subjected to SDS-PAGE and immunoblotting. The viral proteins were detected by a western blot.

3.5 RESULTS

3.5.1 Construction of a recombinant *C. perfringens* vaccine strain carrying HIV-1 Gag (p24 or p55) and HIV-1 envelope (gp120/gp140) genes

A schematic representation of the construction of *C. perfringens* carrying HIV-1 p55 gene is shown in Figure 5. For construction of the recombinant vaccine strain, we used a *C. perfringens*/*E. coli* shuttle plasmid pJRC200, which contains the wild-type cpe gene. The pJRC200 was digested with restriction enzymes to remove the cpe cytotoxicity region, leaving the cpe promoter and a small amount of 5' and 3' sequences for transcription/translation efficiency and mRNA stabilization, respectively (Figure 5). The digested plasmid was then ligated with the p24/p55 ORF amplified from HIV-1 pNL4-3 isolate or gp120/gp140 ORF amplified from HIV-1 ADA isolate. The resulting plasmid was then introduced by electroporation into cpe-negative *C. perfringens* isolate ATCC3624.

Using similar methods recombinant C*lostridium perfringens* carrying HIV-1 envelope gp120 or gp140 genes were constructed. For this purpose, HIV-1 gp120 and gp140 were amplified from full length HIV-1 isolate ADA. After the sequence was confirmed, these were cloned into pJRC20, the *Clostridium perfringens* shuttle plasmid. The recombinant pJRC200 plasmid carrying HIV-1 gp120 or gp140 were subsequently electroporated into cpe negative *C.*

perfringens. Finally the protein expression of these two constructs was also verified by western blot.

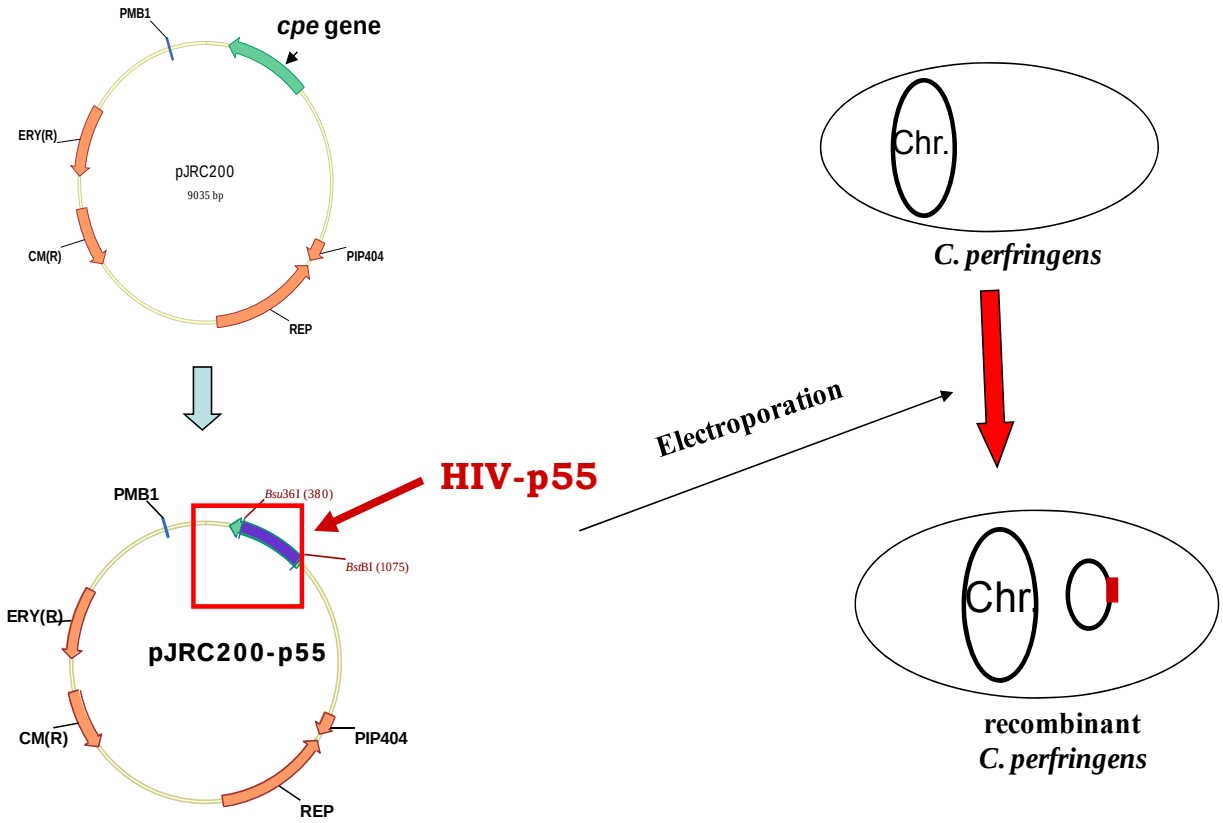

Figure 5. Schematic representation of construction of recombinant clostridia expressing HIV-1 p55

For construction of the recombinant vaccine a *C. perfringens*/E. coli shuttle plasmid pJRC200, which contains the wild-type *cpe* gene was used. The pJRC200 was digested with restriction enzymes to remove the cpe cytotoxicity region, leaving the cpe promoter region and a small amount of 5Vand 3Vsequences .The digested plasmid was then ligated with the HIV p24 or p55 ORF amplified from HIV-1 Pnl4-3 isolate or from pIndieC11 isolate, resulting plasmid, named Cp-24 or Cp-p55 (Gag), was then introduced by electroporation into cpe-negative C. perfringens isolate ATCC3624.

3.5.2 Expression of the HIV-1 Gag and envelope proteins by the recombinant *C. perfringens*

Recombinant *C. perfringens* carrying HIV-1 p24/p55 expression plasmids were grown overnight in MDS media were pelleted by centrifugation and cell lysates were prepared from these cell pellets by sonication. The expression of HIV-1 p24 or p55 by the sporulating recombinant clostridia was quantitated to be 40-50 ug of protein for both HIV-1 p24 and HIV-1 p55 per milliliter (about $1x10^7$ – $1x\ 10^8$ cells) of sporulating culture (Figure 6).

Similarly recombinant *C. perfringens* carrying HIV-1 gp120/gp140 expression plasmids were analyzed for protein expression by a semi-quantitative western blot experiment. In contrast to *C. perfringens* carrying HIV-1 p24/p55, the recombinant clostridia expressing HIV-1 envelope genes (cp120/cp140) did not did not show any expression of HIV-1 envelope proteins in the western blots (data not shown).

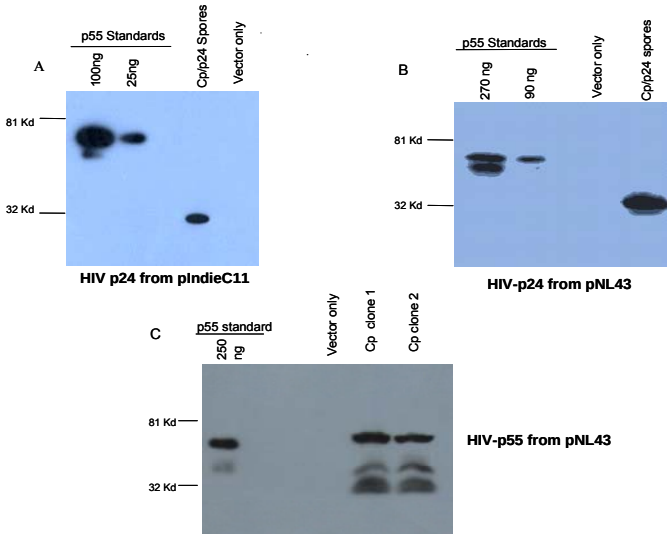

Figure 6. Western blot analysis of recombinant *C. perfringens* expressing HIV-1 p24 from pIndie C (A), HIV-1 p24 (B) or p55 from pNL4-3 (C) proteins.

3.5.3 Purification and expression of HIV-1 virus like particles

HIV-1 virus-like particles (VLPs) were purified from the supernatants of transiently transfected cells by ultracentrifugation (20–60% sucrose gradient) and collected fractions were analyzed for particle composition and stability. Figure 7 shows the expression of HIV-1 Gag gene products in both the cell lysates and supernatant of COS-7 cells transfected with the plasmids encoding various HIV-1 proteins as described elsewhere [193].

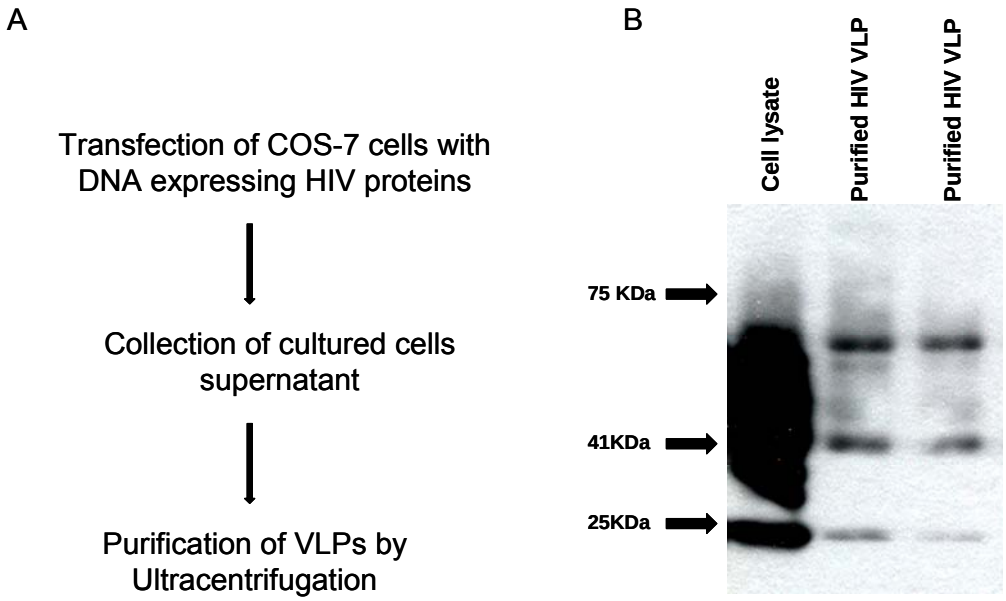

Figure 7. (A) Schematic representation of the construction and purification of HIV-1 VLPs, (B) Western blot analysis of purified HIV-1 VLPs.

Lane 1 is the whole cell lysates, lane 2 and 3 are the two different samples of the purified HIV-1 VLP showing p55, p41 and p24 bands.

3.6 DISCUSSION

The mucosa is the major natural route of HIV-1 transmission and infection, therefore generating HIV-1 specific strong responses at mucosal sites is very important in preventing HIV-1 infections. Administration of antigens to mucosal surfaces might be the best method for inducing mucosal immune responses at local as well as distant sites such as lung and genital sites. Therefore an oral vaccine could trigger immune responses at mucosal sites, which could lead to protection against virus challenge. Since the mucosal layer of the gastrointestinal tract (GI) is naturally exposed to *Clostridium perfringens* in the lumen, these bacteria could present as a vehicle to deliver HIV-1 proteins in a large quantity to the gut-associated lymphoid tissue (GALT) to stimulate mucosal immunity against HIV-1. We have shown that recombinant *C. perfringens* expresses high amount of SIV protein *in vitro,* and we have also shown the evidence of a recombinant *C. perfringens* vaccine construct that produced a high level of HIV-1 proteins *in vitro* under sporulating conditions. Expression of the HIV-1 p55 protein expressed by recombinant clostridia was quantitated to be approximately 40-50μg/ml sporulated bacterial culture (10^8 cfu) which is similar to recombinant clostridia expressing SIV p27 reported earlier [1]. Interestingly, the *p55* gene is twofold larger in size than *p24* or *p27*, yet similar level of expression was obtained, indicating that clostridia is able to handle longer gene inserts for expression purpose. The cpe promoter driven expression of protein accounts for about 15 % of the total *C. perfringens* proteins and this is one of the highest known to be reported in a bacterial vaccine construct. This expression rate is higher when compared with bacteria commonly used for protein expression [194]. In one example of an efficient expression system, *E. coli* expressed human metallothionein 2A as 10-15% of total protein [195]. It has been shown that upon oral feeding, the recombinant *C. perfringens* vaccine carrying SIV p27 construct delivered a large

amount of intact p27 fusion protein to the terminal ileum, indicating p27 protein present in the inclusion body is protected from the harsh environment of the GI tract [1]. Oral delivery of inclusion bodies carrying viral antigens have been shown to induce both systemic and mucosal immune responses without any adjuvant [196]. Therefore, the delivery of intact HIV-1 antigens to the GALT by the recombinant *C. perfringens* should induce strong antigen specific mucosal immune responses.

The failure to obtain recombinant *C. perfringens* expressing HIV-1 envelope proteins may be due to numerous factors. Since these envelope proteins are highly glycosylated and the bacterium lacks the capacity for glycosylation, these recombinant clostridia might not be able to express the non glycosylated form of these proteins. It is also possible that sequences present in HIV-1 envelope may be toxic for bacteria or have bactericidal effect. Therefore the lack of expression of HIV-1 envelope by recombinant *C. perfringens* could be due to lack of glycosylation of these proteins and/or bacteriotoxic properties of these proteins.

Overall, a novel *C. perfringens* based vector expressing HIV-1 Gag was successfully constructed. This recombinant *C. perfringens* also expressed a large amount of HIV-1 Gag under sporulating conditions, that are prevalent in the GI tract and thus presents an attractive mucosal vaccine vector against HIV-1 that can deliver large amounts of HIV-1 proteins to the immune cells present in the GI tract.

4.0 DETERMINE THE ABILITY OF RECOMBINANT *C. PERFRINGENS* EXPRESSING HIV-1 PROTEINS TO INDUCE DENDRITIC CELL MATURATION AND ANTIGEN PRESENTATION TO T CELLS

4.1 PREFACE

This study was performed by Poonam Poonam. Technical help was received from Ruth Helmus.

4.2 ABSTRACT

Dendritic cells (DCs) are present at the sites of antigen exposure and at the inductive mucosal sites such as the gastrointestinal (GI) tract. In this study, the ability of a recombinant *C. perfringens* expressing HIV-1 Gag (*Cp*-Gag) to stimulate T cell mediated immune responses *in vitro* was investigated. Freshly isolated murine bone marrow derived dendritic cells (BMDCs) upon exposure to *Cp*-Gag, up-regulated maturation markers on their cell surface. Furthermore, these mature BMDCs stimulated HIV-1 Gag specific IFN-γ production by immune T cells, demonstrating that the HIV-1 Gag antigen was efficiently delivered to, processed by, and presented on MHC molecules by BMDCs. These findings suggest that HIV-1 Gag from recombinant *C. perfringens* expressing HIV-1 Gag when delivered to DCs enable these DCs to present HIV-1 Gag epitopes to T cells.

4.3 INTRODUCTION

Dendritic cells (DCs) are potent antigen-presenting cells and are an important component of the immune response to pathogens. On exposure to pathogens, DCs mature by up-regulation of costimulatory and maturation markers on their surfaces enabling them to prime and activate antigen specific T cells. DCs also 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 [197, 198]. The $CD8^+$ CTL response to HIV-1 appears essential for limiting viral infection, as displayed by numerous studies of both systemic and mucosal immune responses to HIV-1/SIV [60]. Thus, to generate an effective anti-SIV $CD8^+$ CTL response, a vaccine must induce DCs to present HIV-1 antigen to $CD8^+$ T cells and induce priming of anti-HIV-1 $CD8^+$ CTLs.

A study was initiated to determine whether DCs exposed to recombinant *C. perfringens* expressing HIV-1 Gag proteins (*Cp*-Gag) can prime and activate antigen specific T cells. To study the effectiveness of recombinant to induce a DC-mediated immune response, the functional capacity of T cell stimulatory capacity of DCs exposed to *Cp*-Gag were investigated. The effects of *Cp*-Gag were examined using murine bone marrow-derived DCs (BMDCs). Murine BMDCs were exposed to *Cp*-Gag and examined for surface expression of maturation markers and cytokine production. The function of DCs was also assessed by co-culture of BMDCs with HIV-1 Gag specific T cells and detection of IFN-γ production by these T cells using an ELISPOT assay. These results demonstrate that *Cp*-Gag can efficiently stimulate a DC-mediated Gag-specific immune response *in vitro*, which suggests that *in vivo* immunization might result in HIV-1 Gag specific cellular immune responses.

4.4 MATERIAL AND METHODS

4.4.1 Animals

6-8 weeks old 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.

4.4.2 Antibodies

FITC-α-CD40 (clone L3T4), FITC-α-CD80 (16-10A1), FITC-α-CD86 (GL1), 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. Isotype control antibodies were purchased from the same manufacturers according to the fluorescent conjugate used.

4.4.3 Construction of recombinant *C. perfringens*

Construction of recombinant *Clostridium perfringens* expressing HIV-1 Gag protein has been described in aim 1. Expression of HIV-1 Gag was confirmed in sporulating cultures of the recombinant clostridia by western blot.

4.4.4 Murine bone marrow derived dendritic cells

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

4.4.5 Maturation of BMDCs by recombinant *C. perfringens* expressing HIV-1 Gag protein

Freshly prepared BMDCs (1-2x10^6 cells per 4ml) were incubated with ~2x10^5/ml sporulated, *C. perfringens* expressing Gag (*Cp*-Gag), empty vector *C. perfringens* control, purified Gag protein at a concentration equivalent to that expressed by the recombinant clostridia, and 0.5 μg/ml LPS. The stimulation media were removed after 2 hours via washing and centrifugation and the BMDCs were returned to culture for 22 additional hours. Culture supernatants were then collected and stored at -20°C, whereas the cells were harvested and utilized for further assays.

4.4.6 Flow cytometric analysis of BMDCs

For flow cytometric analysis, DCs were surface stained for CD11c, CD40, CD80, CD86, and MHC class II (I-A^d) at 4°C. After staining, all cells were fixed using 1% paraformaldehyde, and data were collected using a Coulter Epics XL-MCL flow cytometer.

4.4.7 ELISPOT assay

The detection of antigen specific interferon-gamma (IFN-γ) producing cells was performed using mouse IFN-γ ELISPOT Kits from Mabtech. HIV-1-Gag specific splenocytes ($2x10^5$ cells per well) were placed at 37°C for 1 hours, and then BMDCs exposed to *Cp*-Gag or control *C. perfringens* were added to ELISPOT plate wells at a 1:1 or 1:5 – splenocytes: DC ratio, respectively, in a final volume of 200 ul of ELISPOT media (RPMI-1640 with 10% FBS, 1% penicillin/streptomycin, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 10 mM HEPES, and 1 mM sodium pyruvate). DMSO equivalent to the amount present in the peptide pools was also added as background control to the each well. The treatments for all samples were plated in quadruplates. The cells were then incubated at 37°C, 5% CO2 atmosphere for 24h. After incubation cells were washed away and ELISPOT plates were assayed for IFN-γ producing cells as per manufacturer's protocol (MABTECH ELISPOTplus for mouse IFN-γ) kit. The number of spot forming cells (SFC) was counted on an ELISPOT reader. The results are expressed as number of spots per 10^6 cells in stimulated wells minus the number in unstimulated wells.

For HIV-1Gag specific splenocytes; mice were immunized subcutaneously with 10 ug of HIV-1-Gag protein/per mouse along with complete freund's adjuvant. Mice were boosted once

at 10 days with 5 ug of HIV-1 Gag along with incomplete freund's adjuvant. Finally mice were sacrificed at day 14 after a final boost and splenocytes were used in assay.

4.5 RESULTS

4.5.1 Maturation of BMDCs exposed to *C. perfringens* expressing HIV-1 Gag

An *in vitro* experiment was performed to evaluate the interactions of recombinant *C. perfringens* expressing HIV-1 Gag (*Cp*-Gag) with bone marrow derived dendritic cells (BMDCs). In this experiment, it was determined whether BMDC can uptake and process HIV-1 Gag expressed by the recombinant *C. perfringens*. Immature BMDCs were pulsed for 2 hours with lysates of *Cp*-Gag or LPS as a positive control and untreated BMDCs served as a negative control. BMDCs were then washed to remove all bacteria and incubated overnight in culture medium with antibiotics. Following overnight culture, BMDCs were then analyzed for the expression of co-stimulatory molecules - CD40, CD80, CD86, and a DC marker - anti-CD11c. Flow cytometry was performed to determine the intensity of maturation markers on the surface of BMDCs.

The results indicate that the percentage of cells expressing CD40, CD80 and CD86 in BMDCs co-cultured with *Cp*-Gag were very similar to those observed in control LPS-treated BMDCs and higher than control BMDCs cultured in media alone. Furthermore, the mean fluorescence intensity (MFI) of the CD40, CD80 and CD86 expressed by BMDCs treated with *Cp*-Gag were significantly higher than the group where BMDC were not treated with any vaccine ($p<0.05$). Furthermore, BMDC showed higher expression of maturation markers on DC

when treated with *C. perfringens* containing empty vector or LPS (Figure 8). These results demonstrate that recombinant clostridia were able to induce maturation of BMDCs.

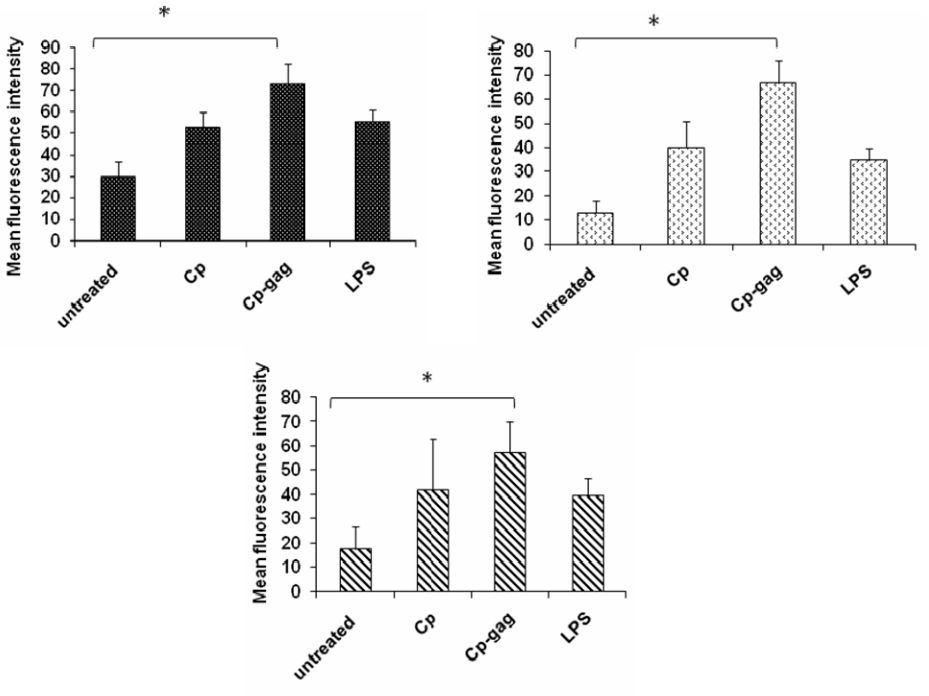

Figure 8. Expression of maturation markers on BMDCs in response to *C. perfringens* expressing HIV Gag (*Cp*-Gag).

BMDCs were cultured with *Cp*-Gag, empty vector control (*Cp*), LPS or BMDC were used without any treatment. Representative figures from 2 independent experiments show average mean fluorescence intensity of expression of maturation markers CD40, CD86 and CD80 on BMDC in response to stimuli as determined by flow cytometry. For statistical analysis *p* values were determined by Student's t-test against untreated cells. $*p<0.05$.

4.5.2 Functional capacity of BMDCs exposed to *Cp*-Gag

The functional capacity of BMDCs exposed to *Cp*-Gag to process and present antigens to T cells was assessed. In this experiment, the BMDCs exposed to *Cp*-Gag were used to restimulate HIV-1 Gag specific mouse splenocytes and IFN-γ secretion by these cells was detected by an ELISPOT assay.

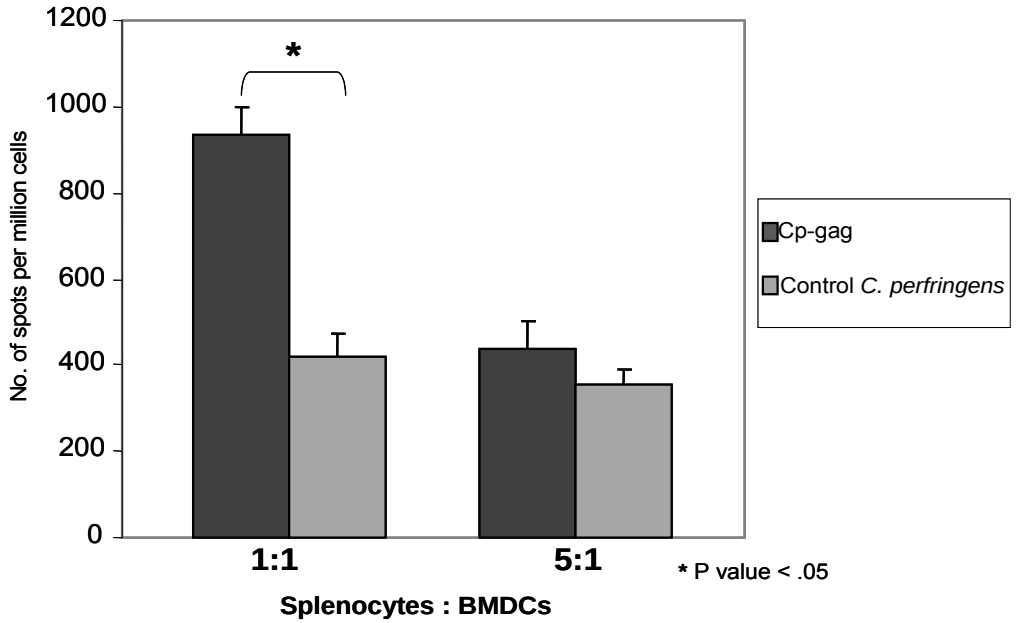

Figure 9. HIV-1 specific IFN-gamma secretion by splenocytes co cultured with BMDCs exposed to *Cp*-Gag or control *C. perfringens* as measured by an ELISPOT assay.

HIV-1 Gag specific murine splenocytes were stimulated with BMDC treated with *Cp*-Gag or *Cp*-empty vector. Two different ratios 1:1 and 5:1 of Splenocytes:BMDC were used in this experiment. IFN-γ ELISPOT results indicate data from 2 independent experiments. Error bars indicate standard error of the mean of samples assayed in triplicate. *p*-value determined by Student's t-test.

BMDCs exposed to lysates of *Cp*-Gag stimulated IFN-γ production by HIV-1 Gag specific splenocytes, indicating that the BMDCs indeed displayed HIV-1 Gag epitopes on MHC molecules and were capable of stimulating an immune response against Gag (Figure 9). The IFN-γ ELISPOT response stimulated by BMDC treated with *Cp*-Gag was about 12 times that stimulated by BMDCs exposed to control *C. perfringens*. It should be noted that the low background IFN-γ response detected by ELISPOT in wells containing BMDCs alone was similar regardless of whether BMDCs were treated with *Cp*-Gag or the control *C. perfringens*. These data demonstrate that the recombinant clostridia expressing HIV-1 Gag delivered HIV-1 Gag protein to BMDCs for efficient presentation to and stimulation of antigen specific T cells.

4.6 DISCUSSION

The goal of this *in vitro* study was to determine whether *Cp*-Gag exposed DCs can induce an immune response against HIV-1 Gag. It has been previously shown that the bacteria based vaccines interact with DCs as shown in the case of *L. monocytogenes* which can stimulate cytotoxicity against antigen-specific target cells *in vitro* [115]. The present study investigated the phenotype and functionality of murine BMDCs following exposure to *Cp*-Gag. Upon exposure to *Cp*-Gag, BMDCs showed an increased surface expression of CD80, CD86, and CD40 when compared to BMDCs exposed to Gag protein alone. However BMDCs exposed to control 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* outer membrane. The splenocytes cultured with BMDC exposed to *Cp*-Gag showed at least 10-fold higher antigen specific IFN-γ production that when cultured with BMDCs exposed to control *C. perfringens*. Overall this *in vitro* study demonstrated maturation of BMDCs following exposure to recombinant clostridia. It also demonstrated that these BMDCs can efficiently present antigen to antigen specific T cells. These results suggest that the DCs exposed to *Cp*-Gag have the potential to prime or activate antigen specific T cells to form a productive immune response *in vivo*.

In a related study, freshly isolated Peyer's patches DC (PPDCs) were exposed to recombinant *C. perfringens* expressing SIV p27 (*Cp*-p27) and up regulation of maturation markers and production of pro-inflammatory cytokines by the PPDCs were observed (personal communication, Ruth Helmus). These results indicated that DCs isolated from PPs could also mature upon interaction with recombinant clostridia expressed antigen and stimulate antigen specific cellular immune cells which suggests that HIV-1 antigen specific immunity could be

generated *in vivo* via delivery of HIV-1 proteins to PPDCs using recombinant clostridia. In addition, it was observed that unlike other bacterial and viral-based vectors, *C. perfringens* does not invade GI cells and therefore does not appear to present its antigens through the classical MHC class I pathway. However, it has been seen that DCs can alternatively process exogenous antigens and present antigens through the MHC class I pathway by a phenomena know as cross-presentation [199, 200]. Because DCs in PPs have been shown to take up SIV p27 protein expressed by the recombinant *C. perfringens*, it is expected that the recombinant clostridia expressing HIV-1 proteins will induce both cellular and mucosal immune responses.

5.0 INDUCTION OF CELLULAR IMMUNE RESPONSES IN THE GUT MUCOSA AGAINST HIV-1 USING A COMBINATION OF RECOMBINANT *CLOSTRIDIUM PERFRINGENS* AND VIRUS LIKE PARTICLES

5.1 PREFACE

This chapter is adapted from a manuscript in preparation. This experiment was accomplished with the technical assistance of Ruth Helmus, Lori Caruso, Dr Yue Chen and Dr Cheng- Li Shen in vaccine administration, sample collection and processing. Flow cytometry was performed with the assistance of Luann Borowski. Dr. Ted M. Ross provided guidance for CpG ODN sequence selection. LT (R192G) or mLT was a kind gift of Dr. John Clemens. The antibodies against CD3, CD4 and CD8 were generous gift from Dr Rosemary Hoffman, of the University of Pittsburgh. Statistical help was provided by Dr. Patrick Tarwater. Portions of this work were presented at the AIDS Vaccine Conference 2006 (*Development of a mucosal vaccine strategy against HIV-1 using Virus like particles and recombinant Clostridium perfringens expressing HIV-1 proteins*. Poonam, P, Chen, Y, McBurney, SP, Ross, TM, and Gupta, P) and at the 2008 Keystone Symposia on HIV-1 Vaccines (*Induction of Polyfunctional T cells in the Gut Mucosa against HIV-1 using a Combination of Recombinant Clostridium perfringens and Virus like Particles*. Poonam, P, Helmus R, Chen, Y, McBurney, SP, Ross, TM, and Gupta, P).

5.2 ABSTRACT

The gastrointestinal tract is a major mucosal target of early HIV-1 infection [37]. Recombinant *Clostridium perfringens* has been shown to deliver SIV/HIV-1 proteins in large quantities to the terminal ileum in order to induce strong mucosal immune responses. Virus like particles (VLPs) has been shown to induce strong mucosal responses when delivered intranasally [141]. Therefore a mucosal immunization strategy using *C. perfringens* and VLPs should be able to induce potent immune responses against HIV-1 in the gastrointestinal (GI) tract and other mucosal tissues. Cellular immune responses play a central role in HIV-1 replication. Therefore, the ability to elicit potent cellular immune responses has become a priority for HIV-1 vaccine candidates. The effectiveness of an immunization strategy against HIV-1 can be measured by its capacity to induce HIV-1 specific $CD4^+$ and $CD8^+$ T cells that can produce IL-2 ,TNF-α and IFN-γ. Also the cytotoxic function of antigen specific $CD8^+$ T cells is important for virus clearance during HIV-1 infection. Therefore in this study HIV-1 Gag specific cellular immune responses induced by recombinant *C. perfringens* expressing HIV-1 Gag (*Cp*-Gag) and VLPs were evaluated. HIV-1 specific mucosal immune responses in both the effector and inductive sites of the GI tract were significantly higher in mice immunized using recombinant *Cp*-Gag and VLPs in heterologous prime-boost approaches compared to mice immunized with either *Cp*-Gag or VLPs alone. These groups also had higher proportions of HIV-1 specific multifunctional T cells. However no significant HIV-1 specific humoral immune responses were detected in the mucosal or systemic compartments of the immunized mice. Mucosal immunization with a combination of *Cp*-Gag and VLPs was successfully able to prime GI tract mucosal immune responses against HIV-1, which may be important to counter HIV-1 infection at the gut at the initial stages of HIV-1 infection.

5.3 INTRODUCTION

Heterosexual transmission accounts for most of the worldwide spread of HIV-1 infection and mucosal sites such as the gastrointestinal (GI) tract are a major target for early HIV-1 infection. Therefore strong immune responses against HIV-1 at the mucosal sites may protect against early infection. Thus an effective vaccine against HIV-1 must induce mucosal immunity to prevent primary infection at the mucosal surfaces of the genital organs and the GI tract. The various sites of the mucosal immune system are interconnected and show a lot of similarities in overall mucosa structure [132]. Therefore immunization of the intestinal mucosal surface could also sensitize other mucosal surfaces at remote sites, namely genital surfaces, and induce both mucosal and systemic immune responses [201]. Mucosal vaccines using oral or nasal routes represent an attractive approach for immunization against HIV-1 infections due to their importance in eliciting protective immune responses both at mucosal and systemic sites; and also due to their ease of administration. Therefore, combining both of these routes for immunization with HIV-1 vaccine candidates should enable the development of protective immune responses against HIV-1 at its major sites of primary infection.

Neutralizing antibodies have shown the ability to block HIV-1 infection [202], but they are difficult to generate. However, cellular immunity plays a critical role in controlling acute infection and maintaining low viral set point. Therefore, protective antiviral cytotoxic T lymphocytes (CTLs), particularly in the mucosal compartment, should be a major component of any immune response induced by an effective vaccine against HIV-1. While inducing cellular immunity at mucosal sites is important for HIV-1 vaccines, which generally are measured by HIV-1 specific IFN-γ secreting T cells; the functionality of immune response induced against HIV-1 also affects the outcome of infection [98]. Generally, functionality is measured by

multifunctional T cells that make two or more cytokines on an individual cell basis and these have been associated with protection in vaccines trials against HIV-1 infection. HIV-1-infected long term non-progressors (LTNP) preferentially maintain HIV-1 specific T cells that produce multiple effector molecules like IL-2/TNF-α/IFN-γ and cytotoxic factors such as CD107a in the same effector cell termed as a multifunctional T cell [203]. However, the information of the multifunctionality of the T cells of the GI tract during HIV-1 infection is limited [101, 204]. It is expected that not just the level of response but also the quality of immunity, including multifunctional $CD8^+$ T cells, in the mucosal tissues will relate directly to the outcome of infection or effectiveness of vaccine-induced immune responses [205]. Thus, examining the functional cellular responses in mucosal tissues such as the GI tract may provide correlations between control of HIV-1 infection and immunity, either natural or vaccine-induced.

The mucosal layer of the GI tract is naturally exposed to bacteria in the lumen; therefore a bacterial vector provides an attractive delivery vehicle for HIV-1 vaccine. *Clostridium perfringens* was selected as a novel vaccine vector, because the unique characteristics of *C. perfringens* make it an attractive model for the development of a mucosal vaccine against HIV-1 [1]. Most importantly, it provides a highly efficient inducible promoter, which has been shown to produce large amounts of target protein in the GI tract, where most of the body's lymphocytes are located. Therefore *C. perfringens* derived vectors can be potent vehicles to deliver immunogens to the lymphoid tissues of the GI tract and may stimulate strong mucosal immune responses.

Intranasal immunization with virus-like particle vaccines (VLPs) provides an alternative approach to induce mucosal immunity. VLPs, or pseudovirions, can be defined as self-assembling, non-replicating, non-pathogenic, genome less particles that are similar in size and

conformation to intact virions. These immunogens can be administered as purified particles or as DNA plasmids expressing the viral proteins to form VLPs *in vivo* [139]. Several successful VLP vaccines have been developed and tested in human trials attesting to the efficacy of HIV-1 VLPs as a promising HIV-1 vaccine candidate [141, 142]. Intranasal delivery of VLPs have been shown as an effective vaccine to induce strong immune responses at mucosal and systemic sites and requires lower doses of antigen than oral delivery [141].

Efforts to develop a vaccine that elicits a mucosal immune response against HIV-1 have not been very successful due to the complex nature of mucosal surfaces and induction of immune tolerance. Adjuvants have been used in mucosal immunizations to overcome immune tolerance. Bacterial toxins, such as the mutated form of heat labile *E. coli* enterotoxins (mLT) and synthetic oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs (CpG ODN) are potent mucosal adjuvants and have been commonly used in animal models [169]. The safety of these adjuvants has been demonstrated in mice, nonhuman primates and humans [48]. The mLT has been used in many studies to enhance Th2 type immune response and CpG-ODN has shown to enhance adaptive Th1 humoral and cellular immune responses in mice and primates [169, 170]. Therefore mLT and CpG-ODN adjuvants were used in the mucosal immunization strategies to overcome immune tolerance and generate strong mucosal immune responses.

In this study a novel mucosal immunization strategy against HIV-1 using recombinant *C. perfringens* and HIV-1 VLPs to generate robust mucosal immune responses against HIV-1-Gag was developed. The results indicate that heterologous prime-boost immunization with oral administration of recombinant *C. perfringens* expressing HIV-1 Gag and intranasal administration of HIV-1 VLPs induce strong antigen specific T cell responses at both mucosal and systemic sites that are targets of early HIV-1 infection.

5.4 MATERIALS AND METHODS

5.4.1 Animals

6-8 weeks old 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.

5.4.2 Vaccine constructs

The construction of recombinant *C. perfringens* expressing HIV-1-Gag proteins and VLP has been described in the chapter 3.

5.4.3 Immunization of animals

The animals were immunized orally by gavages with 150 ug of sporulating *C. perfringens* expressing HIV-1-Gag (oral) and intranasally with 10 ug of HIV-1 VLPs. The interval between successive immunizations was 3 weeks and animals were sacrificed 2 weeks after the final booster immunization. The animals were not fasted or ph neutralized before oral immunization. All procedures used in this study complied with federal guidelines and University of Pittsburgh Institutional Animal Care and Use Committee.

5.4.4 Mucosal Adjuvants

Whole inactivated cholera toxin (CT), cysteine-phosphate-guanine oligodinucleotides (CpG ODNs) and mutant *E. coli* heat-labile toxin (mLT) were used as oral adjuvants in this study. CT and HPLC-purified S-thiolated CpG ODNs were purchased from Sigma. The CpG ODN sequences were: CpG-A=TCCATGACGTTCCTGACGTT; CpG-B=TGACTGTGAACGTTCGAGATGA. The mLT was provided by Dr. John D. Clements and was reconstituted to 1 mg/ml in sterile water.

5.4.5 Isolation of Lymphocytes from Spleen, Mesenteric Lymph Nodes, Peyer's Patches and Lamina propria

Mice were sacrificed 14 days after final immunization and spleens, mesenteric lymph nodes (MLN), and small intestines were removed. Blood samples were collected via heart puncture. Vaginal wash was collected via washing with PBS containing protease inhibitor and stored at -70°C. Serum was separated from blood and was stored at -70°C. Spleens were gently crushed with glass stoppers to release splenocytes, while the red blood cells were lysed with RBS lysing buffer. MLN tissues were gently crushed using glass stoppers to release the cells. Intestinal cells were isolated as described elsewhere with few modifications [206]. Briefly, the intestines were first rinsed with sterile PBS. Peyer's patches (PP) were then carefully removed from the intestinal tissue by first removal of epithelial cells through incubation at 37°C in EDTA-DTE solution [(PBS containing 10% bovine growth serum (HyClone), 1 mM EDTA, and 1 mM dithioerythritol(DTE)], and then PPs cells were released through incubation with collagenase solution (RPMI 1640 containing 10% fetal bovine serum and 1 mg/ml collagenase D) at 37°C

followed by gentle crushing. The released PP cells were subsequently passed through a nylon mesh, spun down and washed with 2% cold RPMI. To isolate lamina propria (LP) cells, the remaining intestinal tissues were cut open longitudinally and made into 0.5-1cm pieces. The epithelial cells were removed through incubation with EDTA-DTE solution and then the tissue pieces were treated with collagenase solution for 30 minutes. Collagenase-treated pieces were gently crushed, and then released cells were subsequently passed through a nylon mesh, spun down and kept on ice in serum-containing medium. Remaining tissue pieces were returned to fresh collagenase solution for additional stirring at 37°C and cell isolation. When all tissue pieces were digested, cells were washed and the cell pellet was resuspended in 12 ml ice-cold 40% isotonic Percoll in PBS, and then 4 ml of cell suspension was distributed equally into three15 ml tubes. Each 4 ml cell suspension was then underlayed with 100 % ice-cold isotonic Percoll. Tubes were then centrifuged at 1700 rpm 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 spun down for 10 minutes with centrifugation at 4°C at 1500 rpm. These single cell suspensions were then used for evaluating HIV-1 specific T cell responses by IFN-γ ELISPOT and flow cytometry assays.

5.4.6 ELISPOT assay

The detection of antigen specific interferon-gamma (IFN-γ) producing cells was performed using mouse IFN-γ ELISPOT Kits from Mabtech. Lymphocytes from spleen, MLN, PPs and LP tissues were used in this assay. Freshly isolated lymphocytes ($2x10^5$ cells/well) were placed on activated ELISPOT plates. These cells were stimulated (in triplicates) with 5 ug/ml/peptide pools of HIV-1 Consensus Subtype B Gag 15-mer peptides overlapping by 11 amino acids (NIH AIDS

Research and Reference Reagent Program). A background control; DMSO equivalent to that in the peptide pools was also added to each well. The treatments for all samples were plated in triplicate, except in case of low yields specifically for LP; in that case duplicate samples were used. The cells were then incubated at 37°C, 5% CO_2 atmosphere for 24 hours. After incubation, the cells were washed away and ELISPOT plates were assayed for IFN-γ producing cells as per the manufacturer's protocol (MABTECH ELISPOTplus for mouse IFN-γ). The number of spot forming cells (SFC) was counted on an ELISPOT reader. The results are expressed as number of spots per 10^6 cells in stimulated wells minus the number in non stimulated wells.

5.4.7 ELISA to detect systemic and mucosal anti-HIV-1 antibodies

96 well ELISA plates were coated with HIV-1 p24 protein (NIH AIDS Research and Reference Reagent Program) overnight. Then serial dilutions of serum or undiluted samples of fecal extracts, vaginal washes, or intestinal washes were made and placed in the wells. These plates were then incubated at room temperature for 2 hours (serum) or at 4°C overnight (fecal extracts, vaginal washes, and intestinal washes). Plates were washed, and AKP conjugated α-mouse IgG, IgG1 or IgG2a antibody or biotin-conjugated α-mouse IgA antibody (BD Biosciences) were placed in the appropriate wells for 1 hour 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. 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma) was used to detect p24-specific antibody, and optical density was read on a plate reader at 405nm. Background values from negative control wells on each plate were subtracted.

5.4.8 T cell subpopulation depletion and enrichment

To further identify the IFN-γ secreting specific T cell subset, either CD4 or CD8 depletion was performed. Single cell suspensions were prepared from freshly harvested LP, PPs, MLN and spleen tissues. The red blood cells were removed by using RBC lysing buffer. Mouse anti CD4 (clone L3T4) or CD8 (clone Ly-2) microbead kits (Miltenyi Biotech) were used to enrich for $CD4^-$ or $CD8^-$ T cells, respectively. For $CD4^+$ or $CD8^+$ T cell depletion, cell suspensions were magnetically labeled with either CD4 or CD8a microBeads. After 15 minutes incubation on ice, the cell suspensions were loaded on a MACS® Column which was then placed in a magnetic field. The magnetically labeled cells were retained in the column. The unlabeled cells were passed through, and collected as the cell fraction that is depleted of either $CD8^+$ or $CD4^+$ T cells. These depleted cell populations were then used in the ELISPOT or flow assay along with the total, non separated T cells. The purity of the recovered cells was confirmed by flow cytometry analysis and was routinely greater than 95%.

5.4.9 Surface and intracellular cytokine staining

FITC-anti-CD107a (clone 1D4B), R-PE-anti-CD8 (clone 53-6.7), APC-anti-IL-2 (clone JES6-5H4), PE-Cy7-anti-IFN-γ (clone XMG1.2), Biotin-anti-TNF-α (clone MP6-XT3), and APC-Cy7-streptavidin were purchased from BD Pharmingen. PE-Cy5-anti-CD3 (clone 145-2C11) was purchased from BioLegend. Isotype control antibodies were purchased from the same manufacturers according to the fluorescent conjugate used. A total of 0.5 -2x10^6 freshly isolated cells were stimulated with 5 ug/ml of HIV-1-B Gag peptide pool in presence of 3 uM monensin and 5 ug/ml of brefeldin A in 200 ul growth media (DMEM with 10% fetal calf serum, 1 mM

sodium pyruvate, 2 mM L-glutamine, 0.025 M 2-Mercaptoethanol, and 1.25 mM HEPES). FITC conjugated antibody against CD107a (clone 1D4B), was also added in each well and the cells were incubated at 37°C, 5% CO_2 for 5 hours. A background control cultured without any peptides was included in each sample. As a positive control, samples were cultured with 50 ng/ml phorbol myristate acetate and 1 μg/ml ionomycin. After the 5 hour incubation, plates were stored at 4°C for overnight. Cells were first stained for surface marker specific antibodies (anti-CD3-PerCP-Cy5.5 and anti-CD8 PE), then fixed and permeabilized with saponin containing FACS solution and intracellular stained with cytokine-specific antibodies (anti-IFN-γ PE-Cy5, IL-2 APC, TNF-α APC-Cy7). Then these cells were washed, fixed, and analyzed by flow cytometry within 12 hours of staining. The proportions of fluorochrome-labeled cells were assessed with a BD Canto flow cytometer and analyzed by FlowJo software (provided by Dr. Mario Roederer, NIH). Data was typically collected from 50,000 lymphocytes.

5.4.10 Flow Cytometry and analysis

Stained cells were analyzed using a BD Canto flow cytometer. FCS files were analyzed using FlowJo software (version 7.2.2). 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. For SPICE analysis, individual mice with no HIV-1-p55-

specific response were excluded. A threshold value of the 75% confidence values of negative percentages for each T cell subset was used.

5.4.11 Statistics

Statistical analyses were performed using GraphPad Prism version 4. Except where noted, *p* values were determined using 1-tail two-sample Student's t-test with unequal variance. Results were considered significant if $p<0.05$.

5.5 RESULTS

5.5.1 Screening of immunodominant HIV-1 Gag peptide pools for use in measuring HIV-1 specific cellular immune responses in immunized mice

There is very little information available on the mouse immunodominant T cell epitopes within the HIV-1 Gag region. Therefore a study was initiated to identify immunodominant T cell epitopes within the HIV-1 Gag region in the mouse, the results of which were then used for subsequent screening of HIV-1 Gag immune responses in immunized mice. Since yields of cells from mucosal tissues of mice are generally limited, the use of a few immunodominant peptides instead of all the peptides spanning the Gag region is beneficial for increasing sensitivity of immune response assays. To identify the immunodominant epitopes, 5 different overlapping peptide pools of 15 mer encompassing the entire HIV-1 Gag region were created from the HIV-1 Gag peptides (Figure 10B). The mice were immunized subcutaneously with purified HIV-1 Gag

protein along with complete Freund's adjuvant and boosted 10 days later with the same antigen in the presence of incomplete Freund's adjuvant (Figure 10A). The mice were sacrificed 14 days after the final boost and splenocytes were harvested and used as responder cells. An IFN-γ ELISPOT was performed by incubating these splenocytes with the HIV-1 Gag peptide pools for 24 hours to stimulate antigen specific T cells. As shown in figure 10C, peptide pool 3 (p24 region) and pool 5B (p7/p6 region) showed higher HIV-1 Gag specific IFN-γ responses when compared to other peptide pool regions, and therefore they were selected for future ELISPOT assays. Also, although pool 4 (p24 region) and pool 5A (p24 and p7 region) showed similar responses, pool 5A was selected for future use due to the inclusion of peptides from both p24 and p7 regions of Gag. Overall we selected the peptide pools 3 and 5B for use as antigen-specific stimulants in all subsequent studies described below.

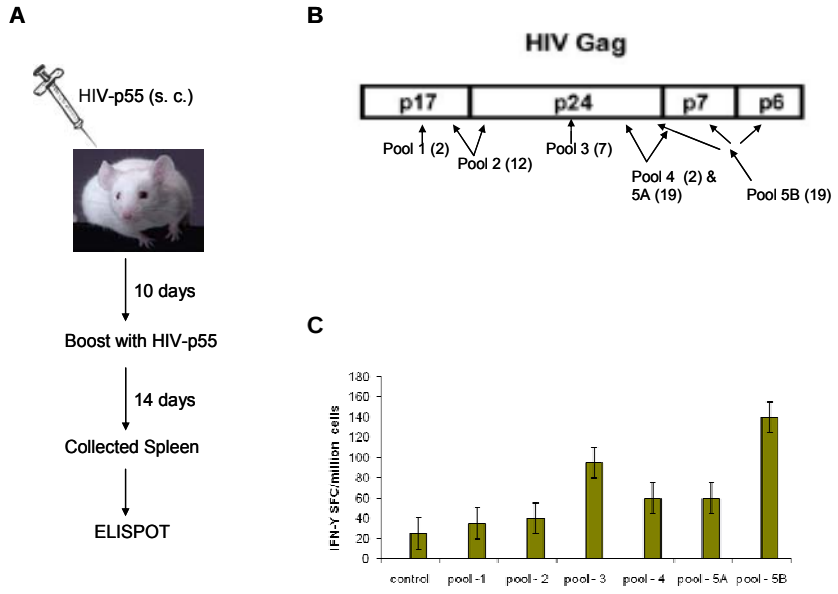

Figure 10. Screening of HIV-1 Gag peptide pools.

(A) A group of 10 mice each were immunized with HIV-1 Gag (p55) protein and (subcutaneously) with adjuvants and a group of 5 control mice immunized with PBS as show. Spleen was harvested for use in ELISPOT assays. **(B)** HIV-1 Gag protein was divided into 5 different peptide pools as shown. **(C)** IFN-γ secreting splenocytes stimulated with the different HIV-1 Gag peptide pools as determined by ELISPOT. Error bars representing standard error of the mean.

5.5.2 Cellular immune responses induced by immunization with recombinant *C. perfringens* and VLPs

Initially a pilot study was performed to evaluate and compare immune responses induced by heterologous versus homologous prime boost vaccination approaches using recombinant *C. perfringens* (CP) and VLPs. This initial study was performed with a total of three groups: (1) both prime and boost were done with VLP (VLP), (2) priming was done with VLP and boosting with CP (VLP/CP) and (3) adjuvant control group in which animals received CT adjuvants only without vaccine (control). Cells from spleen, Peyer's patches (PPs) and mesenteric lymph nodes (MLN) were analyzed for HIV-1 Gag specific IFN-γ secreting cells by incubating with Gag-

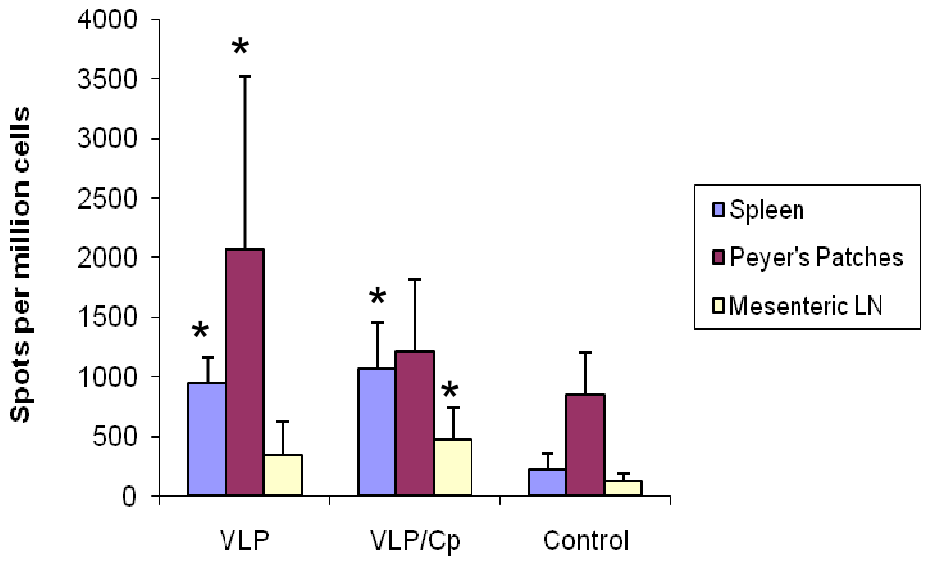

Figure 11. HIV-1 specific IFN-γ secretion by T cells from mice immunized with either VLP alone (VLP), VLP along with *Cp*-Gag (VLP/Cp) or adjuvants only (Control).

Three groups of five mice each were intranasally immunized with either VLP in a prime boost approach (VLP) or with combination of Cp-gag (orally) in a prime boost approach. Immunized mice spleen, Peyer's Patches and Mesenteric lymph nodes were harvested for use in ELISPOT assays. For statistical analysis *p* values were determined by Student's t-test. * p value < 0.05, compared to control groups.

specific peptide pools in an ELISPOT assay. When compared to the adjuvant control group there was a significant increase in the HIV-1 Gag specific T cell response in spleen and PPs of both the VLP and VLP/CP groups, and in MLN of the VLP/CP group (Figure 11). However no significant differences were observed between VLP and VLP/CP groups in the responses from T cells isolated from spleen**,** MLNs and PPs of the immunized mice. However, PPs had the highest number of HIV-1 specific IFN-γ producing cells compared to spleen and MLN cells in all immunized mice. HIV-1 Gag specific humoral immune responses were also evaluated by measuring the IgG1 and IgA subtype of antibodies in sera and mucosal fluids (feces and intestinal washes) from the immunized mice. There were no significant HIV-1 specific humoral immune responses observed in the mucosal or systemic compartments of the immunized mice (data not shown). This study was performed using non optimized doses of cholera toxin (CT) and CpG oligodeoxynucleotides (ODN) as adjuvants. The failure to see a difference between the VLP and VLP/CP immunized groups might be a result of the use of suboptimal doses of adjuvants that was given orally with CP. Therefore a study was initiated to select proper adjuvants and their doses that could induce maximum mucosal immune responses.

5.5.3 Optimization of oral adjuvants

The cholera toxin (CT), mutant heat-labile toxin (LT [R192G], mLT), or unmethylated cytosine-phosphate-guanine oligodeoxynucleotides (CpG ODN) were selected as adjuvants for use with recombinant *C. perfringens*. The optimization of dose and adjuvant capacity of these adjuvants was carried out by delivering them along with recombinant *C. perfringens* expressing SIV Gag (*Cp*-SGag*)* via the oral route to naïve mice. Small groups of mice (3 mice per group) were orally administered with 150 ug/mice SIV Gag (measured by western blot) in the form of bacterial

culture of *Cp*-SGag and varying doses of CT, mLT, or CpG ODN. Control groups consisted of mice vaccinated orally with *Cp*-SGag without any adjuvants. Cells from PPs were assayed for SIV Gag specific IFN-γ production using an ELISPOT assay. As shown in Figure 12 A, the groups that received 5 μg CT, 1 μg mLT, or 50 μg of CpG ODNs demonstrated higher number of SIV Gag specific IFN-γ secreting T cells when compared to the other groups. However within these three adjuvants groups, 1 ug of mLT and 50 ug of CpG-ODNs groups showed superior responses. Surprisingly delivery of *Cp*-SGag with higher doses of CT (10 or 50 ug) or mLT (5, 25 or 50 ug) gave lower cellular responses when compared to delivery with lower doses of CT (5ug) or mLT (1 ug), although this was not statistically significant. IgG1 and IgG2a levels in serum and IgA levels in fecal extract and intestinal wash were low in all groups, with no difference observed between vaccinated mice and the PBS control group (data not shown).

Since both CpG ODN (50 ug) and mLT(1 ug) induced greater cellular responses than CT (5ug) when used with *Cp*-SGag, combinations of different doses of CpG ODNs with mLT were further tested to determine their optimum combination to induce the strongest cellular immune responses. Groups of 4 mice were inoculated orally with *Cp*-SGag with various combinations of doses CpG ODNs with 1 μg mLT, and the cellular immune responses in the PPs were compared with groups of mice that received each adjuvant dose alone or *Cp*-SGag alone (Figure 12 B). IFN-γ ELISPOT assays of cells from PPs showed that 50 μg CpG ODN combined with 1 ug of mLT generated a higher but statistically insignificant cellular immune response than 25 or 100 μg CpG ODN combined with mLT. The group of mice that were immunized with *Cp*-SGag along with 1 ug mLT and 50 ug of CpG-ODNs was the only group that displayed a significantly higher cellular response compared to the groups that received *Cp*-SGag without adjuvants. Therefore this combination of 1 ug mLT and 50 ug of CpG-ODNs were chosen as the adjuvant

dose to be used with recombinant *C. perfringens* in all subsequent oral immunizations. The probable reason for the higher responses seen in the mLT and CpG ODNs adjuvant combination groups compared to groups with either adjuvant alone could be that mLT and CpG ODNs stimulate immune cells through separate signaling mechanisms which are additive in nature.

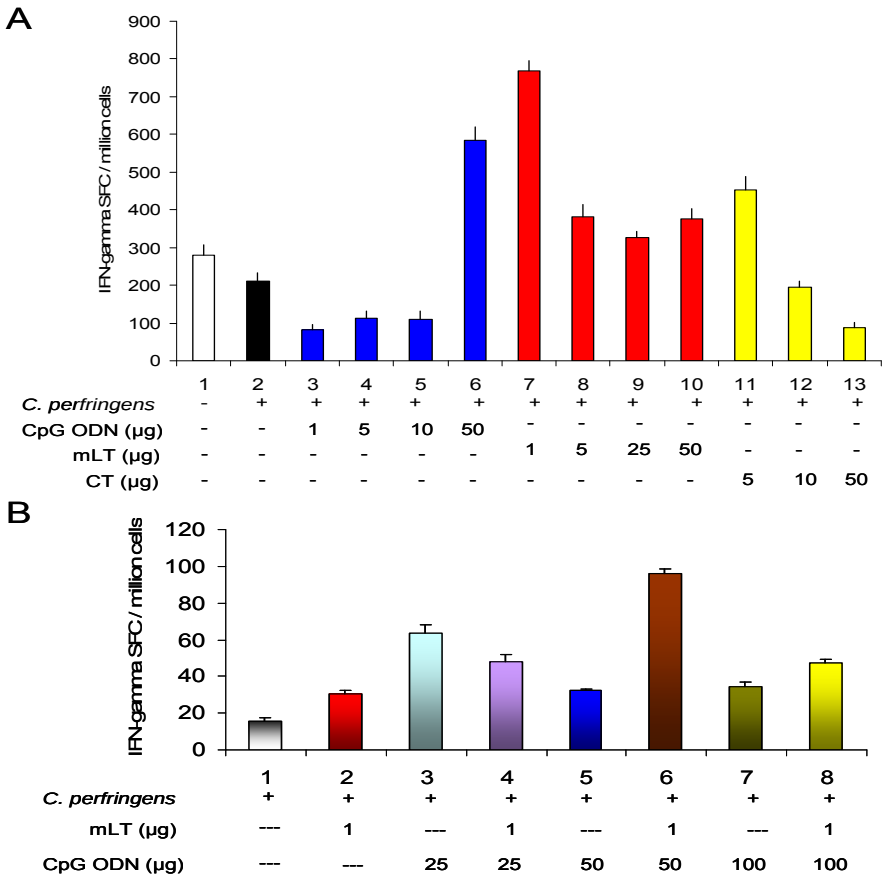

Figure 12. HIV-1 specific IFN- γ secretion by T cells from mice immunized with recombinant *C. perfringens* expressing SIV Gag in combination with various doses of adjuvants.

(A) SIV Gag specific IFN-γ ELISPOT results from PPs, each bar representing average of 3 mice per group with error bars representing standard error of the mean. **(B)** Adjuvants CpG- ODN and mLT was combined in various doses and orally administered to mice along with antigen CP-Gag. This experiment is representative of 2 individual studies. Mean results from the PPs of 4 mice per group are shown with error bars representing standard error of the mean.

5.5.4 Comparison of cellular immune responses induced by homologous and heterologous immunization approaches

The HIV-1-Gag specific T cell responses were evaluated after using homologous (recombinant *C. perfringens* or VLP alone) or heterologous (combining both recombinant *C. perfringens* and VLP) prime boost immunization approaches. The optimized combination doses of mLT and CpG ODN were used along with orally administered recombinant *C. perfringens* expressing HIV Gag (*Cp*-Gag) and intranasally delivered HIV-1 VLPs. Groups of 10 mice were immunized with different prime-boost regimens as described (Table 1), and the resulting systemic and mucosal immune responses were investigated.

Table 1. First immunization schedule

Immunization group	Prime (week 0)	1st Boost (week 3)	2nd Boost (week 6)	3rd Boost (week 9)
PBS	Adjuvant only	Adjuvant only	Adjuvant only	Adjuvant only
CP/CP	*Cp*-Gag	*Cp*-Gag	*Cp*-Gag	*Cp*-Gag
VLP/VLP	VLP	VLP	VLP	VLP
CP/VLP	*Cp*-Gag	*Cp*-Gag	VLP	VLP
VLP/CP	VLP	VLP	*Cp*-Gag	*Cp*-Gag

Mice were primed with either *Cp*-Gag (150 ug/mice) or VLP (20 ug/mice) were boosted thrice at three week intervals with either vector in a homologous manner where the *Cp*-Gag primed group received *Cp*-Gag as boost (CP/CP) and the VLP primed group received VLP as boost (VLP/VLP) or in a heterologous manner where *Cp*-Gag or VLP primed groups were boosted with VLP (CP/VLP) or *Cp*-Gag (VLP/CP) respectively. The control group received adjuvants in PBS without any antigen at each inoculation. Fourteen days after the final booster immunization, mice were sacrificed. Peripheral blood serum, intestinal washes, and fecal samples were collected and examined for humoral immune responses. Cells isolated from spleen,

mesenteric lymph node (MLN), and Peyer's patches (PPs) were examined for cellular immune responses.

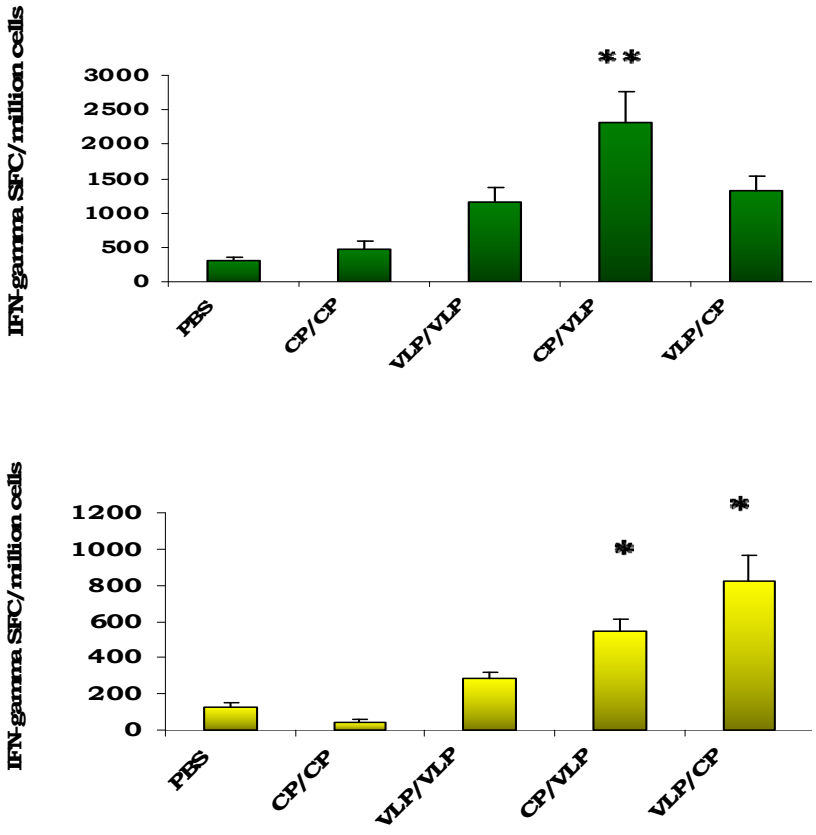

Figure 13. HIV-1 specific IFN- γ secretion by T cells from Peyer's patches (A) and spleen (B) of mice immunized with various prime-boost regimens.

Average HIV Gag specific IFN-γ ELISPOT results from **(A)** Peyer's Patches (PPs) and **(B)** Spleen. Results are shown in bar graph, with each bar representing the average of 6 mice per group and are shown with error bars representing standard error of the mean. For statistical analysis *p* values were determined by Student's t-test with unequal variance. *$p<0.05$,compared to the homologous and adjuvant alone groups. **$p<0.05$ compared to the VLP/CP, homologous and adjuvants alone groups.

An IFN-γ ELISPOT assay was used to determine the number of IFN-γ secreting HIV-1 Gag specific immune cells in the mucosal and systemic compartments of the immunized mice. As shown in Figure 13 in general all heterologous groups which received both *Cp*-Gag and VLP had significantly higher IFN-γ secreting cells in all tissue compartments compared to the

homologous groups. As observed in previous immunization studies, PPs had the higher numbers of HIV-1 specific IFN-γ producing cells compared to spleen. Furthermore, the CP/VLP group showed the highest level of T cell immune responses. The results from this study indicated that the mice primed with *Cp*-Gag followed by boosting with VLPs induced significantly higher levels ($p<0.05$) of HIV-1 specific cellular immune responses in spleen (Figure 13A), and PPs (Figure 13B), compared to either vaccine vector alone .

Low levels of IgG1 antibody responses were detected in sera from mice that were immunized with *Cp*-Gag and VLP in a prime boost approach compared to mice that received either construct alone (Figure 14A). No IgA antibody responses could be detected in mucosal samples from feces and intestinal washes (Fig 14B and C). This suggests that the use of *Cp*-Gag and VLP together in a prime boost (heterologous) approach induces low level of antigen specific IgG1 antibody response in serum, but not in mucosal compartments. Therefore this vaccination approach needs further optimization to obtain higher levels of systemic and mucosal humoral responses.

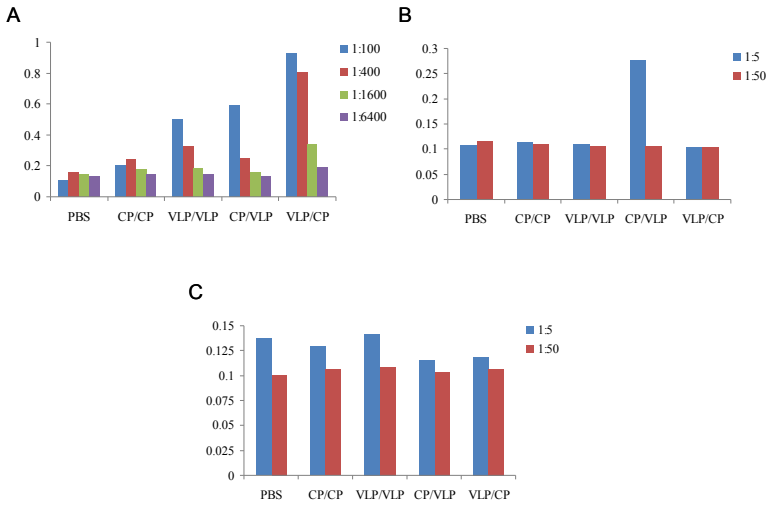

Figure 14. OD405 values for anti-HIV-1 antibodies present in serum (IgG, A), fecal washes (IgA, B) and intestinal washes (IgA, C) of immunized mice.

5.5.5 Cellular immune responses measured in the GI tract lamina propria

The lamina propria (LP) is the effector site of the GI tract immune system and is known to contain a large number of lymphocytes. Therefore induction of antigen specific lymphocytes in the LP is a desired property for mucosal vaccines that target the GI tract. Since LP tissues were not analyzed in the previous immunization study, a second similar immunization study was performed to assay if *Cp*-Gag and VLP prime-boost immunization can induce antigen specific cellular immune responses in the LP. Table 2 describes the immunization schedule, which differs from the previous schedule as shown in table 2, a single VLP immunization was used and the number of booster immunizations was reduced to two.

Table 2. Second immunization schedule

Immunization group	Prime (week 0)	1st Boost (week 3)	2nd Boost (week 6)
Control	Adjuvant only	Adjuvant only	Adjuvant only
VLP1	VLP	PBS	PBS
VLP2	PBS	PBS	VLP
CP/VLP	*Cp*-Gag	*Cp*-Gag	VLP
VLP/CP	VLP	*Cp*-Gag	*Cp*-Gag
CP	*Cp*-Gag	*Cp*-Gag	PBS

Systemic and mucosal tissue samples were used to harvest cells from spleen, PPs, MLN and LPs tissue compartments. Lymphocytes were isolated from the tissues and were assayed for HIV-1 Gag specific IFN-γ production in an ELISPOT (Figure 15). As shown in Figure 15D spleen of mice that were primed first with *Cp*-Gag and then boosted with VLPs (Cp/VLP group) had the significantly higher number of IFN-γ secreting HIV-1 Gag specific immune cells compared to mice that were primed first with VLPs and then boosted with *Cp*-Gag (VLP/Cp group) ($p < 0.05$). The Cp/VLP group also had the highest number of IFN-γ secreting HIV-1-

Gag specific immune cells compared to mice immunized with either *Cp*-Gag or VLPs alone ($p<0.01$). A similar trend was seen in cells from both the PPs and MLNs of immunized mice although the magnitude of the responses in MLN was comparatively lower (Figure 15B, C).

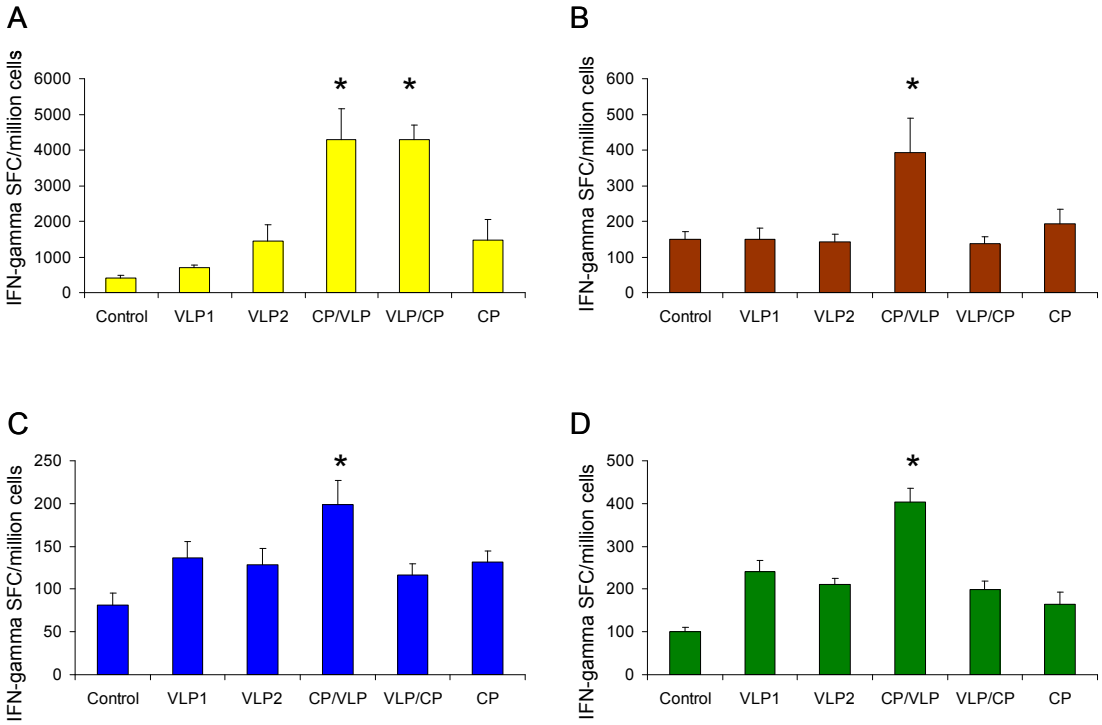

Figure 15. HIV-1 specific IFN- γ secretion by T cells from Lamina propria (A), Peyer's patches (B), MLN (C) and spleen (D) of mice immunized with various prime-boost regimens. * p<0.05 when compared to all other groups.

Average HIV Gag specific IFN-γ ELISPOT results from **(A)** Lamina Propria (LP), **(B)** Peyer's Patches (PPs)**,(C)** Mesenteric Lymph Node(MLN) and **(D)** Spleen (SP). Results are shown in bar graph, with each bar representing the average of 10 mice per group and are shown with error bars representing standard error of the mean. For statistical analysis *p* values were determined by student's t-test. **p*<0.05, compared to all other vaccinated groups.

The LP is an effector site of the gut that has been shown to be important in HIV-1 infections [38]. In the LP of the immunized mice, both the Cp/VLP and the VLP/Cp group induced almost similar numbers of IFN-γ secreting HIV-1 Gag specific immune cells that were significantly higher than that seen in all other tissue compartments ($p< 0.01$). In addition, these CP/VLP and VLP/CP groups significantly increased number of HIV-1 Gag specific IFN- γ from

mice immunized with either *Cp*-Gag or VLPs alone (Figure 15A). It was also observed that the LP tissue compartment had the highest number (4000- 5000 IFN-γ secreting cell/10^6 cells) of IFN-γ secreting HIV-1-Gag specific immune cells as compared to T cell responses in all other tissue compartments (200-400 IFN-γ secreting cell/10^6 cells). However no antibody responses were detected in mucosal and systemic samples from all immunized mice (data not shown).

These data indicate that the priming with *Cp*-Gag stimulates mucosal cellular immune responses in the gut that are improved by intranasal boosting with HIV-1 VLPs and this heterologous prime-boost approach was most effective in the strong induction of antigen specific responses in the LP, an important effector site of the gut.

5.5.6 Measurement of functionality of HIV-1 Gag specific T cells in mucosal and systemic compartments

The ability of T cells to produce multiple cytokines on a single cell basis in response to antigenic stimuli has been shown to be associated with a protective response against infection. Therefore to further explore the potential of *Cp*-Gag and VLPs as a mucosal priming vaccine, the multifunctional quality of cellular responses were measured, after immunization in both the PP, inductive site and LP, the effector tissue. The quality of HIV-1 Gag specific immune responses in these sites were assessed by performing an intra-cellular cytokine staining of cells stimulated with HIV-1 Gag peptides followed by multiparametric flow cytometry analysis

Table 3. Third immunization schedule

Immunization group	Prime (week 0)	1st Boost (week 3)	2nd Boost (week 6)
PBS/ADJ	Adjuvant only	Adjuvant only	Adjuvant only
CP-VLP	*Cp*-Gag	*Cp*-Gag	VLP
VLP-CP	VLP	*Cp*-Gag	*Cp*-Gag

As shown in table 3, in this study there were 3 groups of ten mice, each were inoculated with oral *Cp*-Gag or nasal VLP and. boosted at 3 weeks interval with either *Cp*-Gag or VLP. Mice in control group received only adjuvants in PBS. All mice were sacrificed 14 days after the last booster immunization. Cells were harvested from spleen, PPs and LP tissues of the immunized mice and the presence of antigen specific T cells were evaluated in these cell preparations by both ELISPOT and intracellular staining using multiparametric flow cytometry techniques.

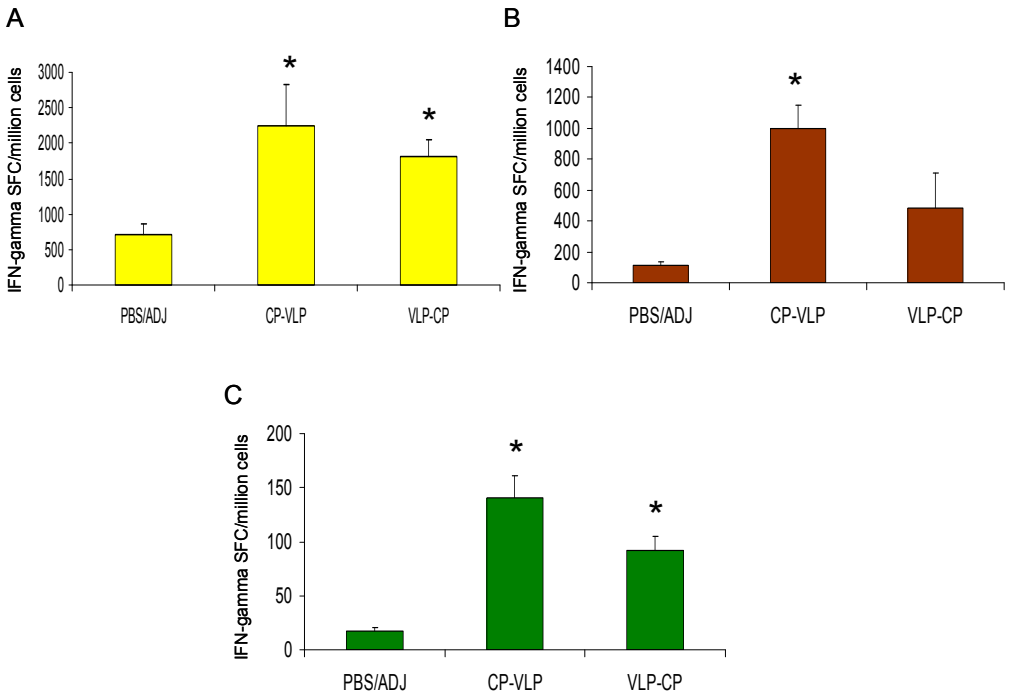

Figure 16. HIV-1 specific IFN- γ secretion by T cells from Lamina propria (A), Peyer's patches (B) and spleen (C) of immunized mice.

Average HIV Gag specific IFN-γ ELISPOT results from **(A)** Lamina Propria (LP). **(B)** Peyer's Patches (PPs**).**and **(C)** Spleen (SP). Results are shown in bar graph, with each bar representing the average of 10 mice per group and are shown with error bars representing standard error of the mean. For statistical analysis *p* values were determined by student's t-test using 2 tailed, unequal variance parameters. *$p<0.05$, compared to control group, where mice received adjuvants only.

The results from the ELISPOT reinforce the findings from previous immunization studies that the HIV-1 specific mucosal immune responses in both the effector (LP) and inductive sites

(PP) of the GI tract were significantly higher in mice immunized using recombinant *Cp*-Gag and VLPs in heterologous prime-boost approaches compared to mice immunized with either *Cp*-Gag or VLPs alone (Figure 16). LP, the effector site of the gut, had the highest number of HIV-1 specific IFN-γ producing cells as compared to spleen and Peyer's patches cells in all immunized mice as measured by ELISPOT. These results further provide evidence to the ability of *Cp*-Gag and VLP prime boost approaches in inducing strong mucosal immune response in the GI tract, one of the major sites of early HIV-1 replication.

To evaluate other functions of the HIV-1 Gag-specific GI tract 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 in these cells. These four immune functions were then detected by multiparameter flow cytometry. Cells were surface stained for CD3 and CD8 to differentiate $CD8^+$ and $CD8^-$ T cells.

5.5.7 Individual cytokine secreting cells in PP and LP

The percentage of cells producing each cytokine in the PP and LP was calculated from flow cytometry results (Figure 17). In both mucosal compartments - PP and LP, the percentage of IFN-γ producing $CD8^+$ cells were highest. Surprisingly the percentage of IL-2 producing cells from all three groups was lowest when compared to other cytokines. In the cytokine response seen in PPs; the CP/VLP group displayed highest IFN-γ and TNF-α production compared to the VLP/CP group (Figure 17A). On the other hand, the VLP/CP group displayed slightly higher percentage of cells expressing IL-2 compared to the CP/VLP or control groups. There was very low expression of the surface marker CD107a. However, the percentage of cells expressing CD107a was slightly higher in the VLP/CP group compared to the other groups. Overall in PPs,

the CP/VLP group displayed slightly higher percentage of cells expressing all cytokines compared to the VLP/CP group.

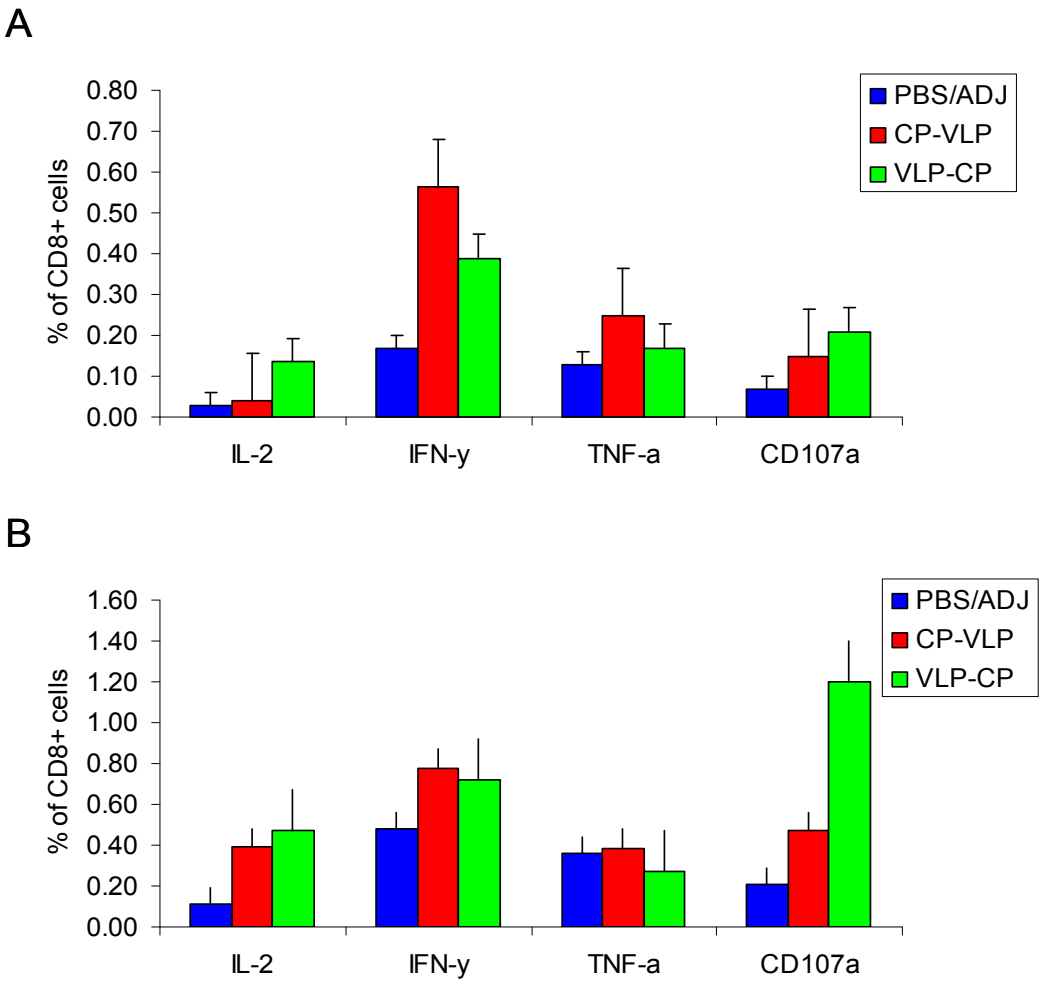

Figure 17. Percentage of CD8$^+$ T cells secreting the indicated cytokines in response to stimulation with HIV-1 Gag peptides in the PPs (A) and LP (B).

HIV Gag-specific IL-2, TNF-α, and IFN-γ and CD107a production from CD8+ CD3+ cells was detected via intracellular staining and samples were analyzed using flow cytometry and percent of cells with any cytokine response was determined. Bars represent the average values from 10 mice per group plus standard error of the mean.

The LP had the higher percentage of antigen specific CD8$^+$ T cell responses to HIV-1 Gag compared to PP (Figure 17B). In contrast to the results from PPs, the LP had higher

percentages of HIV-1 Gag specific cells expressing CD107a on their surface, especially in the VLP/CP group compared to the CP/VLP or control groups ($p<0.05$). IFN-γ production by HIV-1 Gag specific cells in the LP was almost comparable in both the CP/VLP and VLP/CP groups. TNF-α production by HIV-1 Gag specific cells in the LP was also similar in all three groups, even in control group that did not receive any immunogen. It was also observed that in the LP, there was a comparatively higher amount of non-specific cytokine secreting cells in the control group than in the PP. Overall these results demonstrate that mucosal immunization with *Cp*-Gag and VLPs lead to the induction of HIV-1 Gag specific T cell populations that can secrete multiple cytokines in the mucosal tissue compartments of the GI tract.

5.5.8 Measurement of multifunctionality of antigen specific T cells

The multifunctional characteristic (2 or more functions) of the HIV-1 Gag specific T cells from PP and LP were further evaluated. All mice displayed production of at least one of the assayed cytokines in response to HIV-1 Gag in T cells from the PP. There were higher percentages of cells producing two or more cytokines in mice from the CP/VLP and VLP/CP groups compared to those in the control group. The mice from the CP/VLP group had slightly higher numbers of HIV-1 Gag specific multifunctional $CD8^+$ T cells that secrete all three cytokines - IL-2, TNF-α, and IFN-γ and CD107a compared with the VLP/CP or control groups (Figure 18). Overall, the level of multifunctionality (secreting all three cytokines and CD107a) of PP $CD8^+$ T cells was greater in mice from the CP/VLP group compared to all other groups. Surprisingly mice from the control group also displayed lower levels of multifunctionality compared to the other groups.

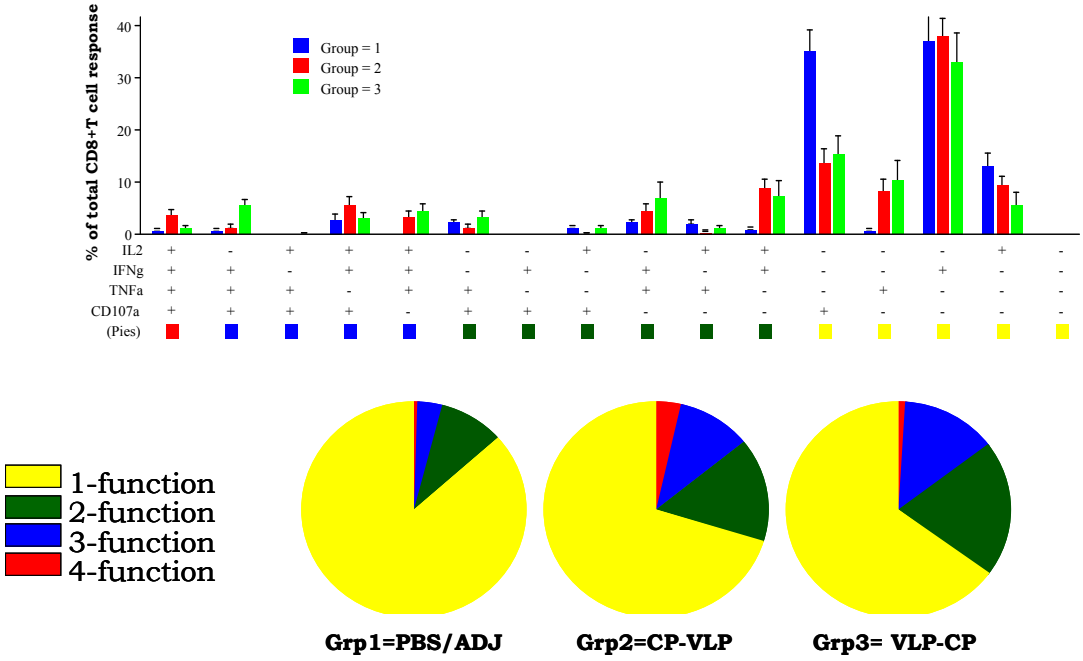

Figure 18. Multifunctional T cell responses in the Peyer's patch an inductive site of the GI tract, of immunized mice.

Data generated from Boolean-gated CD8+ cells from Peyer's patches 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 for each combination of functions is shown with error bars representing standard error of the mean. ***B***, The average percentage of total response at each level of multifunctionality is represented by slices in pie charts.

As observed in the PP, mice in all groups had HIV-1 Gag specific T cells in the LP that produced at least one cytokine (Figure 19). However, in contrast to the PP response, in LP the VLP/CP group was the only group that displayed multifunctionality (secreting all three cytokines and CD107a) of LP $CD8^+$ T cells compared to all other groups. HIV-1 Gag specific cells having all four function were not detected in CP/VLP group as was seen in PPs. Surprisingly control and CP/VLP groups displayed almost comparable level of cells having either 1, 2 or 3 cytokine functions. These results indicate that the T cells in the LP appear to have higher levels of background cytokine production since the control group also had high amounts of non specific cytokine production. The LP is an effector site of the GI tract and therefore is under constant

antigenic exposure in the GI tract which might lead to an overall inflammatory environment producing cytokines and other inflammatory molecules.

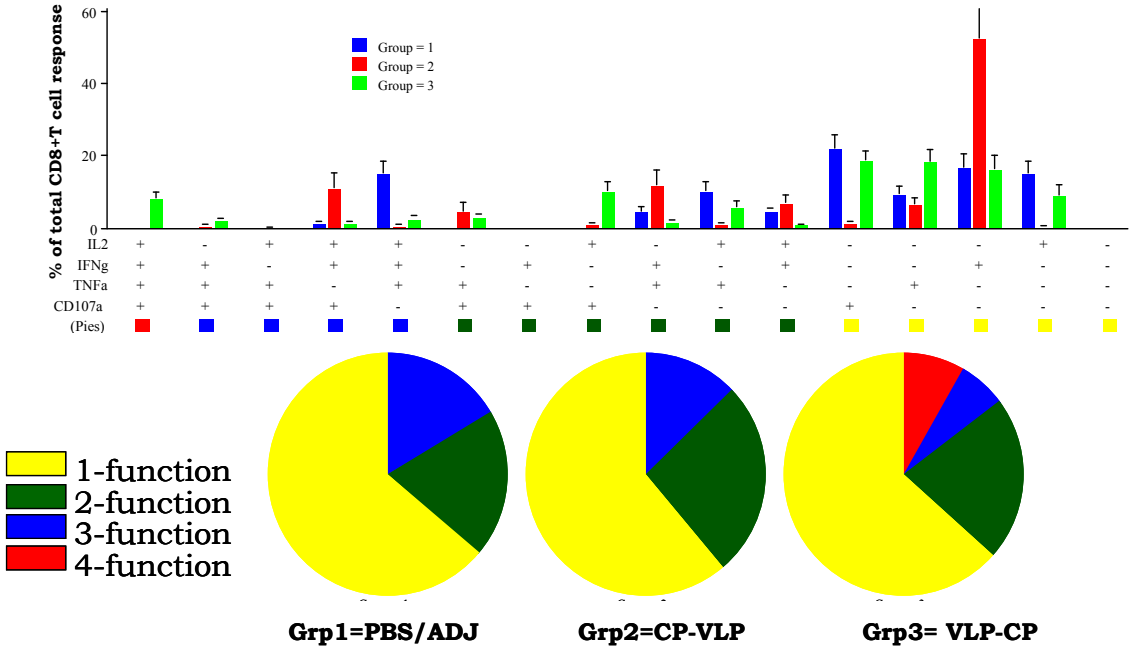

Figure 19. Multifunctional T cell responses in the Lamina propria, an effector site of the GI tract, of immunized mice.

Data generated from Boolean-gated CD8+ cells from 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 for each combination of functions is shown with error bars representing standard error of the mean. *B*, The average percentage of total response at each level of multifunctionality is represented by slices in pie charts.

5.5.9 Characterization of cellular immune responses

Adaptive immunity is mediated mainly by T cells comprised of $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells are also known as T-helper cells and have an important role in the initiation and maintenance of immune responses against pathogens. On the other hand, $CD8^+$ T cells are known as cytotoxic T cells that can directly mediate killing of cells infected with pathogens.

Therefore a study was performed to determine whether immune responses induced by *Cp*-Gag and VLP prime-boost immunization approaches was mediated by CD4+ or CD8+ T cells. Magnetic beads coupled to either CD4 or CD8 antibodies were used to deplete cells expressing them from the cell preparations of spleen, PPs and LP tissues obtained from immunized mice (Figure 20). Then the presence of antigen specific immune responses in these $CD4^+$ or $CD8^+$ depleted cells were assayed by an ELISPOT as described before.

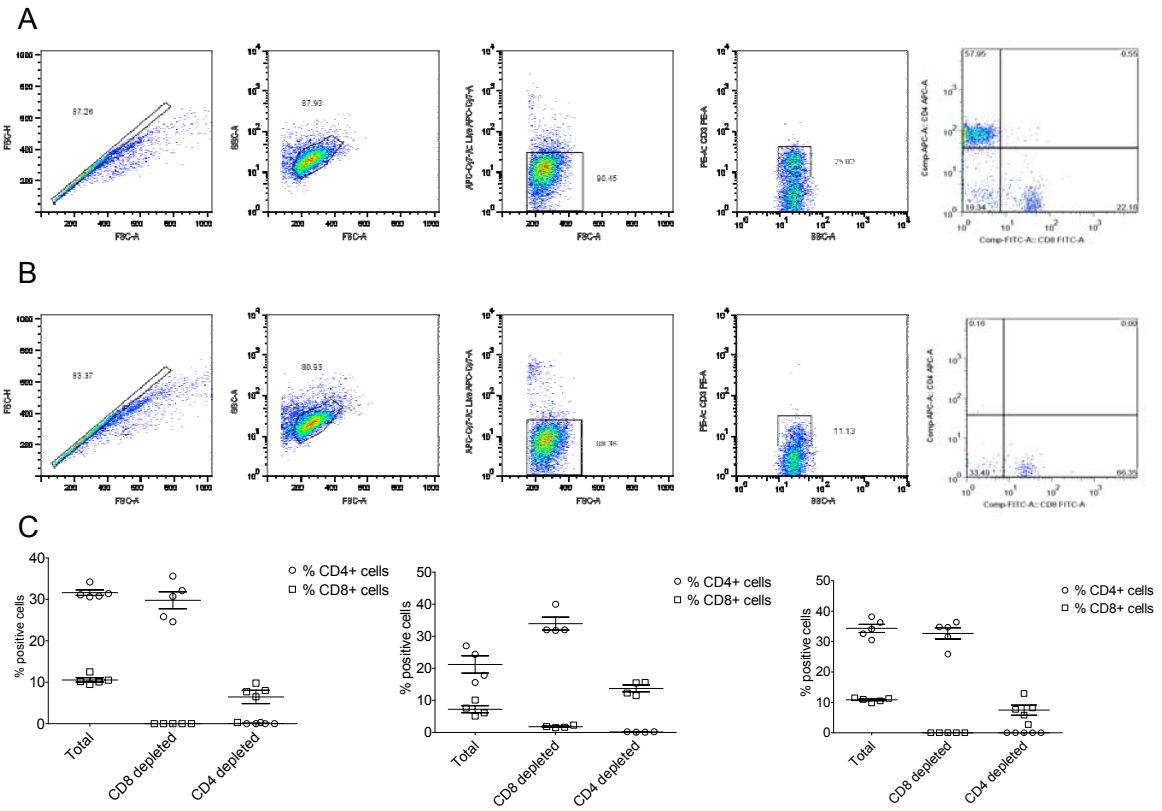

Figure 20. Representative flow cytometry analysis of cells from spleen of immunized mice after negative selection for $CD4^+$ or $CD8^+$ cells using magnetic beads.

(A) Total spleen cell population before depletion, **(B)** CD8+ cell depleted spleen population, and **(C)** Percentages of $CD4^+$ and $CD8^+$ cells after depletion in the adjuvant alone group (left), CP/VLP group (middle) and VLP/CP group (right).

In the spleen, similar numbers of IFN-γ secreting HIV-1 Gag specific immune cells in the total cell population was observed as before, with the CP/VLP group having the highest number

of IFN-γ secreting cell (Figure 21A). The numbers of IFN-γ secreting HIV-1 Gag specific immune cells were higher in the $CD8^+$ cell depleted populations compared to those depleted of $CD4^+$ cells. Surprisingly, depletion of either $CD4^+$ or $CD8^+$ cells led to increased numbers of IFN-γ secreting HIV-1Gag specific immune cells in comparison to the non depleted total cell population in the VLP/CP group only, whereas in the rest of the groups the non depleted total cell population had the highest numbers compared to the $CD4^+$ or $CD8^+$ depleted populations. Therefore these results indicate that there was more contribution of the $CD4^+$ cells to the antigen specific responses seen in the spleen compared to the $CD8^+$ cells.

In the PPs, we found similar results for the $CD4^+$ or $CD8^+$ cell depleted populations as seen in the spleen (Figure 21 B). The CP/VLP group had the highest numbers of IFN-γ secreting HIV-1-Gag specific immune cells in either the total or $CD4^+/CD8^+$ cell depleted populations amongst all immunization groups. In the CP/VLP group, the $CD4^+$T cells generally contributed more to the antigen specific responses compared to the $CD8^+$ T cells. On the other hand, in the rest of the groups both $CD4^+$ and $CD8^+$ depleted cells showed similar levels of IFN-γ secreting HIV-1-Gag specific immune cells. Therefore we found that the CP/VLP had the highest antigen specific responses amongst all immunization groups and the $CD4^+$ cells were the major contributors to this cellular response.

Similarly, the antigen specific cellular responses in the LP were highest in the CP/VLP group, although both $CD4^+$ and $CD8^+$ cells had similar levels of antigen specific responses (Figure 21 C). Also as seen earlier the magnitude of responses was highest in the LP of immunized mice when compared to other tissue compartments and that the Cp/VLP group was best in inducing strong antigen specific responses in this important mucosal tissue compartment.

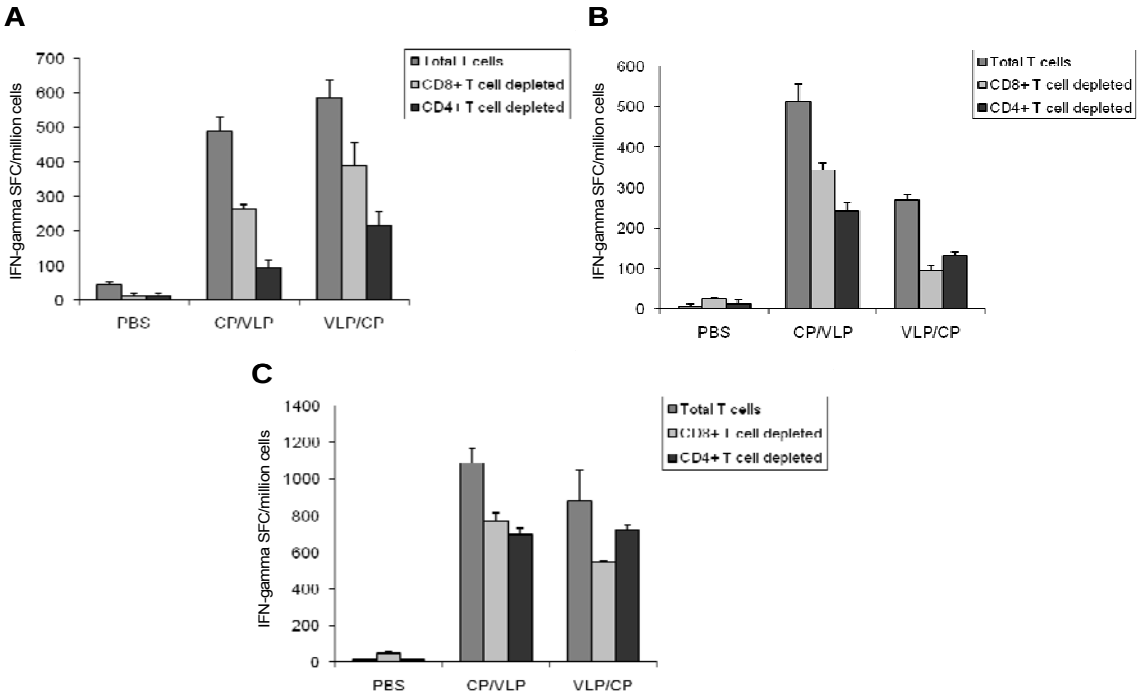

Figure 21. HIV-1 specific IFN- γ secretion by T cell subsets separated using negative selection by magnetic beads from spleen (A), Peyer's patches (B), and Lamina propria (C) of mice immunized with various prime-boost regimens.

5.6 DISCUSSION

Since HIV-1 is largely transmitted via mucosal surfaces and immune cells in the GI tract are primary viral targets during early infection that remains a reservoir during the period of chronic infection [18, 37, 38, 157], induction of mucosal immunity is important for effective prevention of HIV-1 infection and propagation. Stopping infection at the mucosa may ablate infection or lower the severity of resulting infection; however, once the virus has established the infection, it cannot be eliminated from the host [157]. Therefore a vaccine which can prevent virus transmission at mucosal surfaces by inducing strong mucosal HIV-1 specific $CD8^+$ and $CD4^+$ T cells at the port of entry would be an effective HIV-1 vaccine candidate.

C. perfringens has been developed as a vaccine vector against HIV-1 and SIV. It has been shown previously that these recombinant bacteria can deliver intact target protein to the GI tract immune cells. VLPs also present an attractive mucosal vaccine vector and have been shown to induce potent mucosal immune responses. Therefore in this study, a combination of *Cp*-Gag and VLPs was used in a heterologous prime boost immunization strategy. Mucosal vaccination typically requires the use of adjuvant to overcome tolerance and induce an appropriate immune response. Therefore the appropriate mucosal adjuvants and their optimal dose for use with *Cp*-Gag and VLPs were first determined.

Adjuvants have been shown be important for induction of immune responses after mucosal immunizations specially those using the oral route [149]. This is due to the phenomenon of oral tolerance or unresponsiveness of the GI tract immune system against dietary and commensal bacteria, which is important to maintain tissue integrity by preventing harmful inflammatory responses in the intestine. Therefore the use of adjuvants is necessary to induce strong GI tract immune responses as it provides additional signals that induce an inflammatory environment which is required for generation of mucosal immune responses. The two adjuvants used in this study – mLT and CpG ODNs, have been widely used in oral vaccines. The *E. coli* mutated heat labile enterotoxin - mLT or LT (R192G) is a mutant form of LT which has been shown to retain immunogenicity and adjuvanticity with greatly reduced toxicity in animal models, and clinical trials [164, 207, 208]. CpG ODNs are synthetic oligodeoxynucleotides (ODN) that contain unmethylated CpG motifs which mimic the immunostimulatory activity of bacterial DNA [209]. In this study, an additive effect on the immune response was observed when the combination of mLT and CpG ODNs were used as adjuvants and this might be due to the fact that they bind to distinct receptors and induce separate signaling mechanisms.

In the current study, immunization with *Cp*-Gag exclusively stimulated GI tract mucosal cellular immunity, but induced low level of systemic cellular immunity. However, oral priming using *Cp*-Gag followed by an intranasal VLP boost enhanced mucosal immunity and improved systemic cellular immune responses. These results suggest that even when immunity induced by an oral vaccine is at undetectable levels, immunity exists that can be boosted with an unrelated vector. The striking part of this study was the induction of the strongest cellular immune responses against HIV proteins within the LP compartment of the gastrointestinal tract amongst all other GI tissue compartments in the immunized mice.

Higher percentages of vaccine-induced antigen-specific cells that express both IFN-γ and TNF-α have been observed in gut-associated tissue of monkeys who resisted infection as compared to those vaccinated animals who became infected [203]. The $CD8^+$ T cells and the $CD4^+$ T cells that secrete 3+ cytokines, i.e. TNF-α, IL-2 and IFN-γ, have been associated with control of HIV infection. [101, 203]. Therefore, in this study multifunctional mucosal immune responses were also evaluated in the GI tract after the heterologous prime-boost immunizations. This study investigated whether the priming with an oral *Cp*-Gag vaccine followed by boosting with intranasal VLP inoculation could improve mucosal responses in the GI tract. The results presented in this report showed that mice that were first primed with *Cp*-Gag and then boosted with VLP induced stronger systemic and mucosal cellular responses to HIV Gag as compared to control groups in which mice were immunized with either *Cp*-Gag or VLP alone. In addition, multifunctional $CD8^+$ T cells secreting all four cytokines seemed to be higher in mice primed with *Cp*-Gag and boosted with VLP than mice that were vaccinated with only one vector. These results were inherently problematic due to relatively high background levels of staining. In addition, the absence of a live/dead cell marker in the flow staining limits the reliability of the

information obtained from positive intracellular cytokine staining of cells since dead cells are known to give false positive signals due to their inherent auto fluorescence and ability to non specifically bind to antibody conjugates. Therefore, although these results show a trend towards inducing multifunctional T cells by prime-boost heterologous immunization, because of the limitation of the multiparametric flow staining assays they have to be accepted with these caveats.

On the other hand, the characterization of the contribution of $CD4^+$ and $CD8^+$ T cells to the HIV-1 Gag cellular responses observed in the immunized mice provided greater insight into the exact nature of the immune responses elicited by the heterologous prime boost approach using *Cp*-Gag and VLPs. The presence of both $CD8^+$ and $CD4^+$ T cell responses against lentiviruses in the systemic and mucosal compartments has been associated with protection from HIV-1 and SIV infections [98, 105, 210]. The presence of abundant virus specific $CD4^+$ T cells in the GI tract that act as helper cells for virus specific cytotoxic $CD8^+$ T cells can help eliminate the virus infected cells in the GI tract early on during infection. It has been shown that adaptive $CD4^+$ T cells are critical for the generation of effective anti viral $CD8^+$ T cell responses and that long-term protection from disease will be afforded only by T cell vaccines for HIV-1 that provide a balanced induction of $CD4^+$ and $CD8^+$ T cell responses and protect against early depletion of $CD4^+$ T cells post infection [211]. It has also been shown that the presence of both HIV-1 specific $CD4^+$ and $CD8^+$ T cells at the site of mucosal infection is sufficient to control viral replication in the SIV-macaque model [212, 213]. Therefore mucosal HIV-1 vaccines that elicit strong and robust anti viral $CD4^+$ and $CD8^+$ T cells at mucosal sites like the genital and GI tracts that are early targets of HIV-1 infection will provide the best opportunity for protection against mucosal HIV-1 infections.

In this study, it was observed that the antigen specific cellular immune responses that were generated by the heterologous prime boost approach using *Cp*-Gag and VLPs were mediated by not just $CD8^+$ T cells alone, but by both $CD4^+$ and $CD8^+$ T cells. Both T cell subsets contributed generally equally to the overall HIV Gag responses and moreover these responses were highest in the mucosal compartments of the GI tract. Therefore the immunization strategy described here that leads to the development of potent mucosal cellular immune responses might lead to protection against HIV-1 infection at mucosal sites.

6.0 OVERALL SUMMARY OF THE RESULTS

Clostridium perfringens (Cp) has been considered as a vehicle for antigen delivery to the GALT. Recombinant clostridia carrying HIV-1 Gag genes were constructed to express large amount of these HIV-1 Gag protein. After preparation of the vaccine constructs we performed *in vitro* and *in vivo* experiments to evaluate antigen specific cellular and systemic immune responses to recombinant clostridia expressed HIV-1 Gag protein (*Cp*-Gag). Bone marrow derived dendritic cells were observed to mature and stimulate HIV-1 Gag specific T cell responses following exposure to *Cp*-Gag. Since mucosa is an important portal for HIV-1 entry and infection to the human body, *in vivo* experiments to evaluate mucosal immune response induced by *Cp*-Gag and HIV-1 VLPs were performed. Since strong cellular immune responses at mucosal compartments are important in control of HIV-1 infection, my dissertation study was focused on eliciting anti HIV-1 mucosal immune response in order to control HIV-1 infection.

First, immune responses in mice after oral administration of *Cp*-Gag alone were evaluated. Antigen specific IFN-γ ELISPOT revealed very low or almost undetected level of humoral and mucosal T cell immune responses was detected. The vaccination approach was then improved by combining HIV-1 VLPs with *Cp*-Gag vaccine in a prime boost approach. Slightly higher antigen specific immune responses were detected in the orally administered *Cp*-Gag followed by intranasal inoculation of VLPs in a prime boost strategy. The T cell immune response was further enhanced in systemic and mucosal compartments, such as spleen, PP, LP,

and mesenteric lymph nodes by the co-administration of optimized mucosal adjuvants mLT and CpG-ODN along with Cp-Gag and VLP. However, the prime-boost strategy generated strongest cellular immunity in the effector LP tissue in GI tract. Both $CD4^+$ and $CD8^+$ T cell subsets contributed to the overall HIV-1 specific cellular immune responses in spleen and GI tissues, such as PP and LP. Altogether, this dissertation work demonstrates that priming with orally delivered *Cp*-Gag followed by intranasal inoculation of VLP stimulates highest cellular immune response in the mucosal compartments of the GI tract such as PP and LP when compared to the systemic compartment such as spleen. Therefore the immunization strategy with heterologous vectors using oral priming with recombinant *C. perfringens* expressing HIV-1 Gag followed by intranasal VLP boosting leads to the development of potent mucosal cellular immune responses.

7.0 FINAL DISCUSSION

HIV-1 transmission occurs mainly through sexual exposure of mucosal surfaces of the genital tract and rectum to virus and virus-infected cells. Recent studies have shown that mucosal gut associated lymphoid tissue (GALT) is the major target of HIV-1 infection and replication [18, 37, 38, 47, 157]. These findings imply that the induction of strong virus-specific immunity at mucosal sites may be crucial for an effective anti-HIV-1 vaccine. An ideal vaccine must generate both humoral and cellular responses in order to control HIV-1 infection. However, the challenge remains to induce neutralizing antibodies, now major efforts have been made to develop vaccines that can maximize cell-mediated mucosal immunity, especially in the intestine and genital tract. Studies on *rhesus monkeys* have demonstrated that the presence of both anti viral $CD4^+$ and $CD8^+$ T cells at the site of mucosal infection is sufficient to control SIV replication at mucosa.[[212, 213]. Therefore a vaccine which induces strong HIV-1 specific $CD8^+$ and $CD4^+$ T cells at mucosal sites like the genital and GI tract should be able to prevent HIV-1 infection and transmission at mucosal surfaces.

One of the challenges of HIV vaccine design is the complexity of inducing immune responses in the GI tract. It is often difficult to deliver sufficient amounts of vaccine antigen to the relevant GALT inductive tissues, namely PPs or LP. Due to the importance of GI tract mucosal immunity against HIV-1 and SIV [38], a strategy to improve the mucosal immunity at GI tract is desirable. Both oral and rectal inoculation strategies are under investigation for the

purpose of delivering HIV/SIV vaccine antigen to immune inductive tissues of the GI tract [115]. Studies have shown that oral immunization strategies have induced immune responses in the GI tract, whereas nasal immunization strategies have been reported to induce robust immune responses in the respiratory and genitourinary tracts [136-138].

Several studies using invasive bacteria such as *Lactococcus lactis*, *Salmonella enterica* serovar *Typhimurium* and *Listeria monocytogenes* as oral vaccine delivery vectors have been performed [214-220]. *Shigella flexneri* has also been used for intranasal inoculation [221-223]. These studies generated mostly systemic cellular immune response in the spleen, even after oral or nasal deliver. Therefore, in this study a bacterial based vector which can induce strong cellular immune responses in mucosal compartments was explored. A non-cytotoxic, non invasive *C. perfringens* based mucosal vaccine against HIV-1 and SIV was developed. The unique characteristics of *C. perfringens* make it an attractive mucosal vaccine model against HIV-1 and SIV. First advantage is the presence of its inducible and strong cytopathic enterotoxin (*cpe)* promoter. The harsh conditions of the GI tract induce the *cpe* promoter and lead to production of large amounts of *cpe* protein. The strength of the *cpe* promoter is demonstrated by the fact that *cpe* enterotoxin production in the human intestine accounts for up to 15% of total bacterial protein, which accumulates in cytoplasmic inclusion bodies inside the mother cell. Therefore, placing an antigen of interest, like HIV-1 Gag under the *cpe* promoter will enable it to produce large amount of protein in the GI tract. Since the HIV-1 Gag antigen is present in inclusion bodies of recombinant *C. perfringens* expressing HIV-1 Gag (*Cp*-Gag), the HIV-1 Gag protein is protected from stomach acid and degrading enzymes of the small intestine, while the bacterial vector travels through the upper GI tract. This would deliver high amounts of intact viral antigen to inductive immune tissues in the gut. The goal of this study was to deliver HIV-1 Gag antigen

via *C. perfringens* to GI tract mucosal tissues and induce strong immune response against the antigen. To achieve this goal a recombinant *C. perfringens* based vaccine vector was constructed that produced high level of HIV-1 Gag protein under sporulating conditions.

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 the 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 the control of the *cpe* promoter in the *C. perfringens* vector. The incorporation of an envelope protein for expression by *C. perfringens* was considered, since the envelope has been shown to carry antigenic determinants that elicit strong cellular and humoral immune responses in acutely and chronically HIV-1 infected humans, and in HIV-1 vaccinated humans. However, it is realized that envelope protein synthesized from *cpe* promoter will not be glycosylated, 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 envelope gp41 molecule do not require glycosylation-controlled structural constraints for production of neutralizing antibodies and are also quite conserved among all HIV-1 types, thus making gp41 an attractive HIV-1 protein to incorporate into recombinant *C. perfringens*. However, repeated attempts to obtain HIV-1 envelop gene expressing recombinant clostridia were not successful. The reason for lack of envelope protein expression from clostridia was not very well understood. It's possible that sequences present in HIV-1 envelope may be toxic for bacteria or have bactericidal effect. Several laboratories have observed that the glycoprotein components of HIV-1 and other retroviruses have variety of toxic effects on

bacterial cultures [224, 225]. It has been also shown that amino terminal portion of HIV-1 envelope transmembrane (TM) domain contains a highly conserved sequence [226] that is structurally similar to an amino terminal sequence present on the fusion protein of myxoviruses, which has been shown to have high cytopathic effect. Molecular modeling studies of protein sequences identified two positively charged, highly amphipathic amino acid segments in the cytoplasmic tail of the transmembrane (TM) envelope protein of HIV-1 which has a very high antibacterial effect [227]. Therefore HIV-1 envelope TM domains may play an important role in cytopathicity. In other studies it has been shown that some virally derived peptides have antimicrobial properties [228]. These virally derived peptides are designated as lentivirus lytic peptides (LLPs). Like cationic peptides, LLP1 is a highly effective against many antibiotic-resistant microbial pathogens. Moreover, LLP1 readily kills *S. marcescens*, an important organism of ophthalmologic concern which is generally resistant to cationic peptides. Therefore the lack of expression of HIV-1 envelope by recombinant *C. perfringens* could be due to the cytotoxic properties of viral peptides and TM domains of viral envelope proteins like gp140.

The bacteria-based vaccines are thought to interact with DCs and, in the case of *L. monocytogenes*, can stimulate cytotoxicity against antigen-specific target cells *in vitro* [229]. The results of the current study demonstrate that *in vitro* exposure of GI tract mucosal BMDCs to the *Cp*-Gag vaccine leads to effective stimulation of HIV Gag specific cells, indicating that this interaction may support immune response induction in the GI tract.

Another challenge in the development of orally delivered vaccines is oral tolerance. While oral tolerance is an important phenomenon to protect unnecessary inflammatory responses, it presents an obstacle in the development of oral vaccines. There are some successful oral vaccines, but most of them are live attenuated forms of the pathogenic strains. These live

attenuated vaccines administered by the oral route are associated with effective development of systemic and mucosal immune responses, superior protection against pathogen infection, and ease of administration. The use of attenuated strains of pathogens can overcome the tolerogenic response in the GI tract since they mimic natural infection without causing pathogenesis and thus lead to protective immune responses. Although this is an attractive approach, a major drawback of such an approach is the possibility of reversion of the attenuated strain to the wild type pathogenic strain. Therefore alternative ways to avoid oral tolerance and induce strong mucosal immune response need to be explored. Mucosal vaccination typically requires the use of adjuvant to overcome tolerance and drive the induction of an appropriate immune response. The co administration of mucosal adjuvants with recombinant bacterial vaccine vectors is expected to alter the type of immunity induced by oral vaccination. Therefore in the current study strong mucosal adjuvants were selected and their optimal doses were used along with the mucosal vaccine constructs. The combination of 1 μg mLT and 50 ug of each CpG ODNs resulted in the highest percentage of IFN-γ producing T cells when compared with the other adjuvant combinations.

Heterologous prime-boost vaccination has been shown to be an efficient way of inducing T cell responses in animals and in humans. [230]. It was shown that sequential immunization with two different replicating recombinant viral vectors could induce a strong $CD8^+$ T cell response [231]. Further studies in mice have shown that the heterologous prime-boost strategy that combines two different vectors encoding the same antigen is more efficient in inducing cell-mediated immune responses than the use of a single vector and is surprisingly immunogenic with non replicating vectors [123, 124]. Studies in nonhuman primates have revealed that the prime-boost approach induces strong cellular response and can lead to protection against diseases such

as malaria or simian-HIV in primates [61, 125, 232, 233]. In this study heterologous immunization of *Cp*-Gag and VLP through oral and nasal route has been explored. This heterologous immunization in a prime-boost mode induced immune response in both mucosal and systemic tissue compartments. The HIV Gag specific IFN-γ immune responses were highest in the mucosal compartments, PP and LP, compared to the responses seen in the spleen or mesenteric lymph nodes (MLN). It is not very clear why responses at these systemic sites were comparatively low. It is possible that the time points used for analysis may not represent time points when draining LNs contain antigen-specific IFN-γ producing cells. The lower immune responses observed in MLN could be due to the difference in trafficking of PP and LP antigen presenting cells, specifically dendritic cells. LP dendritic cells travel to the MLN to present antigen, whereas dendritic cells from PP primarily relocate from the subepithelial dome (SED) to T and B cell areas of the PP and do not frequently migrate to MLN [234-236]. Therefore, even if the PP dendritic cells were exposed to *Cp*-Gag and they efficiently acquire Gag antigen, the responses they stimulate will not be detectable in the MLN. Higher responses in the MLN and systemic tissues have been observed following oral vaccination with other bacterial-based vectors, and this may be due to the fact that these other vectors deliver protein to cells in the LP through direct infection and subsequent active replication [215, 220, 237, 238]. For example, a *Salmonella*-based vaccine delivers vaccine antigen by entering macrophages in the LP [215, 237].

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 [98, 239]. Therefore, in the current study, multifunctional mucosal cellular immune responses in the GI tract after immunization with the mucosal vaccine constructs were evaluated. Despite low systemic

humoral and cellular responses with this vaccine strategy, strong cellular responses in the inductive (PPs) and effector (LP) sites of the GI tract were observed. Additionally, this study investigated whether the mucosal immune responses that were primed with oral *Cp*-Gag vaccine could be boosted with a subsequent intranasal VLP inoculation. The results obtained indicate that mice that were first primed with *Cp*-Gag and then boosted with VLP induced stronger systemic and mucosal cellular responses to HIV-1 Gag compared with the groups in which mice were immunized with either *Cp*-Gag or VLP alone.

In this study, immunization with *Cp*-Gag and VLP alone could induce very low levels of HIV-1 Gag specific IgA and IgG antibodies in the mucosal and serum samples of immunized mice, respectively. However, IgG1 antibody levels in serum of mice primed with *Cp*-Gag and boosted with VLP were higher than those from mice in control groups, although these were not significant. It is possible to induce a humoral response creating antibodies through the use of adjuvants that promote Th2-type immune responses. The adjuvants utilized in the majority of the current study, CpG ODNs and mLT, are known to promote Th1-type immunity rather than Th2-type responses. The use of cholera toxin (CT) has been recognized as promoting Th2-type immunity, particularly when the CT or CTB subunit is conjugated directly to the antigen being delivered. 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.

A number of studies using viral vectors such as adenovirus, vaccinia virus, papilloma virus, and adeno-associated virus have been reported in which cellular immune responses were observed in mucosal compartments [240, 241]. However, these studies were mostly focused on measuring cellular responses in PP, which is the inductive site of the GI tract. There were only

few studies where HIV-1 specific cellular immune responses were measured in the LP, which is an effector site of the GI tract and is important in early HIV infection. In one such study, an intrarectally delivered modified vaccinia Ankara based vaccine provided high level of antigen-specific IFN-γ secreting cells (800-900 sfc/10^6 cells) in the LP [242]. In the current study, oral immunization of *Cp*-Gag followed by an intranasal boost with VLP produced even stronger antigen-specific IFN-γ secreting cells (3000-4000 sfc/10^6 cells) in the LP. Presence of this strong HIV specific immune response in LP could be very important in controlling viral replication during early HIV-1 infection in the gut.

The results of the current study demonstrate that *in vivo* oral inoculation with recombinant *C. perfringens* primes GI tract cellular immune responses. These findings suggest that recombinant *C. perfringens* based vectors should be considered as a mucosal HIV-1 vaccine vector. Oral administration of vaccines is often preferable to and more cost effective than inoculation strategies requiring sterile needles, so including recombinant *C. perfringens* in a vaccination regimen involving boosting with VLPs would have the added benefit of non needle inoculations. Given the importance of the GI tract in HIV-1 infection, it is vital to pursue development of vaccine vectors such as recombinant *C. perfringens* that may induce effective anti-viral immunity in the gut.

7.1 PUBLIC HEALTH SIGNIFICANCE

Currently there are no vaccines available against HIV-1, while HIV-1 infection has become a global scourge. Therefore the development of a low cost effective HIV-1 vaccine is of immediate need. In this proposal, I have investigated the use of non-invasive bacterial-based protein delivery system directed at the GI tract using recombinant *C. perfringens*. I have explored different mucosal immunization approaches using recombinant *C. perfringens* expressing HIV-1 proteins and HIV-1 VLPs to generate robust mucosal immune responses against HIV-1. The proposed vaccine constructs hold great promise for developing a practical vaccine against HIV-1 due to its safety, low production cost and easy administration.

8.0 FUTURE DIRECTIONS

The current study provides a novel strategy to develop a heterologous vaccine against HIV-1 using recombinant *C. perfringens* expressing HIV-1 Gag protein (*Cp*-Gag) and HIV-1 virus like particles (VLPs) in order to induce strong HIV-1 Gag specific cellular immune responses in mucosal tissues. However through the course of these studies many pertinent questions have arisen that could not be addressed due to constraints of time. In this section I will briefly mention some of the key areas that could be further studied.

Despite the induction of strong cellular immune responses at mucosal tissue compartments such as Peyer's patches (PP) and Lamina propria (LP), weak cellular responses were detected in spleen or mesenteric lymph nodes (MLN). It is possible that the time points used to analyze the HIV-1 Gag specific cellular immune responses in PP and LP may not be optimal for spleen and MLN. Therefore a study could be performed to assay cellular immune responses in the spleen and MLN at different time points after immunization.

Although strong cellular immune responses were generated at mucosal sites, there were very minimal or no humoral responses at systemic and mucosal tissues compartments. Therefore, a study should be performed to elicit humoral immune responses using the current heterologous approach. One of the approaches could be the use of adjuvants which are known to induce Th2 type immune responses that are known to be important for induction of humoral immune responses. The adjuvants used in this study - CpG ODNs and mLT are known to promote Th1

type rather than Th2 type responses. The use of CT has been recognized as promoting Th2-type immunity, therefore it can be included as another adjuvant to enhance humoral responses induced by the current heterologous vaccine approach.

Although the multiparameter flow cytometry analysis showed trends towards the generation of polyfunctional CD8 T cells in animals immunized with *Cp*-Gag and VLPs, it needs to be re-examined with better gating strategy to evaluate induction of polyfunctional CD8 T cells in response to vaccination with *Cp*-Gag and VLPs.

Recently the SIV-p27 gene was inserted into the chromosome of *C. perfringens* where the viral gene is expressed under the control of cpe gene promoter and thereby negating the use of antibiotic resistant plasmids carrying the gene of interest under the *cpe* promoter. The safety of this vector has been further rendered by inactivating two more toxin gens, alpha and theta. Therefore this *C. perfringens* vector would be safer than the *Cp*-Gag used in the current study.

The incorporation of various viral proteins into a vaccine could be beneficial since the production of a broader immune response against a variety of viral proteins is thought to aid in protection from disease progression. Therefore other HIV-1 genes like *env, pol,* and *nef* could be placed under control of the *cpe* promoter in recombinant *C. perfringens* and these recombinant clostridia could be tested for induction of HIV-1 specific immune responses against these proteins. It is possible that the mixture of recombinant *C. perfringens* expressing multiple HIV-1 proteins would be able to induce broad and strong mucosal and systemic immune responses against HIV-1, which might be important for protection against HIV-1 infection.

A challenge experiment will help in assessing whether mucosal T cell immune responses against HIV-1 Gag in the GI tract are protective against a mucosal HIV-1 infection. This can be done in initially in mice with intra-vaginal challenge with recombinant vaccinia virus expressing

HIV-1 Gag (rVV-Gag). Studies are going on in our laboratory to establish vaccinia challenge model in mice and measure efficacy of protection conferred by vaccination with *Cp*-Gag and VLPs in a prime boost mode. If the *Cp*-Gag and VLPs in a prime boost mode is found to be protective in mice, this could be extended to monkey model. Similar immunization studies could be performed using recombinant *C. perfringens* expressing various SIV genes in the SIV-macaque model to induce strong mucosal and systemic immune responses against SIV and to determine the ability of these responses to protect against a mucosal challenge with a pathogenic SIV, which is the closest animal model of HIV/AIDS. Furthermore, if it is demonstrated that these vectors can induce protective immune responses in the SIV-macaque model, then clinical trials can be planned to assess the safety and immunogenecity of recombinant *C. perfringens* based vectors in humans.

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