Cervicovaginal Inflammatory Cytokine and Chemokine Responses to Two Different SIV Vaccines in Female Mauritian Cynomolgus Macaques

By

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ABSTRACT

Human immunodeficiency virus (HIV) -1 infects CD4+ T lymphocytes and activated CD4+ T cells are preferentially targeted. This posed great challenges in developing an effective prophylactic HIV-1 vaccine because candidate HIV vaccine are likely to activate immune system and generate more target cells. Studies have shown that vaccine vectors and route of immunization can differentially activate immune system. The pro-inflammatory and chemotactic cytokines produced by the activated immune cells can propel the cycle of immune activation, target cell recruitment, and enhance infection. However, the effects of vaccine immunogen on immune activation and mucosal inflammation have not been studied. This study aims to evaluate the effect of vaccine immunogen on cervico-vaginal inflammatory cytokine and chemokine levels in Mauritian cynomolgus macaques. Using a customized 14-plex cytokine/chemokine panel, I evaluated the cervico-vaginal cytokine and chemokines during immunization and boosts of two vaccines delivering different immunogens, the PCS vaccine and the Gag/Env vaccine. The PCS vaccine delivers 12 20-amino acid peptides overlapping the 12 simian immunodeficiency virus (SIV) protease cleavage sites (PCS) and the Gag/Env vaccine delivers full Gag and Env proteins. The results show that the PCS vaccine immunization and boosts induced lower level increase of a few pro-inflammatory and chemotactic cytokines, and the effect is short-lived. In contrast, the Gag/Env vaccine induced a persistent increase of multiple cytokines and chemokines with higher magnitude. Thus, it is important to consider the effect of vaccine immunogen on mucosal inflammation when developing and evaluating candidate HIV vaccines.

DEDICATION

As always, to God, and to my family, Dennis, Virginia, Shiela, and David.

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1. INTRODUCTION

*1.1.*Human immunodeficiency virus

1.1.1. Global Burden

Over three decades after it was first discovered, the human immunodeficiency virus (HIV) type-1 pandemic remains a global public health challenge [1]. By the end of 2017, the World Health Organization (WHO) reported an estimated 36.9 million people globally were living with HIV while 35.4 million had died from AIDS-related illnesses since the beginning of the epidemic [2]. In 2017 alone, 1.8 million new infections were recorded, 59% of which occurred in women from Sub-Saharan Africa [2]. Alarmingly, young women in sub-Saharan Africa continue to be disproportionately infected with HIV-1 [3]. Currently, young Sub-Saharan women are twice more likely than men, to be infected with HIV-1 mostly due to their cultural, social and economic vulnerabilities [4]. The advent of antiretroviral therapy (ART), however, has provided a silver lining. For the first time, the number of AIDS-related deaths dropped below one million (940 000) and by the end of 2017, 2.3 million more people living with HIV started accessing ART [2].

In spite of the improvements in access to treatments and preventive measures, global ambitions of a 90-90-90 treatment target by 2020 (90% of people living with HIV will know their status, 90% of whom will receive treatment, and 90% will have viral suppression) [5], seems to be far from realization. Currently, it is estimated that only 75% of people living with HIV know their status, only 79% of whom were receiving ART, and only 81% were virally suppressed [2].

It is evident that while treatment is critical in improving the longevity and quality of life for infected individuals, it is highly unlikely to eliminate the pandemic by itself. More aggressive

forms of prevention are needed to augment the success of ART. Further efforts in research and better knowledge translation are necessary to fuel more innovative developments in prevention.

1.1.2. Brief history and origin of HIV

In 1981, the first cases of HIV infection were reported when a group of young homosexual men, who presented with an opportunistic type of pneumonia infection – *Pneumocystis jejunii* (formerly *P. cranii*) and a rare form of cancer called Kaposi's sarcoma in New York and San Francisco, USA [6, 7]. By the following year, the Center for Disease Control and Prevention (CDC) coined the term AIDS – acquired immunodeficiency syndrome – to describe the new disease that typically infected intravenous drug users and men who had sex with men (MSM) [8]. The causative agent of AIDS was later determined to be a retrovirus, now known as Human Immunodeficiency Virus (HIV) [9-12]

HIV has two major strains, the more common and pathogenic strain HIV-1, and HIV-2 found primarily in West Africa [9-11, 13]. The introduction of HIV-1 and HIV-2 infection into the human population likely occurred through zoonotic transmission of closely related simian immunodeficiency viruses from chimpanzees (SIV_{cpz}) and sooty mangabeys (SIV_{SM}), respectively [14, 15]. Cross-species infection likely arose from exposure of human mucous membranes to SIV-infected body fluids and raw meat during bush meat hunting [16]. HIV-1 is further classified into 4 groups, M, N, O, which originated from independent zoonotic transmission events from chimpanzees, and P, which originated from gorillas [17-21]. Although both strains follow the same pathogenesis, the worldwide pandemic is mainly attributed to HIV-1 Group M while HIV-1 Groups N, O, P are primarily confined in West Africa [22-24]. In contrast with HIV-1, the HIV-2 strain is less infectious and results in slower disease progression [25-27].

1.2. HIV Virology

1.2.1. HIV Structure and Genome

HIV is a member of the *Retrovirodae* family, *Orthoretrovirodae* subfamily and belongs to the genus *Lentivirus*. It is an enveloped virus that is roughly spherical in appearance with an approximate diameter of 120nm [28]. The HIV viral envelope is formed by a lipid bilayer derived from the host cell membrane during virus budding. HIV envelope glycoprotein trimers are distributed along the surface lipid bilayer and are made up of a glycoprotein cap, gp120 and a stalk, gp41. These trimers are the primary structures for attachment and fusion of virus envelope to the target host cell membrane. Meanwhile, the inner surface of the bilayer is lined with matrix proteins p17. The cone-shaped core consists of capsid protein p24 that encloses two positive sense RNA strands. Also found inside the virus core are three viral enzymes, the integrase (IN), protease (PR), and reverse transcriptase (RT) and five accessory proteins: viral infectivity factor (Vif), viral protein R (Vpr), viral protein unique (Vpu), negative regulatory factor (Nef) and transactivator of transcription (Tat) [29].

The two single stranded RNA (ssRNA) strands that make up the HIV genome are about 9-10 kb long and contains three genes *gag, pol, env* encoding major structural proteins and six genes *vif, vpr, vpu, nef, rev* and *tat* encoding regulatory and accessory proteins [29, 30]. The Gag precursor protein encoded from the *gag* gene is cleaved by PR into matrix (p17; MA), capsid (p24), nucleocapsid proteins (p6 and p7), while the polymerase (*Pol)* precursor protein is cleaved into the three viral enzymes PR, IN, and RT. Meanwhile, a polyprotein gp160, is encoded from the *env* gene and subsequently cleaved to surface glycoprotein, gp120 and transmembrane glycoprotein, gp41. Several viral regulatory and accessory proteins are also encoded by the HIV genome namely: Tat, Rev, Nef, Vif, Vpr, and Vpu [31, 32].

1.2.2. HIV Life cycle

The viral life cycle of HIV-1 can be summarized into 7 major stages and these are: i) binding and entry ii) fusion, iii) reverse transcription (RT), iv) nuclear entry and integration, v) replication, vi) protein translation/assembly, and vii) budding.

The initial step in the HIV-1 life cycle is binding and entry into the target cell. Viral gp120 subunit binds to CD4 receptors on the surface of CD4+ T lymphocytes or macrophages resulting in a conformational change that exposes co-receptor binding domain of gp120 [33]. This conformational change allows for interaction between target cell CCR5 or CXCR4 chemokine co-receptors (for R5- or X4- tropic strains) and the gp120-CD4 complex [33]. This stable co-receptor binding enables the N-terminal end of the Env gp41 fusogenic subunit to penetrate the target cell membrane leading to a conformational change in gp41 that brings the viral envelope and target cell membrane in closer proximity [34]. The second stage of HIV life cycle is the fusion of the viral envelope and host cell membrane that results from the insertion of gp41. The viral core is then delivered and subsequently uncoated into host cell cytoplasm to release the viral RNA [35].

Following fusion and uncoating of the viral core and once inside the cytoplasm, the HIV RT converts the viral RNA into double stranded DNA through a process called reverse transcription and forms the reverse transcription complex (RTC)[34]. Afterwards, the RTC with the proviral DNA combines to forms the pre-integration complex (PIC) that facilitates transportation of viral cDNA into the host nucleus where the DNA is integrated into the host cellular genome by viral integrase [34, 36].

Once integrated, the proviral cDNA can either undergo active transcription or enter latency [37, 38]. Active transcription of the HIV provirus is initiated and accelerated by viral Tat but makes

use of host transcription factors such as NF-kB for transcription to viral mRNA [39]. The newly transcribed viral mRNA transcripts are then exported out of the nucleus and subsequently translated to polyproteins by host cell ribosomes in the cytoplasm. Viral protein Rev facilitates the transport of viral mRNA from the nucleus to the cytoplasm [40]. p17 (MA) then initiates viral assembly on the inner surface of host cell membrane and is mediated by the Gag polyprotein and Gag-Pol-Pro polyprotein [41]. The virus core is assembled from gag-pol protein interaction with the plasma membrane, Env deposition on the membrane and packaging of Vif, Vpr and Nef, and the genomic viral RNA into immature spherical virions [28, 42]. Upon budding or the release of immature virions from host cells, the polyprotein Gag-Pol undergoes further cleavage by viral protease (PR) to produce structural proteins MA, CA and NC and viral enzymes IN, RT and PR [43]. This mature HIV is now fully capable of starting another replication cycle in a new target cell.

1.3. T cells in HIV-1 infection

1.3.1. CD4+ T cells

CD4+ T cells play a major role in the generation of host immune responses particularly in the adaptive arm of immunity. First, it serves to bridge the innate and adaptive immune responses through recognition and binding of pathogen-derived antigens or vaccine immunogens presented by the major histocompatibility complex (MHC) II of antigen-presenting cells (APCs) [44]. Innate signaling processes such as recognition of PAMPs through PRRs, pro-inflammatory cytokines and chemokines secretion, and the expression of co-stimulatory molecules like CD40, CD80, and CD86 on APCs can all serve as necessary secondary signals in the activation of naive CD4+ T

cells [45]. Following binding of cognate antigen and co-stimulatory signals from innate immune cells, naive CD4+ T cells then differentiate into T helper cells that can mediate the humoral and cell mediated arms of host adaptive immunity [44]. In the presence of macrophage-secreted IL-12 , naive CD4+ T cells differentiate into T helper (Th) 1 cells that secrete IL-2, IL-12, TNFβ, and IFNγ and enhance cellular responses against intracellular pathogens [46-48]. On the other hand, IL-4 secreted by basophils and mast cells can induce CD4+ T cells to differentiate into Th2 cell types that produce IL-4, IL-5, IL-6, IL-10, and IL-13 which are known co-stimulants of B cell differentiation into antibody-producing plasma cells [47, 49, 50]. Since both Th1 and Th2 cells produce IL-12 and IL-4 respectively, these cells also contribute to the cycle of T cell proliferation and differentiation [48]. CD4+ T cells are also known to produce other cytokines and chemokines after antigen stimulation, such as IL-1β, IL-17, MCP-1, MIP-1α/β, RANTES, IL-8, MIG, IP-10 CXCL11, CCL22, GM-CSF and TNFα, among others [51] that can increase vasodilation, vascular permeability and further increase immune cell migration [52, 53]. During HIV-1 infection, the perpetuation of this cycle of activation and localization of immune cell targets is also facilitated by the virus itself [54].

1.3.2. CD4+ T cell as HIV-1 target

The decline and eventual depletion of CD4+ T lymphocytes is a defining characteristic of HIV infection. HIV-1 preferentially infects and replicates in activated CD4+ T cells [55]. The gp120 unit of HIV-1 Env primarily binds to cluster-of-differentiation (CD) 4 markers on CD4+ T cells, subsequently allowing co-receptor binding to CCR5 or CXCR4 chemokine co-receptors on host cells [56]. Increased HIV-1 cellular entry has been observed in plasma and endocervical CD4+ T cells with increased expression of metabolic and activation markers, Ki67 and CD69, indicating virus preference for actively cycling cells [57]. Further, in HIV infected individuals, more HIV

viral DNA are typically found in HIV-specific memory CD4+ T cells throughout disease progression [58]. An activated cellular state seems to be favored due to increased accessibility to elevated amounts of host nucleotides and transcription factors, creating a more conducive environment for proviral DNA synthesis and transcription [59, 60].

Unlike in the non-lytic infections of macrophages [61], HIV-1 infection leads to the destruction of CD4+ T cells. In addition to the release of viral progeny at the plasma membrane and the cytopathic effects of HIV-1 infection, other mechanisms can lead to the destruction and depletion of CD4+ T cells. HIV-1 infected cells expressing surface Env can also induce syncytia formation and bystander apoptosis in uninfected neighboring CD4+ T cells through gp41-hemifusion and autophagy [62, 63]. Depletion of CD4+ T cells, specifically T helper cells and HIV-1 specific memory CD4+ T cells, predominantly takes place in the lamina propria of the gastrointestinal tract, severely affecting host defenses in the gut [64, 65]. The weakened immunity due to CD4+ T cell depletion leads to microbial translocation and elevated plasma levels of microbial products, and which contributes to the persistent systemic immune activation [66].

Unlike other pathogens, HIV-1 clearly usurps the host immune responses to its advantage. This continuous cycle of immune cell activation, infection and depletion contributes to immune dysfunction, ultimately leading to immunodeficiency and susceptibility to opportunistic infections [67, 68]. This can pose a great challenge for HIV-1 vaccine development since immunization also contributes to immune activation and proliferation that can further replenish the target cell population.

1.3.3. CD8+ T cells

$CD8^+$ T lymphocytes are key components of the host cellular mediated immune responses and play a pivotal role in controlling HIV-1 replication and delaying the eventual onset of disease [69]. Similar to CD4+ T cells, activation of resting CD8+ T cells also involves antigen-specific TCR (T cell receptor) and MHC-co-receptor binding, as well as cytokine signaling. During primary infection, the TCR of CD8+ T cells recognizes and binds endogenously processed antigens presented on MHC class I molecules of infected host cells and APCs [44]. Pro-inflammatory cytokines such as IL-2, IL-12, IL-21, and type I IFN produced by Th1 cells and innate immune cells like macrophages also induces the activation and differentiation of CD8+ T cells as well as the contraction of effector cell pool after infection [46, 47, 70, 71]. During an infection, activated CD8+ T cells have the capacity to respond and eliminate pathogens by differentiating into cytotoxic effector cells and long-term antigen-specific memory CD8+ T cells [72]. Short-lived Cytotoxic T lymphocytes (CTLs) eliminate infected host cells by secreting perforin and granzyme molecules leading to cell lysis or CTL mediated apoptosis [73]. In addition to anti-viral IFNγ, activated CTLs are also known to secrete chemokines such as MIP-1α, MIP-1β, and RANTES [73, 74]. Following primary infection, about 95% of CTLs go through a contraction phase and undergo rapid apoptosis, but a pool of long-term antigen-specific memory CD8+ T cells are established and rapidly proliferates upon re-exposure [69, 75].

In the case of HIV-1 infection, multiple studies have demonstrated the significance of HIV-specific CD8+ T cell responses in the control of infection and disease progression. An early in vitro study in the late 1980s, provided evidence of CD8+ T cell suppression of HIV-1 replication [76]. In an NHP/SIV model, CD8+ T cell immune responses were shown to restrict SIV replication as a result of immunization with a Cytomegalovirus (CMV)-vector based SIV vaccine [77]. Similarly, in two

separate studies on Rhesus macaques, experimental depletion of CD8+ T cells resulted in significant increase in SIV replication in both acute and chronic infections [78, 79]. It has also been demonstrated that long term non-progession in SIV-infected rhesus macaques correlated with efficient killing of infected host cells by granzyme secretion of CTLs [80].

In HIV infected individuals, the emergence CD8 T cell responses coincide with initial control of primary viremia during acute infections [81, 82]. In both long-term nonprogressors (LTNPs) and elite controllers (ECs), long-term viral load control was associated with higher frequency and polyfunctionality of CD8 T cells, including potent cytotoxic responses and secretion of IFN-γ, TNF-α, MIP-1β, and IL-2 [83]. Similar to what was observed in SIV/NHP models, elite controllers and LTNPs also exhibit increased granzyme and perforin production associated with suppression of HIV replication and apoptosis of infected cells [84-86]. Furthermore, in HIV-1 infected individuals, the control of viral replication and delayed disease progression correlated with the breadth of Gag-specific CD8+ T cell responses [87]. Taken together, these studies suggest that further understanding of cellular responses during HIV-1 infection is crucial in improving strategies for infection control and treatment.

1.4. HIV Pathogenesis

1.4.1. HIV Transmission

A thorough understanding of the dynamics of HIV-1 transmission is crucial to the development of preventive strategies against HIV-1 infection. It is known that HIV-1 infection is primarily transmitted through contact with contaminated bodily fluids either by horizontal and vertical routes

of transfer. Horizontal transmission of HIV-1 can occur via unprotected penile-vaginal/anal sexual intercourse and accounts for majority of HIV-1 infections [88]. The virus can also be transmitted horizontally by the parenteral route, such as through needle sharing among intravenous drug users (IDUs), via percutaneous injury in a hospital setting, or through inoculation with infected blood during blood transfusions [63]. On the other hand, vertical transfer of the virus can occur from mother to child while *in utero*, during child birth, or via breastfeeding.

During sexual intercourse, the mucosal lining of the female/male genital tract or the anal and rectal surface is exposed to HIV-1. Interestingly, the risk of transmission through anal intercourse is greater than through vaginal intercourse [89]. The disparity between observed risks is likely due to the different physical and immunological landscapes of these two epithelial surfaces. The rectal epithelium is rich with HIV-1 target cells and is lined with microfold M cells that have high affinity to foreign antigens [90] while the vaginal epithelium is not. In addition, disruptions in the rectal epithelium are more common than in the vaginal mucosa [91] which could allow easier access by HIV-1.

In the female genital tract (FGT), HIV-1 entry often involves interaction with innate immune cells. Scavenging Langerhans cells (LCs) in the vaginal and endocervical epithelia are able to bind HIV Env gp120 and the binding often resulted in degradation during low viral concentrations [92]. However, at high viral loads the LCs end up either presenting bound or internalized HIV-1 to T lymphocytes in the mucosa or transporting the virus to the lymph nodes which is rich in susceptible immune cell targets [93, 94]. This aids in the systemic spread of HIV-1 and the establishment of viral reservoirs in secondary lymph organs [95, 96]. There is also evidence that HIV-1 can go through epithelial cells, a process called transcytosis, to cross the genital epithelium and reach submucosal target cells [97].

1.4.2. Stages of HIV-1 infection

The progression of HIV-1 disease is conventionally divided into disease stages by monitoring blood CD4+ T cell count, viremia (viral load), antiviral antibodies, as well as the presence of overt disease symptoms [98-100]. Depletion of CD4+ T cells is the hallmark of HIV pathogenesis and is therefore monitored in order to effectively diagnose and direct treatment decisions in HIV-infected individuals [99].

The first 2 weeks of infection is called the eclipse phase when viremia is still undetectable. During this stage, there is rapid viral replication in the absence of an effective immune response at the site of infection [101]. Although this stage offers a "window of opportunity" to eradicate early infection by the founder population, the lack of characteristic symptoms makes it difficult to do so [102].

Following the eclipse phase, a sharp increase in viremia of about 10^7 copies/mL or more occurs around 2-4 weeks after infection [98, 103]. The acute phase of infection often lasts about 12 weeks and is accompanied by a depletion of CD4+ T cell count. Due to the rapid viral replication, there is higher risk of transmission during this stage [63]. At peak viremia, HIV-specific host antibody and cellular mediated immune responses begin to appear [98, 104], preceded by a cytokine "storm" [105]. During this stage, the first clinical signs of HIV infection manifests as flu-like symptoms [106, 107].

The beginning of the latency or asymptomatic phase of chronic infection is marked by a partial rebound in CD4+ T cell count. At this stage, viral replication is controlled initially by HIV-specific CTL responses and later by HIV-1 specific humoral responses, to a set point viremia of about

100,000 copies/mL [81, 100]. This chronic phase can last approximately 10 years and is characterized by gradual decline in CD4 T cell count and slow increase in viremia.

In general, when CD4+ T cell count falls to lower than 200 cells/μL of blood, disease enters the final stage of infection [108]. This stage, AIDS, is characterized by severe immunodeficiency leading to susceptibility to a diverse range of opportunistic infections [68]. Eventually, in the absence of proper interventions, death occurs due to AIDS-related complications.

1.4.3. Natural Immunity and Altered HIV-1 Pathogenesis

It has been widely documented that there is heterogeneity in susceptibility to HIV-1 infection and disease progression within a population [109-111], as seen in host responses to other infectious pathogens. There are groups of individuals who are known to remain healthy for long periods of time in the absence of treatment. Long-term non-progressors (LTNPs) are defined as HIV-1 infected individuals who, while receiving no treatment, present with low viral RNA in plasma while maintaining CD4+ T cell counts of >400 cells/mL for more than 7 years after infection [109, 110]. A subgroup of LNTPs are able to maintain remarkably low viral loads of <50copies/mL were defined as elite controllers, [111].

Several studies have also shown that not all sexual exposures to the virus lead to HIV-1 infection. There are groups of individuals who, in spite of frequent high risk exposures to HIV-1, remain uninfected [112-114]. This population considered to be HIV-1 resistance includes HIV-exposed seronegative (HESN) commercial sex workers (CSW), uninfected infants born to HIV-1 infected mothers, HIV-1 sero-discordant couples, hemophiliacs who remain uninfected after exposure during blood transfusion and at risk IDUs [115-120].

In both LTNPs and HESN populations, several genetic and immune factors have been associated to altered disease progression and natural immunity. Polymorphisms in host genetics, particularly in the HLA (Human leukocyte antigen) class I and class II loci have been shown to play significant roles in both protection against HIV-1 infection and control of HIV-1 pathogenesis [121-125]. For example, several studies have shown that HLA-B*57:01, HLA-B*58:01, HLA-B27 and HLA-B51 are enriched in LTNPs which prompted further studies on the epitopes of these alleles [126-128]. Individuals who express these specific types of HLAs are able to mount a more robust, diverse and polyfunctional HIV-specific cellular immune responses, often directed at conserved Gag epitopes that result in a more efficient control of viremia [129]. Deletions in the CCR5 co-receptor gene (CCR5Δ32) were also detected in HESNs, hence were resistant to infection by R5-tropic HIV-1 strains [130, 131].

Studies in Kenyan female sex workers (Pumwani sex worker cohort) have shown that specific HLA alleles were associated with natural resistance or susceptibility to HIV-1 infection [121-123, 132, 133]. Further studies were conducted to investigate Gag CD8+ T epitopes of two HLA class I alleles associated with HIV resistant women or with susceptibility to HIV-1 infection [134]. The protective allele, A*01:01 only recognizes 3 Gag epitopes, whereas the allele associated with susceptibility, B*07:02, can recognize 30 epitopes [134]. These results propounded the idea that a broad immune response might not necessarily be beneficial for protection and instead may promote HIV-1 infection [122, 123, 133-135].

In addition to strong HIV-specific cellular responses, higher levels of immune quiescent CD4+ T cells, were also associated with protection in HESNs [136-138]. These cells had lower expression of cellular surface markers of immune activation, thus less likely to support productive HIV

replication compared with the activated immune cells [131, 139-141]. However, these cells were able to mount normal cytokine responses following antigen stimulation [137].

1.5. HIV-1 susceptibility

Several factors are known to influence the risk of HIV-1 transmission. Notably, biological factors such as high plasma viral load (VL) as well as high endocervical and seminal VL can increase transmission risks [142, 143]. Similarly, social and behavioural factors including the number of sexual partners, low condom use rates, experience with sexual violence and drug use can increase risk of HIV-1 transmission [144, 145]. Meanwhile, male circumcision and proper condom use, and administration of pre-exposure antiretroviral drugs are known to decrease the transmission risk of HIV-1 [72, 145].

In addition to social behaviors, factors that threaten the integrity of host mucus membrane such as presence of genital inflammation, fluctuations in the microbiota profile, hormonal changes during menses cycle, and bacterial/viral co-infections, all likely influence chances of mucosal infection with HIV-1 [132]**.** Some sexually transmitted diseases such as syphilis and genital herpes are known to increase the risk of infection, mainly because infection can lead to open sores and damage in the mucosal epithelial layer allowing the influx of HIV and other bacterial and viral pathogens [146-148]. This can in turn induce a cascade of immune activation and inflammatory response that can further increase the presence of susceptible target cells. Similarly, studies have shown that bacterial vaginosis (BV), or the disruption of normal vaginal flora, can increase risk of HIV-1 infection by up to 60% [98, 124] primarily by causing mucosal inflammation, disturbance of protective low pH of the genital tract and damage to vaginal lining [123, 148].

The fluctuation of hormones during menstrual cycle also influences HIV-1 susceptibility. The luteal phase of the menstrual cycle is characterized by high progesterone levels, vaginal epithelium thinning, and decreased protective immune function in the female genital tract (FGT) [149]. The mucosal surface of the FGT functions as the primary physical and immunological barrier against a multitude of infections, including HIV-1 [147]. During the luteal phase when the progesterone level is high, the cervico-vaginal epithelium layer is thinner making it more susceptible to infection [150, 151]. Multiple studies have shown that the use of exogenous progesterone in hormonal contraceptives is associated with increased HIV susceptibility [152-154]. Similarly, it has been demonstrated that topical progesterone can increase the risk of infection in an NHP/SIV model [155]. Increased susceptibility to SIV/SHIV infection was also observed during the luteal phase of the menses cycle in non-human primates, which may be due in part to the thinning of the vaginal epithelia [101, 151, 156].

However, it is also important to note that some studies fail to show differences in vaginal immune cell population, immune cell activation, as well as inhibition of HIV-1 infection between follicular phase and luteal phase of women [148] and instead demonstrated increase in mucosal inflammation markers particularly MCP-1/CCL2, and retention of susceptible immune cell targets in the genital tract during the follicular phase [157]. Overall, these findings suggest that both the availability and activation of immune cell targets, as well as the integrity of physical barriers that restrict access to these target cells, play a role in the perceived risk of HIV infection.

1.6.HIV-1 and host Immune Responses

The human immune system is comprised of the innate and adaptive arms. The innate immunity is

rapid but non-specific, while the adaptive immunity operates through specific antigen recognition and establishment of memory responses.

1.6.1. Innate Immune Response to HIV-1

The innate arm of the host immune response is the first line of defense against invading pathogens, such as HIV-1 and includes a combination of anatomical barriers, innate immune cells, and other non-specific physiological responses[158]. It is known that the type of immune responses mounted during initial infection impact the adaptive immune responses responsible for control of disease progression [158, 159].

Antigen-presenting cells (APCs) such as DCs and macrophages, are also crucial to host innate immunity against HIV-1. They link the innate and adaptive arms of host immunity mainly by mediating adaptive immune cell activation, differentiation, and proliferation. APCs link innate and adaptive immune system mainly through antigen processing and presentation, providing co-stimulatory receptor signals, and through production of cytokines that are necessary for T and B cell activation, differentiation into effector cells, and clonal expansion [160]. Since DCs and macrophages express surface CD4 and CCR5, they are also targets for HIV-1 infection and are known to act as major tissue reservoirs during chronic infection [161, 162]. Pathogen-recognition receptors (PRR), such as toll-like receptors (TLR) 7 and 8 are also found on APCs, can detect viral particles through pathogen-associated molecular patters (PAMPs) such as HIV-1 ssRNA [163]. Binding of PAMPs to cognate PRR leads to a cascade of intracellular signaling events resulting in the production of cytokine, chemokines and anti-microbial peptides [164, 165].

In addition, other innate immune cells such as natural killer (NK) cells also play a major role in host innate immune response to HIV-1 [166, 167]. NK cells have cell surface receptors called

Killer immunoglobulin-like receptors or KIR that are encoded by highly polymorphic genes and bind host HLA-I molecules [168]. NK cells recognize infected or dysfunctional host cells due to lower or lack of HLA I expression their cell surface thereby initiating antiviral effector functions such as direct killing of target cells by exocytotic lytic activity and induction of apoptosis [169, 170]. Multiple population studies have shown that specific KIRs are over-represented during acute HIV-1 infection [158] while others are associated with delayed disease progression [171]. However, studies have shown that HIV-1 Tat and Env proteins are also capable of inhibiting NK effector functions which likely explains the observed decline in IFNγ-producing NK cells during chronic HIV infection[172-175]. The influence of innate immune response in resistance or susceptibility to HIV-1 infection is also supported by genetic studies. Specific KIR and KIR-HLA-I alleles/genotypes were shown to be associated with either resistance or susceptibility to HIV infection in the Pumwani cohort [133] .

Exposure to pathogens and the subsequent activation of innate immune cell also results in the release of soluble proteins messengers that can enhance immune cell activation or influence HIV-1 replication, as previously discussed [176]. For example, IL-12, IL-15, and IL-21 have both anti-viral and chemoattractant properties, particularly for NK cells [174, 175].

Although increased production of pro-inflammatory cytokines as biomarkers of inflammation has been linked to increased HIV-1 susceptibility, conflicting findings exists on whether specific cytokines are associated with either susceptibility or protection. Some studies have shown that *in vitro* levels of secreted macrophage inflammatory protein I alpha (MIP-1α) and beta (MlP-lβ), and RANTES are associated with inhibition of HIV-1 infection [74, 177]. Furthermore, higher levels of RANTES were observed in cervico-vaginal lavage samples of HESNs compared with infected controls suggesting the possible role in protection against infection [178]. Meanwhile, other

studies have demonstrated that high levels of MIP-1α, MlP-lβ and other chemoattractant cytokines can also increase recruitment of target cells [179]. There seems to have a consensus, however, on the role of IL-1β, IL-6 and TNFα in upregulation of HIV-1 replication [180]. Altogether, the secretion of a variety of cytokines and chemokines during innate immune response plays significant role in shaping the host adaptive immune response, hence influencing the resolution of infection.

1.6.2. Adaptive Immune Responses to HIV-1

Antigen specificity and memory sets the adaptive immune response apart from the innate immune responses. The adaptive immune response is antigen specific, and consists of T cell-mediated immune response, and the humoral immune response, which is mediated by B cells and antibodies [44, 181].

Both humoral and cell-mediated adaptive immune responses are induced after exposure to HIV-1. HIV-specific humoral responses begin after antigen binding of B cells and its subsequent differentiation into plasma B cells to produce antibodies [182]. Plasma antibodies are typically detected approximately 1-3 months after infection, referred to as seroconversion [104]. Neutralizing Antibodies (nAbs) against HIV-1 primarily recognize the viral Env protein gp120 and gp41, and are found in most infected individuals [182]. However, efforts to induce these responses through immunization have failed thus far [183, 184]. Other HIV-1-specific immunoglobulins (Ig) capable of inducing cytotoxicity via innate immune cells have also been observed in infected individuals [185].

Several studies have demonstrated the significance of HIV-specific CTL responses in the control of infection during acute infection, as well as in LTNPs and ECs, as previously mentioned [81-83, 86].

HIV disease progression is marked by an increasing exhaustion of CD4+ T cell responses. Over the course of HIV infection, particularly during the chronic stage, the decline in CD4+ T cell count is accompanied with decreases in immune function and proliferative responses over time [186]. Depletion of CD4+ T cells during HIV-1 infection is Depletion of CD4+ T cells during HIV-1 infection is considered due to the infection, cytopathic effects and release of viral progenies [63]. HIV-1 Env antigen presentation on infected CD4+ T cells also induces bystander apoptosis through syncytia formation, gp41-mediated hemifusion, and autophagy inducing cell death of neighboring uninfected CD4+ T cells [62, 63]. In the acute phase of HIV infection, rapid viral replication results in a massive depletion of CD4 T cells and Th17 cells in the gut-associated lymphoid tissue (GALT) and mucosa-associated invariant T cells in the GI tract [64, 187]. This is accompanied by enterocyte apoptosis which consequentially increases gut tissue permeability to microbial products, resulting in further immune cell activation and inflammation [65]. Preferential HIV infection of activated immune cells, particularly CD4+ T cells result in further weakening of host immunity. This self-perpetuating cycle of immune activation and viral replication eventually results in clonal exhaustion of T cell memory pools and the subsequent increase in viremia as disease progress [103, 188, 189]**.** Nevertheless, several studies have shown that the presence of HIV-1 Gag-specific CD4+ T cells that are capable of proliferating and secreting IL-2 or IFNγ correlated with better than CTL responses, with controlled viremia and delayed onset of disease [190, 191]. Vaccine trials that aimed to induce cellular immune responses will be discussed in a later section.

1.6.3. Host Inflammatory Response

Inflammation is an essential part of the host immune response arising from pathogen exposure or tissue injury [49]. The host inflammatory response is generally divided into 4 phases: i.) the primary recognition of infecting pathogen by innate immune cells ii.) localization of immune cells iii.) clearance of pathogen iv.) the eventual resolution of inflammation and return to homeostasis [51]. During both innate and adaptive immunity, the regulation of inflammatory response involves complex interactions between host immune cells and soluble factors including inflammatory cytokines and chemokines [50]. Primary induction and establishment of inflammatory response is triggered by the innate recognition pathways like via PRRs that identify and signal presence of infection [122]. In early immune responses, this recognition of infection eventually results in the activation of innate immune cells and the production of various pro-inflammatory cytokines/chemokines [192]. For example, upon antigen stimulation, monocytes and macrophages secrete RANTES, IL-1α/β, IP-10, IL-6, and IL-8 that increase vasodilation, vascular permeability and immune cell migration [53]. Other immune cells present in the genital mucosa such as NK cells and dendritic cells (DCs) also produce IL-6, IL-1α [53], IL-1β, MIP-1α/β, IL-8, and RANTES [193, 194]. In particular, IP-10 and RANTES are known chemoattractant of T cells, NK cells, and dendritic cells, while IL-8 attracts neutrophils [71, 195]. In addition to promoting migration of monocytes [196], IL-6 also stimulates the differentiation of B lymphocytes to plasma cells [195], the activation of cytotoxic T lymphocytes, and is thought to have both pro- and anti-inflammatory effects through the STAT3 signaling pathway [197].

Adaptive immune cells can also contribute and intensify the inflammatory response and its consequences. Cytokine signaling by innate immune cells can in turn recruit and activate naïve CD4+ and CD8+ T cells of the adaptive immune cells [198] as well as help dictate T cell

differentiation into effector and memory subsets, as previously described. Activated CD4+, CD8+ T and B lymphocytes are also known to secrete pro-inflammatory cytokines IL-6, IL-8, IP-10, IL-1α/β, β-chemokine RANTES, MIP-1α, and MIP-1β [53, 74, 199]. MIP-1α and MIP-1β, although previously attributed to protection from HIV-1 infection in HESNs, and by competitively binding to CCR5 in vitro [74], are also known to be chemotactic for HIV/SIV targets like CD4+ T cells, monocytes, macrophages, DCs and as biomarkers for genital inflammation have been associated with increased risk for viral acquisitions in vivo [179].-Following the cascade of immune activation and the eventual elimination of pathogen, is the resolution of inflammation and clearance of dead cells and debris. Ideally, an efficient inflammatory response would be rapid and also self-limiting [51]. However, if the inflammatory response is not properly resolved, inflammation can lead to persistent infection and tissue damage, as observed during chronic inflammatory disorders [49]

During HIV-1 infection, host inflammatory responses particularly at the mucosal surface, plays a major role in susceptibility and disease progression. It has been demonstrated that exposure to HIV-1 glycoprotein, gp120, alters epithelial barrier function and enhances its permeability [47]. It does so mainly by directly stimulating epithelial cells to produce inflammatory cytokines such as TNFα, IL-1β, IL-6, IL-8, MCP-1, and IFNα that are known to cause epithelial damage. Similarly, mucosal inflammatory responses stimulated after intra-vaginal SIV exposure in Rhesus macaques was found necessary for productive viral infection [200]. In cases of HIV uninfected women where mucosal cytokines were elevated, a distinct mucosal proteomic profile indicative of altered epithelial barrier integrity was also observed linking mucosal inflammation to epithelial damage and target cell localization [201, 202]. In the same study, it was also observed that increased levels of IL-1β and IL-8 were associated with neutrophil-associated protease expression and neutrophil

migration. Neutrophils are known to play a significant role in endometrial damage and repair during the menses cycle [203].

In addition to a weakened epithelial barrier, mucosal inflammation also strongly influences the presence of various HIV-1 target cells in the FGT [204]. In healthy individuals, the vaginal epithelium is almost devoid of immune cells with limited quantities of intraepithelial lymphocytes, often restricted to the basal epithelial layers or the inner lamina propria [101]. However, studies have shown that presence of mucosal inflammation is associated with an increase in both frequency and activity of immune cells [205]. During an infection, cytokines and chemokines, as biological indicators of inflammation, play an essential physiological role in the recruitment of activated immune cells [192]. As previously mentioned, IP-10, MIP-1α, and MIP-1β have chemoattractant properties that recruit T cells, macrophages and dendritic cells [179]. Likewise, during SIV infection in Rhesus macaques, pro-inflammatory cytokine production resulted in CD4+ T cell recruitment [146] while inhibition of chemokine production prevented migration of immune cells to the FGT [200]. Similarly, genital levels of pro-inflammatory cytokines and chemokines such as (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, have been implicated not just in recruitment, but in the activation and differentiation of immune cells as well [206]. Since HIV replication largely depends on the presence of target cells, the level of immune cell activation and monocyte differentiation to macrophages and DC, the recruitment, activation, and differentiation may increase transmission.

As previously mentioned, although an inflammatory response is necessary to resolve infection, persistent inflammation can alter the dynamic environment of the female genital tract [66]. In the case of HIV-1 infection, inflammation allows for a mucosal environment that is conducive to viral replication and productive infection. For example, pro-inflammatory cytokines have been shown

to induce nuclear factor (NF)-KB expression which is a transcription factor that directly up-regulates HIV replication by binding to HIV-long terminal repeats (LTR) [207]. Incidentally, the presence of mucosal inflammation in the FGT has also been associated with increased susceptibility to HIV-1 infection [179]. In both the CAPRISA002 study and CAPRISA004 tenofovir 1% gel trial, elevated levels of at least 3 pro-inflammatory cytokines MIP-1α, MIP-1β, IL-8, IL-1β, IL-1α, TNFα in CVL samples were associated with a 3-fold increase in HIV risk of infection [179, 208]. Coincidentally, implied lack of basal mucosal inflammation in HESN women as a consequence of reduced basal immune activation has been associated with reduced risk of HIV-1 acquisition [137, 209]. Altogether, these findings indicate that mucosal inflammation plays a significant role in HIV transmission and pathogenesis. Therefore, mucosal inflammatory response to potential preventive and treatment strategies must be carefully considered.

1.7. HIV-1 Treatment and Prevention

1.7.1. HIV-1 Treatment

Early in the HIV epidemic, treatment of HIV infection mainly focused on AIDS-related diseases and addressing opportunistic infections because there was no anti-retroviral drugs (ARV) [210]. Although there is still no cure for HIV/AIDS, the development of ARV for therapy (ART) drastically changed the landscape of HIV infection, from a fatal disease to a more manageable chronic illness [211]. A combination of antiretrovirals with different mechanism of actions are used for a stronger and more effective viral suppression, referred to as highly active antiretroviral therapy (HAART) [212]. These drugs are typically categorized based on molecular mechanisms used to disrupt the virus life cycle and include nucleoside-analog reverse transcriptase inhibitors

(NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase inhibitors, protease inhibitors, fusion inhibitors, nucleus entry inhibitors, and co-receptor antagonists [210].

HAART regimens have been proven effective for reconstitution of the host immune system, by drastically inhibiting viral replication and reducing plasma viral loads to below limits of detection (<50 RNA copies/mL) [210]. However, the success of this strategy largely depends on adherence to therapy. Unfortunately, risk of non-adherence and discontinuing therapy due to adverse effects and toxicities associated with HAART remain a serious concern [212]. ARV-associated adverse reactions range from chronic and non-life-threatening conditions such as anemia and diabetes, to acute but fatal side effects such as severe hypersensitivity, hepatotoxicity and nephrotoxicity [213-215]. While ARV treatment can effectively reduce viral load, these side effects, coupled with the other issues such as emergence of drug resistance, chronic inflammation, accessibility to ART in developing countries, and viral latency, remain significant obstacles for the complete eradication of HIV-1 and highlights the need for a more effective strategy in combating HIV infection [216].

1.7.2 HIV-1 Preventive Strategies

Currently, there are three main approaches to the prevention of HIV infection in which the most suitable choice depends on the dynamics of the target population. These may include strategies for high risk individuals pre-exposure, post-exposure and strategies for the general population involving low-risk individuals. In 2015, WHO recommended the offering pre-exposure prophylaxis (PrEP) to all persons at substantial risk of HIV infection [89]. PrEP is recommended in order to prevent HIV-1 infection among high-risk but uninfected individuals such as commercial sex workers, MSMs and IDUs [217]. PreP is a strategy used to reduce chances of infection prior to exposure and involves using a combination of ART drugs [218] such as Tenofovir either once

daily or topically in the form of a vaginal gel. In 2010, the first randomized clinical trial results were released showing effectiveness of oral PrEp (tenofovir/emtricitabine) among MSMs [219]. Multiple clinical trials have since been conducted in different populations including the CAPRISA 004 (safety and effectiveness of 1% tenofovir vaginal gel) in women (54% lower), and together these studies suggest PrEP is highly effective if taken regularly [220]. Clinical trials also continue to demonstrate that effectiveness of PrEP is significantly associated with adherence [220]. As with other HIV treatment and interventions, success largely depends on the knowledge of potential users, their willingness to use it, and the potential barriers and facilitators to its uptake is critical to its success [221]. Post-exposure phrophylaxis (PEP), on the other hand, is a prevention strategy recommended among individuals after an exposure to HIV either in an occupational or non-occupational setting such as accidental (percutaneous) needlestick injury for health care professionals, victims of sexual assault or after unprotected sexual encounters [72]. Prevention in the general population level or low-risk individuals typically includes treatment of sexually transmitted infections, condom use, and voluntary male circumcision (VMC) [222].

1.8.HIV-1 Vaccine Development

Although great progress in HIV-1 prevention and treatment strategies have reduced global incidences of HIV infection in the recent years, it has fallen short in reaching the worldwide goal of HIV-1 eradication. Around the world, the rate of new HIV infections has only declined by an estimated 18% from 2010. Meanwhile new infections are on the rise in about 50 countries including those in Northern Africa and the Middle East (1). Evidently, the best hope of ending the HIV-1 pandemic remains to be an effective HIV vaccine.

1.8.1. Previous Vaccine Trials

Historically, vaccines have been successful at eliminating diseases. However, after decades of effort and investment in vaccine development, there is still no licensed HIV-1 vaccine available. This is largely due to its unique viral characteristics and immunologic challenges that continue to hinder the successful development of an effective HIV prophylactic vaccine [223, 224]. The use of live attenuated HIV virus and inactivated virus have been ruled out due to significant safety concerns with mutation risks and possible reversion to pathogenic strain [225, 226]. On the other hand, whole killed viruses failed to induce both broadly neutralizing antibodies and CTL responses [226]. In contrast with traditional vaccine strategies, modern approaches are typically aimed at inducing humoral and/or cellular immune responses using plasmid DNA vaccines, protein subunits, and the use of recombinant viral vectors [227].

Lessons learned from both pre-clinical and clinical efficacy trials, are excellent resources that can be exploited for further innovation and testing of potential mechanisms of protective immunity [228]. To date, there have only been 6 HIV vaccine candidates evaluated in Phase IIb/III clinical trials while 2 more are ongoing as of 2016 with expected results in 2021 and 2022. The first two vaccine trials were designed to evaluate the efficacy of gp120 subunit vaccine aimed at inducing humoral responses [229] while the next three were T lymphocyte-based HIV-1 vaccines [227]. However, none of the candidate vaccines tested in these trials were effective [229]. The RV144 clinical trial evaluated a heterologous prime-boost regimen of recombinant canarypox vector (ALVAC) expressing *gag*, *pol*, and *env* as prime and a gp120 protein boost (AIDSVAX) [230]. This was the only trial that showed modest, but encouraging efficacy of 31.2% [183]. The vaccine had potential to induce both humoral and cellular immune responses because the selected immunogens in combination. However, this prime-boost regimen was only able to induce non-

neutralizing and weakly neutralizing antibodies [183]. The immune correlates identified in this study underscore the complexity of vaccine-induced protective immune responses. Together with previous efficacy studies, these findings have provided valuable lessons to improve design of future vaccine trials.

1.8.2. HIV- 1 and Vaccine Immunology

The vaccines to infectious pathogens are designed to generate long-lasting, antigen-specific immune memory that can rapidly respond upon re-exposure to a specific pathogen [231]. Active immunization mimics natural infection to induce host immunity against a specific pathogen without actually causing disease. Similar to a natural infection, upon primary exposure to the vaccine immunogen, peptide-specific T lymphocytes undergo TCR selection and subsequent clonal expansion eventually leading to differentiation into effector T cells or long-lived memory cells [232].

Because HIV-1 preferentially targets activated CD4+ T cells, and ongoing infection establishes hyper immune activation [46, 233]. This immune activation then leads to secretion of pro-inflammatory cytokines and chemokines that further perpetuates the immune activation and infection cycle [55, 57, 198]. Thus, it is particularly challenging to develop vaccines to HIV-1. An HIV-1 prophylactic vaccine would inevitably need to engage the immune system, consequently activating CD4+ T cells. The activation of the immune system by the immunogens in the vaccine would result in proliferation and differentiation of CD4+ T cells, thus potentially providing extra targets for infection. Furthermore, since majority of HIV-1 infection are initiated at mucosal surfaces, and elevated genital inflammation being associated with increased HIV-1 acquisition

[179, 234], immune activation at the mucosal site needs to be considered in designing effective HIV vaccine. Thus, rational design of a prophylactic HIV-1 vaccine must consider the delicate balance between excessive activation of CD4+ T cells and the induction of protective host immune responses, especially at the mucosal surfaces [235].

Multiple studies have evaluated factors that influence the magnitude of immune activation induced by a vaccine. For example, the use of recombinant live viral vectors, such as adenovirus, lentivirus, and cytomegalovirus can induce more potent host cellular and humoral immune responses, than non-replicating vectors [236]. The magnitude of induced cellular responses by immunization can also be increased by boosting with a heterologous recombinant virus vector [237]. Moreover, there is evidence that recombinant vaccine vectors can, to an extent, determine the specificity and proliferation capacity of memory T cells induced by a vaccine [238, 239].

In addition, the presence of pre-existing vector-specific immunity may influence vaccine efficacy, as observed in the STEP vaccine trial, where an increase in susceptibility in vaccinated participants was linked to pre-existing immunity to the vaccine vector- recombinant Adenovirus serotype-5 (Ad5) [240]. Follow-up studies showed increased activation of CD4+ T cells in the gut of vaccinated individuals in the STEP and Phambili trials [241]. This was later confirmed in a non-human primate study, where rAd5 vaccination resulted in increased in CD4+ T cell activation in the gut mucosa leading to increased SIV acquisition [242]. Therefore, increased T cell activation due to vector-induced immunity should be an important consideration in the development of HIV-1 vaccine.

Previously, parenteral administration of vaccines alone was not considered to induce antigen-specific immune responses in mucosal tissues [243]. However, multiple studies in both human and

animal models have demonstrated that systemic vaccination can also induce mucosal immune responses. For example, it has been shown that a single intramuscular immunization with recombinant Ad HIV-1 Env vaccine was able to induce both systemic and mucosal Env-specific antibody and CD8+ T cell responses in humans [244]. Similarly, intramuscular immunization with lentiviral-vector vaccine can also induce robust antigen-specific cellular immune responses in the gut mucosa of mice [245]. The presence of vaginal anti-polio virus and salivary anti-influenza antibodies were also detected following intramuscular immunization with inactivated polio virus [246] and trivalent influenza vaccine [247, 248], respectively. The understanding that systemic immunization can effectively induces mucosal immune activation further underscores the importance of evaluating mucosal immune responses to candidate HIV vaccines.

Although no study has directly evaluated the impact of HIV-1-derived immunogens on mucosal inflammation, it has been previously reported that immunization with full length HIV-1 Gag/Pol/Env immunogens induced higher magnitude of cellular immune responses than vaccines containing only conserved HIV-1 regions [75]. In addition, long peptide immunogens are known to be highly immunogenic containing many T cell epitopes, and as in the case of HPV vaccines that can induce strong and broad CD4+ and CD8+ T cell responses [249]. Furthermore, it has also been shown that extending the length of peptide immunogens can lead to a more sustained host T cell responses compared with short peptide immunogens [250]. These studies raise the question as to whether short peptides of conserved HIV-1 regions can be better vaccine immunogens by providing a balance between generating immune responses that are protective, and responses that can lead to susceptibility. Thus, mucosal immune activation and inflammation induced by the immunogens of a candidate vaccine needs to be evaluated during the development of a prophylactic vaccine.

1.8.3. The PCS Vaccine

Learning from the natural immunity observed in HIV-1 exposed seronegative female sex workers from Pumwani [134], a novel vaccine targeting the peptides surrounding the 12 protease cleavage sites was developed (the PCS vaccine) [135, 251, 252]. HIV protease cleaves Gag, Gag-Pol and Nef precursor polyproteins at the 12-protease cleavage sites in a highly specific and temporally regulated process to generate mature and infectious viral progenies [135]. It was proposed that a vaccine focusing immune response on these short, highly conserved sites that are essential for HIV protease mediated viral maturation, could destroy viral infected cells and limit immune activation compared with full HIV immunogens [134, 135] .

To test the efficacy of the PCS vaccine in a nonhuman primate and SIVmac251 model, the 12 20-mer peptides overlapping each of the 12 PCS of SIVmac239 were delivered in a recombinant vesicular stomatitis virus (rVSV) and in nanoparticles. VSV was chosen as vaccine vector in this study for its proven safety in the Ebola vaccine (EBOV) as well as its lack of pre-existing human immunity [253]. NANO formulations were also used to deliver the PCS peptides. The immune responses to this vaccine were evaluated in a Mauritian *Cynomolgus macaque* model because of their relatively simpler MHC genotypes, and the disease progression pattern that resembles HIV infection [254]. A previous study has shown that the PCS vaccine can induce mucosal antibodies against SIV immunogens in the female genital tract of macaques [135]. However, the influence of the PCS vaccine on mucosal inflammation has not been evaluated. The work of thesis sought to evaluate the mucosal inflammatory responses generated by the PCS vaccine in comparison with a vaccine targeting full length Env and Gag proteins.

2. RATIONALE

The current focus of HIV vaccine development enterprise, is to induce robust and long lasting protective immune responses [184]. Because CD4+ T cells, an important part of adaptive immune system are also the main targets of HIV-1, developing an effective HIV vaccine is more challenging than for other infectious pathogens. Studies showed that immune activation and inflammation promote HIV infection (reviewed in [48]). The activated CD4+ and CD8+ T cells produce many proinflammatory cytokines [57] that in turn create more targets for HIV infection [255]. Vaccines activating T cells would have the potential to increase HIV target cells and the susceptibility to HIV infection [44]. Thus, vaccine induced immune activation and inflammation should be studied in HIV vaccine research [44].

Inflammation activates and attracts HIV target cells and the elevated cervico-vaginal inflammation has been associated with higher risks of HIV-1 acquisition [74]. Recently, genital inflammation has also been shown to reduce the efficacy of anti-HIV microbicides [55]. An effective HIV vaccine would be expected to induce effective anti-HIV immune responses at the same time avoiding vaccine induced inflammation, especially at the mucosal level. Live viral vectors are known to induce host immune activation that may enhance susceptibility to HIV-1 infection [2, 3]. However, the potential induction of mucosal inflammation by vaccine immunogens has not been evaluated. Therefore, the potential effect of vaccine immunogen-induced mucosal inflammation needs to be investigated.

This study evaluated and compared the effect of different vaccine immunogens on cervico-vaginal inflammatory cytokines of female Mauritian cynomolgus macaques (MCM). The macaques were immunized and boosted with different immunogens delivered by vesicular stomatitis viral vector

and NANO formulations. The vaccine immunogens included: a. full-length Gag and Env of SIVmac239; b. 12 20-amino acid peptides, each of them overlapping one of the 12 protease cleavage sites of SIVmac239 packaged into 12 different rVSV.

3. HYPOTHESIS AND OBJECTIVES

3.1. Hypothesis

The PCS vaccine delivering 12 20-amino acid peptides would induce fewer mucosal inflammatory cytokines/chemokines with lower magnitude in the cervico-vaginal mucosa of female cynomolgus macaques than a vaccine delivering full Gag and Env proteins of SIVmac239.

3.2. Objectives

a. Evaluate levels of mucosal cytokine/chemokines after immunization and boosts
b. Compare levels of mucosal cytokine/chemokine between different vaccine groups following immunization

Studies have shown that multiple cytokines and chemokines are secreted after CD8+ and CD4+ T cells are stimulated with immunogens [55, 57-59, 74]. It is expected that immunizations with Gag/Env vaccine will induce more cytokines and chemokines with higher magnitude than the PCS vaccine since the full Gag and Env immunogens contains far more T cell epitopes than the 12 20-amino acid peptides. Because mucosal immune response can be induced after vaccination through intramuscular route [244, 245, 252], we analyzed the inflammatory cytokines of the cervico-vaginal washes during immunizations to determine whether different immunogens in the vaccines differentially influence inflammatory cytokines and chemokines at cervico-vaginal mucosa. The

knowledge gained through this study will help to rationally design immunogens for an effective HIV vaccine.

4. MATERIALS AND METHODS

4.1.Ethics Statement

The non-human primate experiments were approved by the University of Wisconsin IACUC protocol (G005765) in accordance with the US Animal Welfare Act and following the recommendations of the National Research Council *Guide for the Care and Use of Laboratory Animals, 8th Edition* and the Weatherall report, *The Use of Nonhuman Primates in Research*. The Wisconsin National Primate Research Center is fully accredited by AAALAC under the University of Wisconsin, Division of Vice-Chancellor for Research and Graduate Education.

4.2.Animal Model

A total of 24 colony-bred female Mauritian cynomolgus macaques (*Macaca facicularis*) (MCMs), age 6-7 years old, were used in the study (Bioculture (Mauritius) Ltd.). All animal work and sample collection were conducted at Wisconsin National Primate Research Center (WNPRC). Briefly, during the course of the study, the non-human primates within the same experimental group were pair-housed in the standard stainless-steel primate cages with visual and auditory access to other conspecifics. Rooms were maintained at 65-70^{o}F with a 12 hour light and 12 dark cycle, starting at 0600 hours. Daily standard NHP chow with fruits or vegetables was provided with foraging enrichment devices and activities provided at least weekly. All animals were monitored for health

or welfare issues at least twice daily. One monkey from the Gag/Env group (Monkey ID: cy0782) had to be euthanized due to severe illness unrelated to the vaccine.

MHC typing was performed by the Wisconsin Nonhuman Primate Research Centre Genetics Services as described previously [252]. Those with the same MHC haplotypes were grouped to form several MHC-based subgroups. To ensure the monkeys with different MHC haplotypes are evenly distributed in 3 different experimental groups the monkeys of each MHC-based subgroup were randomly assigned to three experimental groups. Similarly, animals were also screened for infections and constantly monitored for hormone levels, menses schedule, age and weight. Similar to MHC typing, differences with regards to hormone, menses levels and weights were considered when assigning monkeys to vaccine groups to ensure even and random distribution. The three experimental groups were: the PCS vaccine group, the Gag/Env vaccine group, and the control group.

4.3.Vaccines and Immunization Scheme

Monkeys in the PCS vaccine group were immunized with twelve 20-mer peptides of the twelve protease cleavage sites (PCSs) delivered by recombinant vesicular stomatitis virus (rVSVpcs), or in biodegradable nanoformulation boosts (NANOpcs). Each of the 12 PCS peptides were delivered by a separate virus while all 12 nanoformulated PCS peptides were combined in one mixture for the NANOpcs boost. Collectively the rVSVpcs and NANOpcs are referred here as the PCS vaccine. Monkeys in the Gag/Env group were immunized with the full length Gag and Env immunogens delivered with VSV virus (rVSVgag and rVSVenv) and as DNA packaged in

nanoparticles (NANOgag/env) for boost. The monkeys in the control group were immunized with empty VSV wild type vector (VSVwt) and sterile water.

A final dose of 1×10^7 pfu/animal was used for all rVSV immunizations and was administered through the intramuscular route in the hind legs (quadriceps muscle). Specifically, the final dose was composed of $1x10^6$ p.f.u./PCS type for the PCS group, while 6×10^6 pfu each for rVSVgag and rVSVenv for the Gag/Env group. For Boost 4, a higher dose of $1x\ 10^8$ p.f.u./rVSV type/animal was used. NANO boosts were administered through intranasal application in 600µl liquid. For NANOpcs boost each animal in the PCS group received 50µg/PCS peptide. For NANOgag/env boost each animal in the Gag/Env vaccine group received 500µg of Gag and 500µg of Env DNA in nanoformulations. The immunization timeline (Fig. 1) for all three vaccine groups started with a prime at week 0 followed by boosts at week 6 (rVSVs + NANOs), week 16 (NANOs), week 51 (rVSVs) and week 72 (rVSVs).

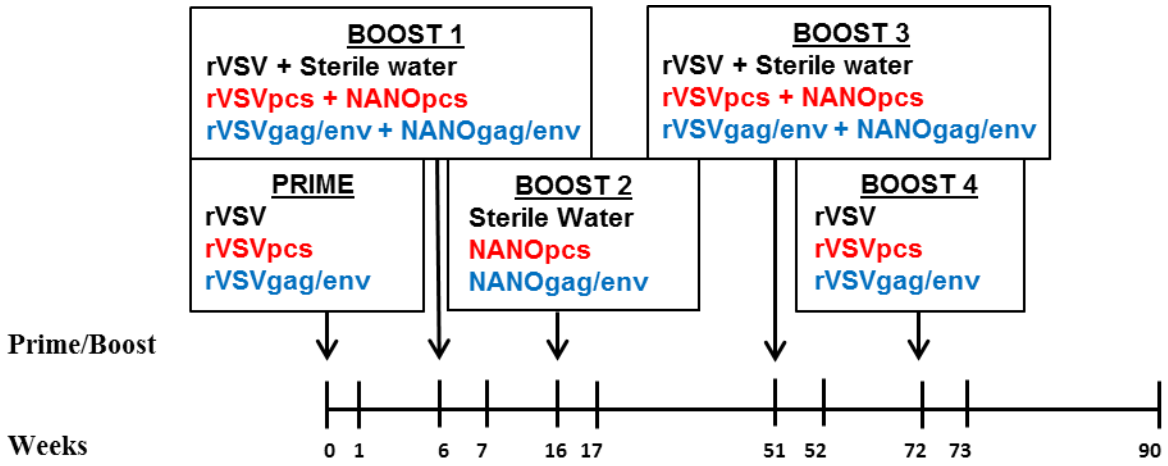

Fig. 1. Immunization and sampling scheme. Prime and boosts were given to three groups of 8 female MCMs on the indicated weeks. CVL samples were collected on all time points. Control group received rVSV vector and/or sterile water (black font), PCS group received rVSVpcs and/or NANOpcs (red font), Gag/Env group received rVSVgag/env and/or NANOgag/env (blue font). rVSV, rVSVpcs and rVSVgag/env were administered intramuscularly. NANOpcs and NANOgag/env boosts were administered intranasally.

4.4.Construction and Generation of rVSVpcs vaccine and NANOpcs Boost

To prepare the rVSVpcs vaccine, nucleotide sequences of SIVmac239 (Los Alamos National Laboratory HIV database) encoding 20 amino acids overlapping 12 PCS [MA(p15)/CA(p27), CA(p27)/p2, p2/NC(p8), NC(p8)/p1, p1/p6gag, NCgag-pol/TFP, TFP/NCgag-pol, p6gag-pol/Protease(p10), Protease(p10)/ RT(p66), RT(p51)/RNase(p15), RNase(p15)/integrase (p31) and Nef] were synthesized and cloned into a pUC(-)MCS plasmid (BlueHeron Biotech), then subcloned in a pATX VSV-G plasmid (modified from a gift of John Rose, Yale University School of Medicine). Details of these methods were previously described [252]. Briefly, rVSVpcs plasmids were transfected into HEK293T cells and then passaged in VeroE6 cells to obtain virus stock which was then purified, concentrated, and stored at -80°C as previously described [252].

The NANOpcs used to boost immune responses in the PCS vaccine group, twelve 20mer peptides overlapping the 12 PCS of SIVmac239 (GenScript) were encapsulated in a biodegradable nanoparticle system of chitosan and dextran sulfate (CS:DS), as previously described [251, 252] Briefly, the formation of the CS: DS were achieved simply by adding 0.825 ml of an aqueous DS solution (1.875 mg/ml) to an equal volume of an aqueous CS solution (0.625 mg/ml) under magnetic stirring. The formation of CS:DS nanoparticles was dependent on ionic interaction between their opposite charges on CS (+) and DS (-).Each PCS peptide was encapsulated in the nanoparticles, by dissolving in either the anionic (DS solution) or in the cationic phase (CS solution) according to isoelectric point (pI) (DS solution: for pI lower than 7). Each peptide was encapsulated into separate nanoparticles, then pooled together for one single dosage containing 50μg of each peptide. The freeze-dried nanoparticle formulations were stored at 4ºC, and reconstituted in water just before the immunization.

4.5.Construction and Generation of rVSVgag/env Vaccine

The preparation of rVSVgag/env vaccine was done using similar methods, as rVSVpcs. Briefly, each SIVmac239 full-length Gag and Env coding sequences were synthesized and cloned in a Blue Heron pUC(-)MCS plasmid (BlueHeron Biotechnology, Bothell, WA, USA) then sub-cloned into the pATX VSV-G (rVSV vector). These were then packaged into rVSVgag and rVSVenv viruses (rVSVgag/env) after confirmation with sequencing, as previously described [252]. Supernatants from VeroE6 cell cultures infected with the rVSV vaccines were previously analyzed by Western blot to test for SIV protein expression. For the NANOgag/env DNA vaccine, separate DNA constructs coding for full-length Gag and Env, pVAX1-Gag and pVAX1-Env were each packaged into separate DNA vaccine nanoparticles, collectively termed NANOgag/env, as previously described [252].

4.6.Sample Collection

Weekly cervico-vaginal lavage (CVL) samples were collected at each time point starting from Day 0, as indicated in the immunization schedule detailed in Fig. 1. On time points when immunization falls on the same day as sample collection, CVL samples were collected before the administration of vaccines. Specifically, the vaginal lumen of sedated animals were irrigated with 2-20mL of phosphate buffered saline (PBS) non-traumatically using a needleless syringe. The fluid was gently flushed five times using the same syringe repeatedly. Ultimately, 2-4mL of the CVL fluid was collected. The CVL samples were then frozen and shipped from Wisconsin to Winnipeg. All sample aliquots were stored in a -80ºC freezer until use.

4.7. Coupling of Capture Antibodies to Magnetic Beads

A Bio-Plex Amine Coupling Kit was used to couple 20 µg of capture antibody for each cytokine/chemokine to 1.25×10^7 109 Bio-Plex Pro™ fluorescently-dyed magnetic COOH Beads, which was then diluted to a stock concentration of 5×10^6 beads/mL according to the manufacturer's instructions. Briefly, stock uncoupled beads were vortexed for 30 seconds and aliquoted into 4 coupling reaction tubes of 100µL volume each. The tubes were then placed into a magnetic separator for 60 seconds. While on the magnetic separator, the stock diluent was carefully removed as to not disturb the beads. The same steps were repeated using 100µL of wash buffer. After washing, 10uL of 25mg/mL S-NHS (N-hydroxysulfosuccinimide) and EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) were added to the beads followed by incubation on a rotator for 20 minutes at room temperature. After incubation, tubes were then washed with 150µL PBS, pH 7.4, twice, allowing for separation between beads and diluent between each repeat, using a magnetic separator. 100µL of PBS were then added to the tubes, followed by addition of 6µg capture antibody. The total volume for each tube was brought up to 500µL using PBS, vortexed, and incubated at room temperature for 2 hours on a rotor. Following incubation, tubes were again placed on magnetic separator and diluent was carefully removed, as in previous steps. Another PBS wash was then performed on the coupled beads, followed by addition of 250µL blocking buffer for resuspension. Tubes were vortexed at medium speed for 15 seconds and incubated on rotor for 30 minutes at room temperature. Following incubation, diluent was again removed as before, followed by addition of 150µL of storage buffer. The bead concentration was then determined using a hemocytometer. All coupled beads were stored at a 4ºC refrigerator.

4.8. Optimization of 14-plex Cytokine/Chemokine Assay

The standard curves were generated by preparing eight four-fold serial dilutions of the pooled 14 cytokine and chemokine proteins diluted in 1% BSA (Bovine Serum Albumin) in PBS. Briefly, the standard curves plotted by the Bio-Plex Manager software were generated using recombinant cytokine/chemokine proteins and corresponding antibody pairs (Table 1). To check for possible cross-reactivity between capture antibodies purchased from different companies, a seven-plex assay was initially developed from same-sourced capture antibodies. Subsequently, the other coupled bead types were added to complete the 14-plex cytokine/chemokine assay. Optimization of the final standard curve range and concentrations, as well as overall assay consistency after the addition of each coupled bead type were attained by keeping the intra- and inter-assay coefficient of variation (%CV) at less than 20% and 30% respectively, while the ratio of observed to expected protein concentration (% recovery) for each cytokine was kept between 70-130%, with no bead counts of less than 50 per well, as recommended. Specificity of the custom assay was validated using BioRad Bio-plex Pro 27-plex human cytokine/chemokine protein standards (Table 1).

4.9.Multiplex Cytokine and Chemokine Assay

Cytokines and chemokines of CVL samples were quantified using a custom 14-plex assay (Bio-Plex) based on a previously published Bio-Plex assay method [252] with some modifications. The final cytokine/chemokine panel consist of 14 (pro- or anti) inflammatory cytokines/chemokines including Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), Monocyte Chemoattractant Protein-1 (MCP-1), Macrophage Inflammatory Protein-1 alpha (MIP-1α), Macrophage Inflammatory Protein-1 beta (MIP-1β), CCL5/RANTES, Interleukin (IL)-1α, IL-8,

IL-10, IL-17, IL-1β, IL-6, IP-10, Tumor Necrosis Factor alpha (TNF-α), and Interferon gamma (IFN-γ), to quantify the inflammatory response in the CVL samples (Table 1). Cytokines and chemokines that were not detected in ≥50% of the samples were excluded from the heat map analysis (TNF-α, IL-1α, IL-10). 50µL of the capture antibody-coupled magnetic complex containing approximately 420,000 beads (1 in 600 dilutions) were loaded on each well of a 96-well Bio-Plex ProTM 105 Flat bottom plate and washed twice (MAG2x cycle) on a BioRad Bio-Plex® Pro II Wash Station Microplate Washer.

Frozen CVL samples were thawed at room temperature shortly before use, and vortexed for 30 seconds. 50µL of undiluted CVL sample or standards was then added to each well of a 96-well plate and incubated while shaking at high speed (~1000rpm) for 30 seconds, then at medium speed (~300rpm) for one hour at room temperature. The assays were performed according to manufacturer's instructions using assay buffer used as general diluent and blank controls. Following the incubation, the plate was washed thrice (MAG3x) with 100µL of wash buffer. After washing 30 µL of the 14 detection antibodies (in 1µg/mL concentration each) was added into each well, followed by 30 minutes incubation on the plate shaker. Incubations and MAG3x washes were performed each time a reagent was added to plate. BioRad 1x stock of Streptavidin-Phycoerythin diluted at 1:100 in assay buffer was then added at 50uL per well, followed by incubation with agitation at room temperature for 10 minutes. After the final wash, beads were resuspended in 125µL of Assay Buffer, agitated for 10 seconds and analyzed on a Bio-Plex® 200 System (Bio-Rad). Protein concentration of samples (in pg/mL) were generated and reported by the Bio-Plex Manager 6.1 software (BioRad) using measured fluorescence intensity signal from each bead.

4.10. Total Protein Quantification

In order to account for the possible dilution variations introduced during CVL sample collection, the total protein concentrations were used to normalize cytokine/chemokine concentrations in the samples. The total protein concentration of the CVL samples was quantified using a NanoOrange® protein quantitation kit (Thermo Fisher Scientific) according to manufacturer's protocol, in order to determine the final cytokine/chemokine levels in the CVL samples per total protein (pg/mg). The kit had sensitivity between 10ng/mL and 10µg/mL, which encompassed the range of total protein contained in each CVL sample. Briefly, standard BSA solution diluted with the commercial NanoOrange working solution was used to generate a standard curve. The NanoOrange working solution was prepared by mixing a NanoOrange commercial diluent with distilled water and adding a NanoOrange commercial reagent. Fluorescence was measured using a Spectrophotometer Microplate Reader (Spectramax ®) at an excitation at 485 nm and capturing emission at 590 nm, as recommended. All samples were prepared and run in duplicates with triplicate dilutions.

4.11. Materials and Reagents

The capture-detection antibody pairs and protein standards, as well as reagents used in this study are listed on Table 1. The antibody pairs and protein standards were purchased from Novus Biologicals Canada/R&D Systems[a], Thermofisher Scientific[b], and Souther Biotech[c]. Reagent kits and magnetic beads used for 14-plex assays were purchased from Bio-Rad Laboratories.

4.12. Statistical Analysis

Graphing and statistical analysis were performed using GraphPad Prism® 7 statistical software. Matched paired analyses were done using Wilcoxon matched-pairs signed-rank paired *t* test. Statistical analyses between study groups using unpaired comparisons were performed using the Mann-Whitney *t* test. *P* values lower than 0.05 were considered to be statistically significant.

Protein Standards	Capture Antibodies	Detection Antibodies
Recombinant Human CXCL8/IL-8 Protein[a]	Human CXCL8/IL-8 MAb[b]	Human CXCL8/IL-8 MAb, Biotin-labeled[b]
Recombinant Human CCL5/RANTES Protein[a]	Human CCL5/RANTES PAb[b]	Human CCL5/RANTES MAb, Biotin-labeled[b]
Recombinant Human GM-CSF Protein[a]	Rat Anti-Human GM-CSF- Unlabelled[c]	Rat Anti-Human GM-CSF-BIOT[c]
Recombinant Human Interferon Gamma Protein[b]	Human IFNγ MAb[b]	Human IFNγ MAb, Biotin-labeled[b]
Recombinant Human IL-1 beta/IL-1F2[a]	Human IL-1beta/ IL-1F2 Antibody[a]	Human IL-1beta/IL-1F2 Biotinylated Antibody[a]
Recombinant Human IL-6 Protein[a]	Human IL-6 MAb[b]	Human IL-6 MAb, Biotin-labeled[b]
Recombinant Human IL-10 (aa 19-178) Protein[a]	Rat Anti-Human IL-10-Unlabelled[c]	Rat Anti-Human IL-10-BIOT[c]
Recombinant Human IL-17A Protein[a]	Human/Primate IL-17/IL-17A Antibody[a]	Human/Primate IL-17/IL-17A Biotinylated Antibody[a]
Recombinant Human CXCL10/IP-10[a]	Human IP-10/ CXCL10/CRG-2 Antibody[a]	Human IP-10/CXCL10/CRG-2 Biotinylated Antibody[a]
Recombinant Human CCL2/MCP-1 Protein[a]	Human MCP-1/CCL2/JE Antibody[a]	Human MCP-1/CCL2/JE Biotinylated Antibody[a]
Recombinant Human CCL3/MIP-1 alpha protein[a]	Human MIP-1α/CCL3Antibody[a]	Human MIP-1α/CCL3 Biotinylated Antibody[a]
Recombinant Human CCL4/MIP-1 beta Protein[a]	Human MIP-1β/CCL4 Antibody[a]	Human MIP-1β/CCL4 Biotinylated Antibody[a]
Recombinant Human TNF-alpha Protein[a]	Human TNFα MAb[b]	Human TNFα MAb, Biotin-labeled[b]
Recombinant Human IL-1 alpha[a]	Human IL-1 alpha/IL-1F1 Mab[a]	Human IL-1 alpha/IL-1F1 Biotinylated[a]
Other Reagents and Buffers		
BioRad Bio-Plex® Pro™ Human Cytokine Standards 27-plex		
BioRad Streptavidin-PE		
BioRad Bio-plex® Amine Coupling Kit		
BioRad Bio-Plex® Calibration Kit		
BioRad Bio-Plex® Pro™ Magnetic COOH Beads		
BioRad Bio-Plex® Validation Kit		
NanoOrange Protein Quantitaion Kit[b]		
Wash Buffer (PBS + Tween 20)		
Assay Buffer (PBS + 1% BSA)		

Table 1. Materials and Reagents. A table listing the capture, detection antibodies, and protein standards used in the customized 14-plex cytokine/chemokine bead assay, and other kits.

5. RESULT

5.1.Fold changes in cervicovaginal inflammatory cytokines relative to the pre-immunization baseline induced by prime and boosts with different modalities of vaccines

To evaluate whether different modalities of vaccines influence cervico-vaginal mucosal inflammation cytokines we assessed the fold change of the cytokine level after prime and each of the boosts. A heat map (Fig. 2) based on the group median was generated to show the fold change of the cytokine and chemokine level in comparison with their level at pre-immunization (Week 0 baseline).

We also compared the levels of CVL inflammatory cytokines after prime and each of the boosts to the pre-immunization baseline to determine whether these changes in cytokines result in the significant difference between the cytokine level of baseline and that after prime and each of the boosts.

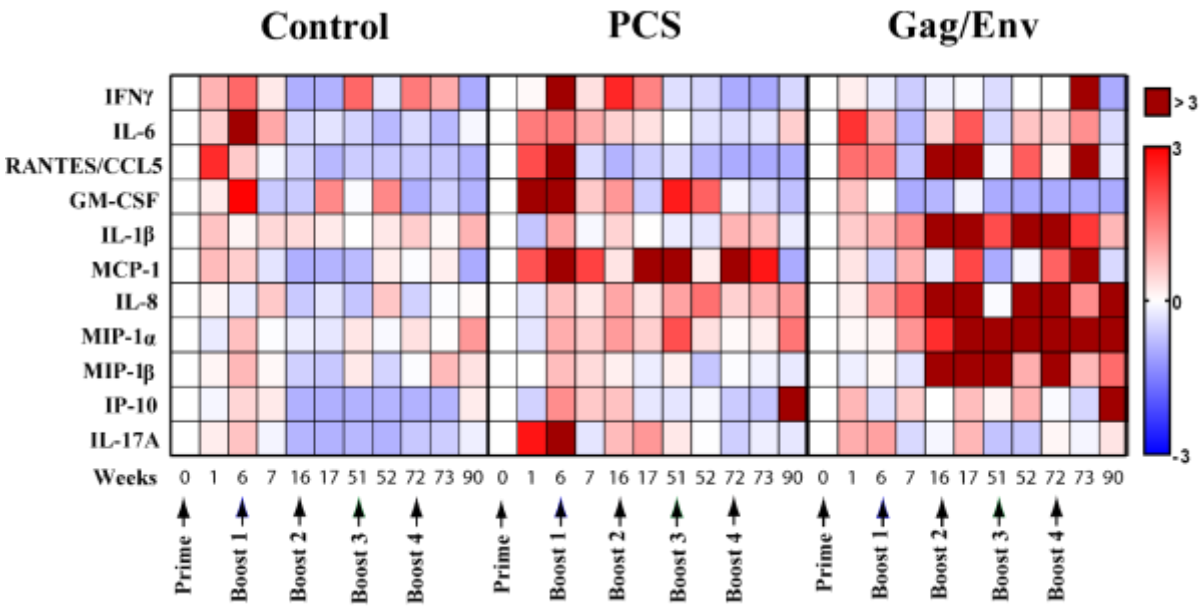

Fig. 2. Median cytokine/chemokine fold-change levels at each time point relative to group baseline (Week 0) in all 3 vaccine groups. The fold change was calculated as the ratio of the concentration to the baseline where baseline was set to 0. Arrows indicate each immunization.

5.1.1. Prime vaccination increased the levels of CVL cytokine and chemokines

The heat maps shows a general increase in the CVL cytokines and chemokines in all 3 vaccine groups (rVSVpcs, rVSVgag/env and control-rVSVwt) after prime based on the fold increase of almost all cytokines (Fig.2). A 3-fold increase in IL-6 (3.19) levels was observed in the control group six weeks after prime (Fig. 2). During the same period, more than 3-fold increase in IFNγ (4.6), RANTES (4.8), GM-CSF (3.4, 3), MCP-1 (4.4), and IL-17A (3.3) levels were observed in the CVLs of monkeys in the PCS vaccine group (Fig. 2). For the Gag/Env vaccine group IL-6 level was increased 2.4 fold while a lower level of increase was observed for other cytokines. Comparison of the cytokine levels before and after prime (Fig.3-5), VSV vector immunization resulted in the significant increase of cervico-vaginal mucosal RANTES (P=0.0156, week 1) and IL-10 (P=0.0156, week 6*)* (Fig3.), rVSVpcs-immunization resulted in a significant increase of MCP-1(P=0.0078, week 6) (Fig.4), and prime with rVSVgag/env did not result in any significant change in cytokine levels.

This increase of multiple cytokines after prime is likely induced by the replication competent VSVs. We also observed the immunogen effect on cervico-vaginal cytokines by rVSVpcs as more than 3 fold increase in the levels of 6 cytokines after rVSVpcs prime. The lower level increase of the pro-inflammatory cytokines in the Gag/Env vaccine group after prime could be due to the slower replication rate of rVSVgag/env virus carrying larger inserts of full Gag or full Env, than the short 20-amino acid PCS peptide immunogens.

5.1.2. Boosting with combined VSV and NANO-delivered immunogens induced higher levels of multiple cytokines and chemokines in the Gag/Env group

Ten weeks after Boost 1, a marked increase in several CVL inflammatory cytokines was observed in the Gag/Env vaccine group with 3 to 5.9 fold increase in RANTES (4.1), IL-1β(5.9), IL-8(5.3), and MIP-1β(3.3). MIP1-α was increased 2.5 fold (Fig. 2). Boost 1 resulted in significantly higher than pre-immunization level in IL-1β (*P*=0.0234), IL-8 (*P*=0.0078), and MIP-1α (*P*=0.0313) in this group (Fig. 2-3). An initial decrease in RANTES levels (*P*=0.0391) was also observed at week 7 (Fig. 3).

Boosting with rVSVpcs and NANOpcs appeared to have only modest influence on cervico-vaginal cytokines of the PCS vaccine group, with higher than baseline level of MCP-1(2.23 fold) and IFNγ(2.5 fold) was observed (Fig. 2).

After boosting with VSV vector and water, the majority of the CVL cytokines in the control group were lower than their pre-immunization levels. This resulted in the significant lower than baseline level in GM-CSF (*P*=0.0313, week 7), RANTES (*P*=0.0391, week 16), IP-10 (*P*=0.0391, week 16), and IL-17A (*P*=0.0234, week 16) (Fig. 3-5).

5.1.3. Boosting with NANO-delivered immunogens differentially increased CVL cytokine and chemokine levels

Only NANO formulated immunogens or water were used for Boost 2. For the Gag/Env vaccine group boosting with NANOgag/env induced marked increase (4.55 to 12.96 fold) in RANTES (6.64), IL-1β (12.3), IL-8 (4.55), MIP-1α (7.69), and MIP-1β (4.63) a week after the boost (Fig.

2). This resulted in significantly higher than baseline levels of RANTES (*P*=0.0391), MIP-1α (*P*=0.0156), IL-8 (*P*=0.0078), and IL-1β (*P*=0.0156) (Fig. 3). Furthermore, the elevation of MIP-1α (4.84), and MIP-1β (12.96) was persisted 35 weeks after the boost (Fig. 2). Whereas, the level of GM-CSF decreased and was significantly lower (*P*=0.0156) than the pre-immunization baseline 35 weeks after boost 2 (Fig. 5).

Boosting with NANOpcs only resulted in more than 3-fold increase (4.37) (Fig. 2) and a significant higher than pre-immunization level in MCP-1 (*P*=0.0078, a week after the 2nd boost) in the PCS vaccine group (Fig. 4). This higher than baseline level of MCP-1 was maintained 35 weeks after the boost (fold increase: 3.43; *P*=0.0234) (Fig. 4). Whereas, boosting with NANOpcs decreased RANTES and resulted in significant lower than baseline level (*P*=0.0391, a week after the boost; *P*=0.0234, 35 weeks after the boost) (Fig. 3).

As expected water had no effect in boosting cervico-vaginal cytokine level in the control group, and the lower than baseline levels of RANTES (*P*=0.0156), IP-10 (*P*=0.0078), MCP-1 (*P*=0.0391), MIP-1β (*P*=0.0391), and IL-17A (*P*=0.0156) were similar to their levels at the boost (Week 16) except for the decrease in RANTES levels (Fig. 3-5).

5.1.4. Further boosting induced elevated cytokine and chemokine levels in the Gag/Env group

The 3rd boost with rVSVgag/env and NANOgag/env resulted in a pronounced increase in several CVL inflammatory cytokines in the Gag/Env vaccine group. More than 8-fold increase in IL-1β(14.33, 14.43), IL-8(41.67, 4.7), MIP1α(6.68, 10.4) and MIP-1β(8.37) one week or 21 weeks after boost 3 (Week 52 and 72, respectively), with more than 40 fold increase in IL-8 a week after the boost (Fig. 2). The boost maintained significantly higher than pre-immunization levels of MIP-

1β (*P*=0.0156), IL-1β (*P*=0.0156), IL-8 (*P*=0.0156), and MIP-1α (*P*=0.0156) one week after Boost 3, as well as of MCP-1(*P*=0.0313) and MIP-1α (*P*=0.0313) levels 21 weeks after Boost 3 (Fig. 3-4).

Further boosting the monkeys in the PCS vaccine group with rVSVpcs and NANOpcs appears to have little effect on majority of the cytokines except for MCP-1, which was 3.3 fold higher than pre-immunization baseline 21 weeks after the 3rd boost, while RANTES level was lower than at pre-immunization (*P*=0.0391) (Fig. 2-3).

Boosting with VSV vector had little effect on the cervico-vaginal cytokine level of the control group, though the levels of IP-10 (*P*=0.0234, a week after boost 3; *P*=0.0078, 21 weeks after boost 3) and RANTES (*P*=0.0313, a week after boost 3) remained lower than the pre-immunization level (Fig. 3).

5.1.5. Higher dose of vaccine induced persistent elevated levels of CVL cytokines and chemokines in the Gag/Env group

After a higher dosage of rVSVgag/env boost, a strong increase in several CVL inflammatory cytokines (RANTES, 3.22; MCP-1, 4.94; MIP1α, 5.11) was observed in the Gag/Env vaccine group (Fig. 2). Specifically, one week after Boost 4, there was an 11-fold increase in IFNγ (11.33) and close to 5 fold increase in MCP-1(4.94) that were not observed after previous immunizations. Boost 4 elevated several cytokines (IL-8, 3.7; MIP1α, 9.67; IP-10, 9.64) for 18 weeks, and the levels of MIP-1α (*P*=0.0313, a week after Boost 4) and IP-10 (*P*=0.0313; 18 weeks after Boost 4) were significantly higher than pre-immunization levels (Fig. 2-3).

Boosting with higher dose of rVSVpcs did not elevate CVL cytokines in the PCS vaccine group, in fact, majority of the CVL cytokines remained lower with a moderate fold increase of MCP-1 (2.73) one week after Boost 4, and by week 90 (18 weeks later) only the level of IP-10 (3.05) and MIP1α(1.62) were higher than the pre-immunization baseline (Fig. 2). RANTES levels remained lower than pre-immunization levels one week after Boost 4 (P=0.0391) until Week 90 (P=0.0391) (Fig. 3).

The higher dose of VSV vector had little influence on the CVL cytokines of the control group. RANTES (P=0.0156) and IP-10 (P=0.0078) at Week 73 and MCP-1 levels (P=0.0078) at Week 90, were significantly lower than pre-immunization levels (Fig. 3).

The data showed that immunization and boosts with different modalities of vaccines influenced inflammatory cytokines in the cervico-vaginal mucosa. Immunization and boosts with the Gag/Env vaccine resulted in the increase of a broader spectrum and higher magnitude of CVL inflammatory cytokines. This elevation of multiple pro-inflammatory cytokines in the cervico-vaginal mucosa persisted 18 weeks after the final boost. Whereas, immunization and boosts with the PCS vaccine do not appear to have much effect on the cervico-vaginal mucosal inflammatory cytokines.

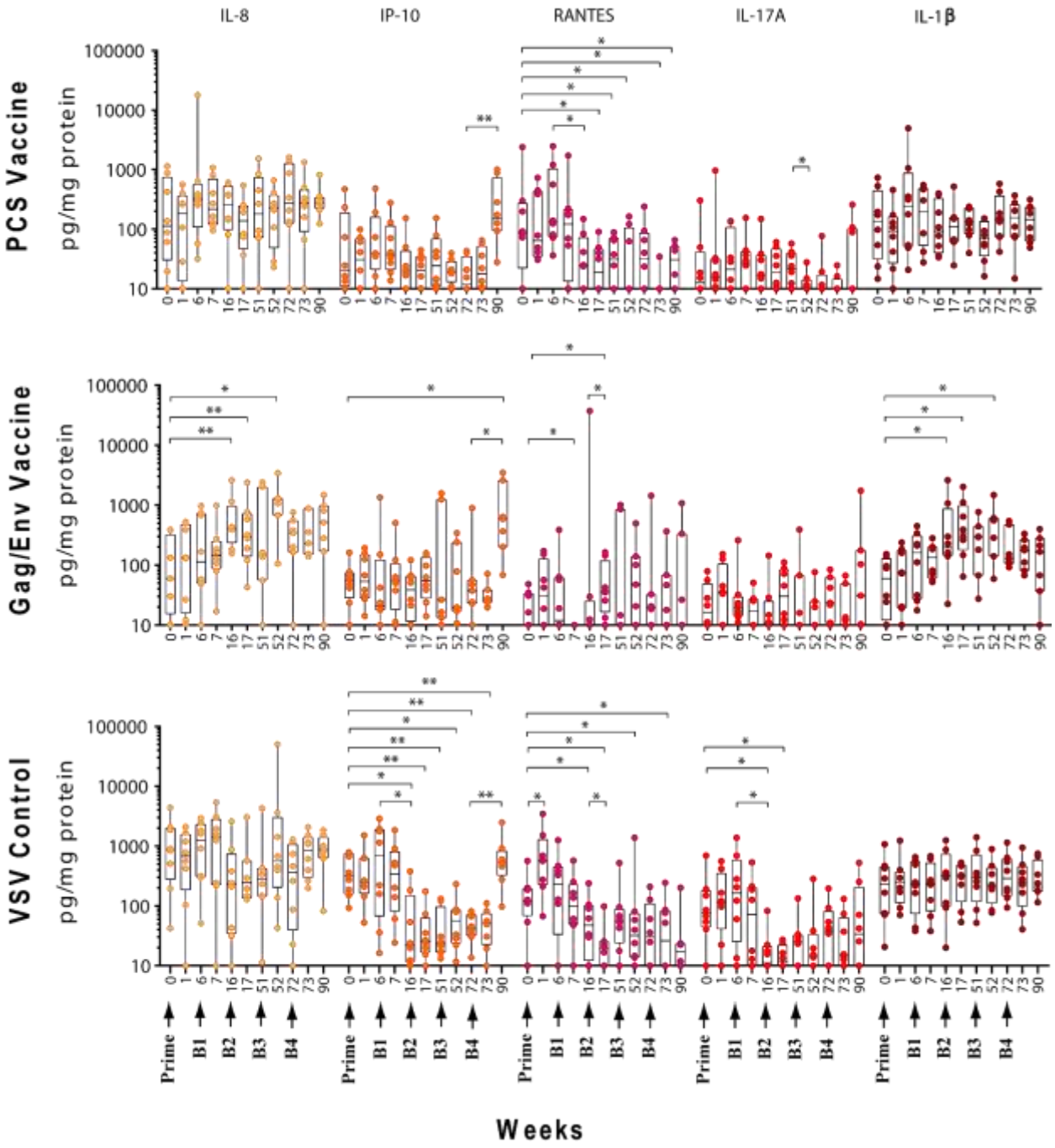

Fig 3. Significant changes in the CVL levels of IL-8, IP-10, RANTES, IL-17A, and IL-1β after immunization and multiple boosts. Data presented as values from individual monkeys with median and range. Arrows indicate immunization. *p<p0.05, **p<0.01, and ***P<0.001.

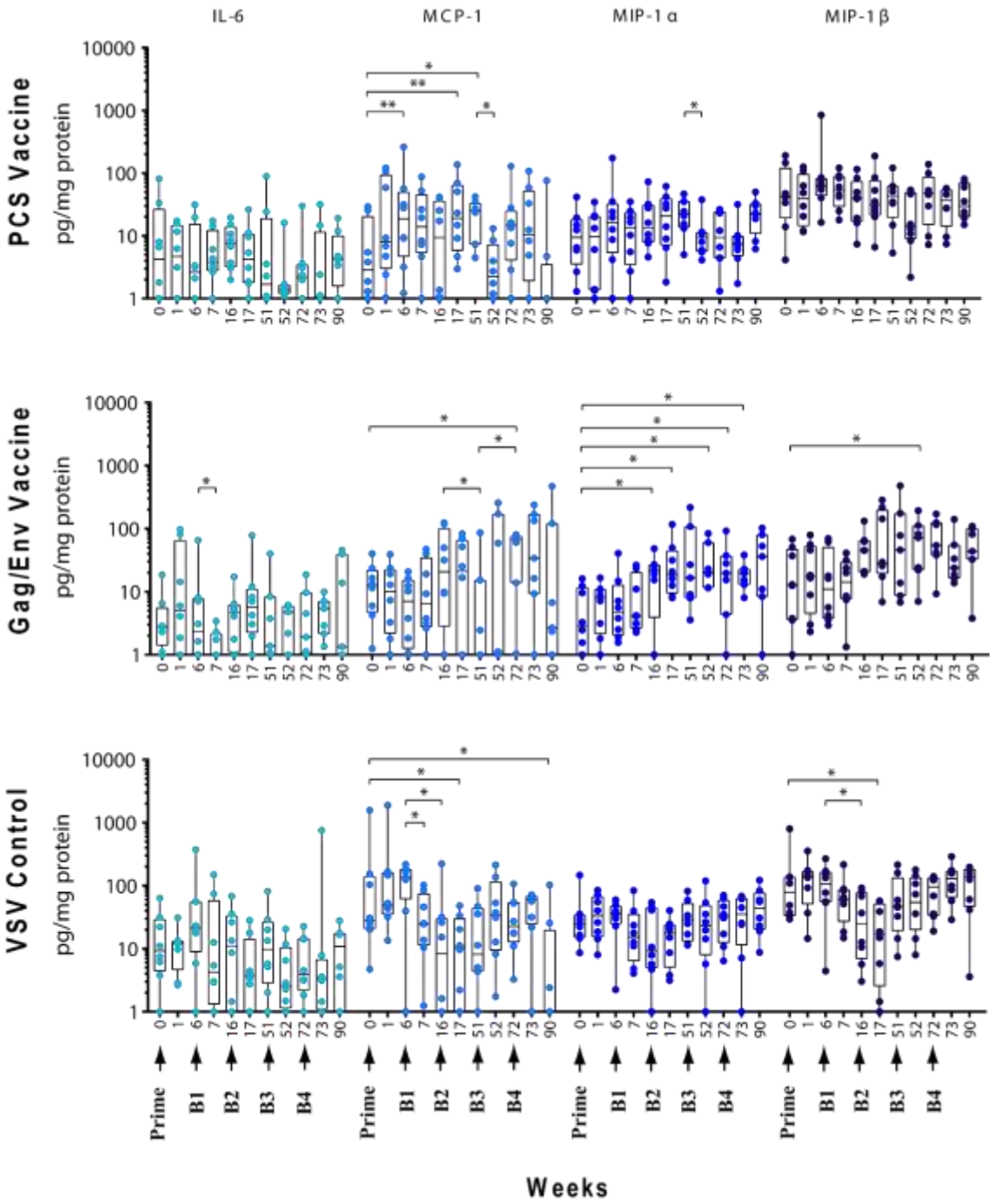

Fig 4. Significant changes in the CVL levels of IL-6, MCP-1, MIP-1α, and MIP-1β after immunization and multiple boosts. Data presented as values from individual monkeys with median and range. Arrows indicate immunization. *p<p0.05, **p<0.01, and ***P<0.001.

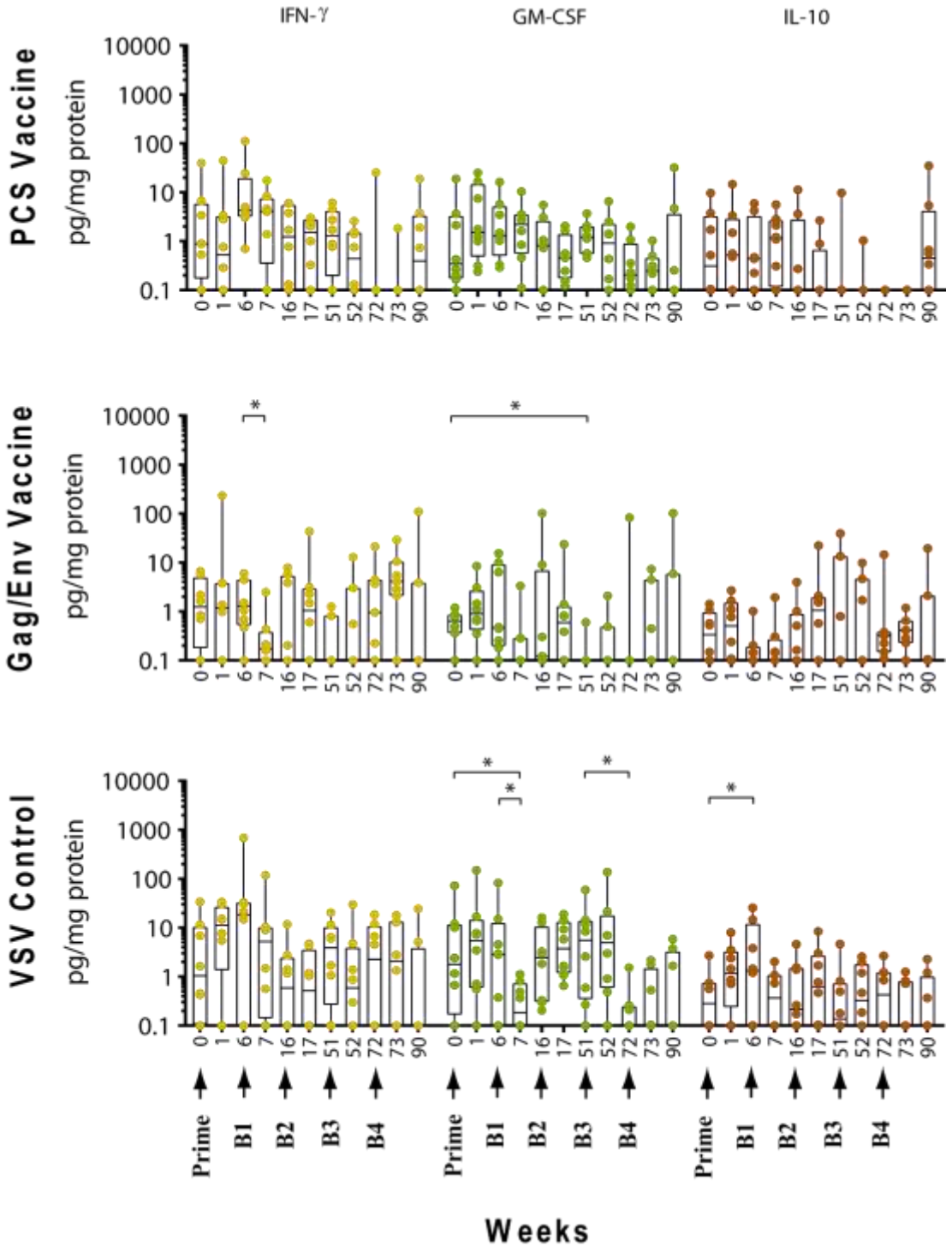

Fig. 5 Significant changes in the CVL levels of IFN-γ, GM-CSF, and IL-10 after immunization and multiple boosts. Data presented as values from individual monkeys with median and range. Arrows indicate immunization. *p<p0.05, **p<0.01, and ***P<0.001.

5.2. The effect of boost with different modalities of vaccines on CVL cytokine level

We also analyzed the levels of CVL cytokines/chemokines at weeks following each boost compared with their pre-boost levels using Wilcoxon Paired Matched t-test (Fig. 3-5). This is to determine the effect of each boost with different modalities of vaccines on the CVL inflammatory cytokine levels of the 3 VSV groups.

5.2.1. CVL cytokine and chemokine levels decreased after boosting with VSV vector and water

Prime with VSV vector and rVSVpcs increased the levels of several cytokines and chemokines. However, no further increase was observed with subsequent boosts. In fact, further boosts with VSV vector resulted in significant decrease in the levels of many CVL cytokines and chemokines. Transient and non-significant increase in the levels of some cytokines were also observed (IL-8, IP-10, IL-17A, IL-6, MCP-1, MIP-1α, MIP-1β, IFNγ, GM-CSF, and IL-10). The significant decrease in most of CVL cytokine and chemokines was observed after the first boost (VSV vector and water). These include MCP-1 (P=0.0156, Week 7; P=0.0391, Week 16), GM-CSF (P=0.0469), IP-10 (P=0.0391), MIP-1β (P=0.0234) and IL-17A (P=0.0156) compared to their levels at the time of Boost 1 (Week 6) (Fig. 3-5). Additional boosts with an empty VSV vector did not induce further significant changes in CVL cytokine levels except for the significant decrease of GM-CSF (P=0.0469) at Week 72 (after Boost 3) and RANTES (P=0.0234) at Week 17 (after Boost 2), as well as the significant increase in IP-10 levels (P=0.0078) at Week 90 after a higher dose of the VSV vector for Boost 4 (Fig. 3). However, the IP-10 level at Week 90 was not significantly higher than the level prior to immunizations (Week 0). These results show that

boosting with the same VSV vector (and/or sterile water) did not maintain the transient increase in CVL inflammatory cytokines observed after prime. The significant decrease in CVL cytokine levels after multiple boosts with VSV vector may be the results of host immune response to the replicative VSVs. Previous studies have shown that anti-VSV antibodies were rapidly induced after immunization.

5.2.2. CVL cytokine and chemokine levels decreased after boosting with the PCS Vaccine

Similar to the Control group boosting with the PCS vaccine significantly decreased the levels of RANTES (P=0.0156, after Boost 1), IL-17A (*P*=0.0391), MCP-1 (*P*=0.0234), and MIP-1α (*P*=0.0156, after Boost 3) (Fig. 3-4). The only significant increase was observed in IP-10 (P=0.0078) at Week 90 after boosting with a higher dosage of rVSVpcs. However, the IP-10 level at Week 90 in the PCS vaccine group was not significantly higher than that of pre-immunization (Fig. 3). Transient insignificant increase was also observed for GM-CSF, IFNγ, IP-10, IL-6, MIP-1α, MCP-1, IL-17A, IL-1β, and IL-10. Boosting with a higher dose of rVSVpcs (Boost 4) induced a modest increase of CVL cytokines, but was not significantly higher than the levels of pre-immunization. These results showed that repeated exposure to the PCS immunogen through multiple boosts did not induce further increase in CVL inflammatory cytokines after prime and instead significantly decreased cytokine levels in the PCS vaccine group.

5.2.3. CVL cytokine and chemokine levels increased after boosting with the Gag/Env Vaccine

In contrast to the PCS vaccine group and the Control group, boosting with the Gag/Env vaccine resulted in the elevated levels of multiple cytokines and chemokines. Although significant increase was only observed in RANTES (P=0.0313, Boost 2), MCP-1 (P=0.0469, Boost 3) and IP-10 (P=0.0156, Boost 4) compared to their levels at the time of the boosts (Fig. 3-4), a general increase after multiple boosts was observed in IL-8, IL-1β, MCP-1, MIP-1α, and MIP-1β. Only the levels of IL-6 (P=0.0391, Boost 1), IFNγ (P=0.0156, Boost 1) and MCP-1 levels (P=0.0313, Boost 2) were significantly decreased compared with their levels at boosts (Fig. 4-5). These results show that repeated boost with the Gag/Env immunogen induced and maintained higher level of most CVL inflammatory cytokines in the Gag/Env vaccine group. As the result the Gag/Env vaccine significantly elevated CVL cytokine levels compared to that of pre-immunization.

5.3.The effect of immunization and boost on the difference of CVL inflammatory cytokines/chemokines between vaccine groups

To determine whether immunization with different modalities of vaccine resulted in significant differences or changes in the mucosal inflammation cytokines and chemokines between the vaccine groups, the levels of cervico-vaginal inflammatory cytokine/chemokines were compared between the 3 VSV groups after prime and each of the boosts. The analysis showed that immunization and boosts with different modalities of vaccines changed the relative differences of multiple CVL cytokines/chemokines among the vaccine groups (Fig. 6-9).

Prior to immunization (Week 0), CVL levels of IP-10 (P=0.0104, and P=0.0006), IL-8 (P=0.007), MCP-1 (P=0.0205), MIP-1α (P=0.0499, and P=0.0019), RANTES (P=0.0104), MIP-1β(P=0.0207), and IL-17A (P=0.0499) in the control group were significantly higher than that of the PCS vaccine and/or the Gag/Env vaccine groups (Figs 6-9). Only the level of RANTES in the PCS vaccine group was higher than that of the Gag/Env vaccine group (P=0.0104) (Fig. 8G).

Several notable changes in the relative levels of cytokines and chemokines among the VSV vector control, the PCS vaccine and the Gag/Env vaccine groups were observed after immunizations and boosts. The significantly higher levels of IP-10 in the Gag/Env vaccine group than that of the PCS vaccine group was only observed after Boost 2 (Week 17) (P=0.0207) (Fig. 6F) with nanoformulated immunogens (NANOgag/env). It appears that the change is due to the increase of IP-10 after NANOgag/env intranasal boost, whereas, no increase in IP-10 levels were observed after NANOpcs intranasal boost. The significantly higher IL-1β levels of the Gag/Env vaccine group than that of the PCS vaccine group was also only observed after Boost 2 (NANOgag/env) (P=0.0379) (Fig. 7D) and the difference of IL-1β between the two vaccine groups was enhanced after Boost 3 (NANOgag/env and rVSVgag/env) (P=0.0379) (Fig. 7E). Thus, the nanoformulated Gag and Env DNA vaccines appear to influence cervico vaginal mucosal IP-10 and IL-1β levels. Whereas, intranasal boost with NANOpcs increased MCP-1 and resulted in the significantly higher MCP-1 levels in the PCS vaccine group than that of the Gag/Env group (P= 0.0471) 35 weeks after the Boost 2 (Fig.7J).

The altered relative levels of IL-8 (Fig.6. I-L), MIP-1α (Fig.8. A-F), RANTES (Fig.8. G-L), among the groups appear to occur due to repeated immunization with different immunogens. Repeated immunizations with the Gag/Env vaccine lead to higher level of these cytokines/chemokines in the Gag/Env vaccine group than that of the PCS vaccine group. However, multiple immunization and

boosts with different immunogens does not appear to alter the relative levels of IL-6, MIP-1β, and IL-17A among the three groups of animals (Fig.9).

In summary, multiple immunization and boosts with different immunogens altered the relative level of many cytokines and chemokines in the cervico-vaginal mucosa among the three groups of animals during the course of immunization. By Week 90, 28 weeks after the last boost, significant differences in IP-10 IL-1β, RANTES, MIP-1α, MCP-1 and IL-17a were no longer observed among the three groups of animals. Except that IL-8 (P=0.0281) (Fig.6L) and MIP-1β (P=0.0499) (Fig. 9I) of the Control group remain significantly higher than that of the PCS vaccine group.

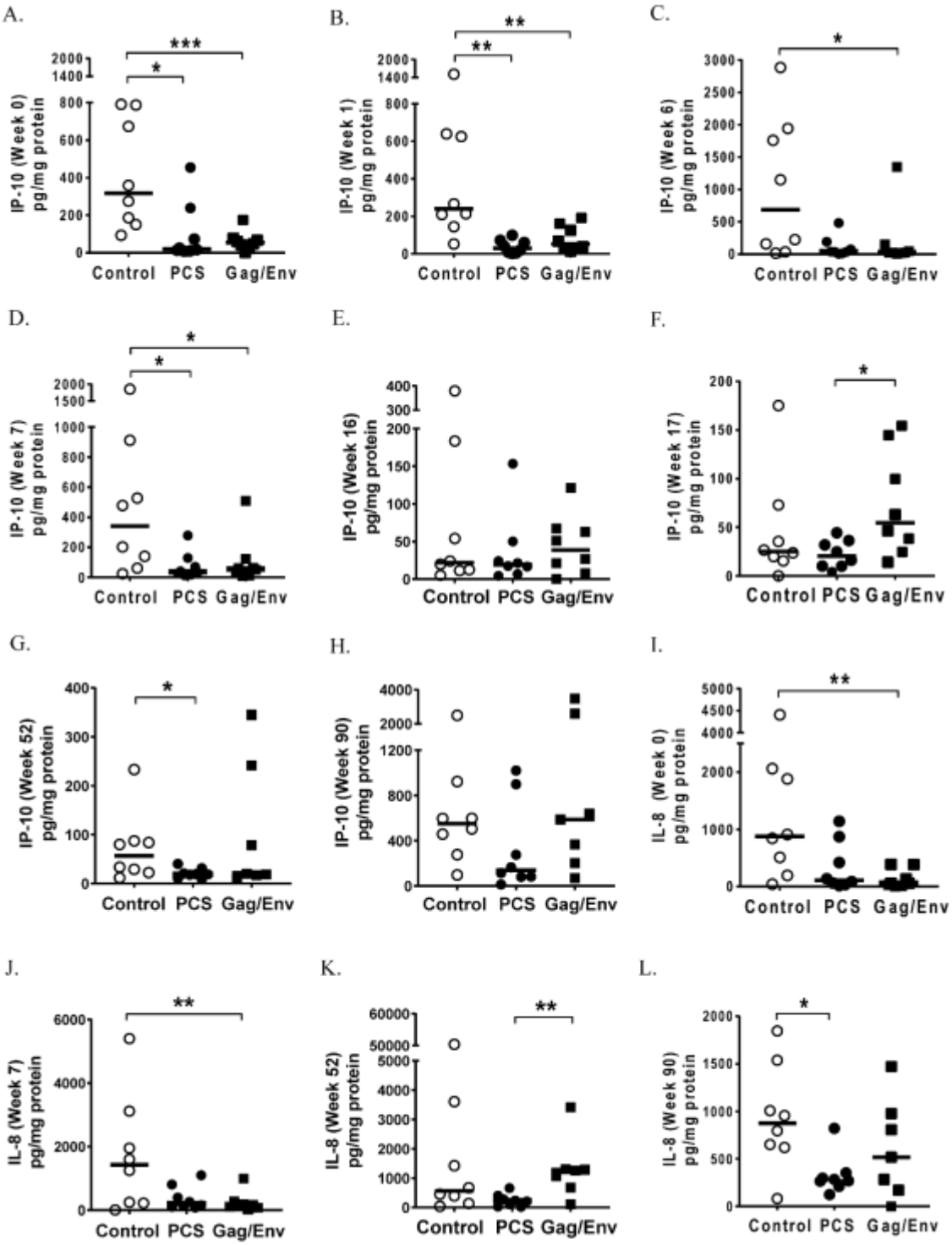

Fig 6. Significant differences in CVL levels of IP-10 and IL-8 among the 3 vaccine groups at different time points including Week 0 and Week 90. Data presented as values from individual monkeys with median. (A-F) IP-10 (G-L) IL-8. *p<p0.05, **p<0.01, and ***P<0.001. Non-significant differences between groups were not included.

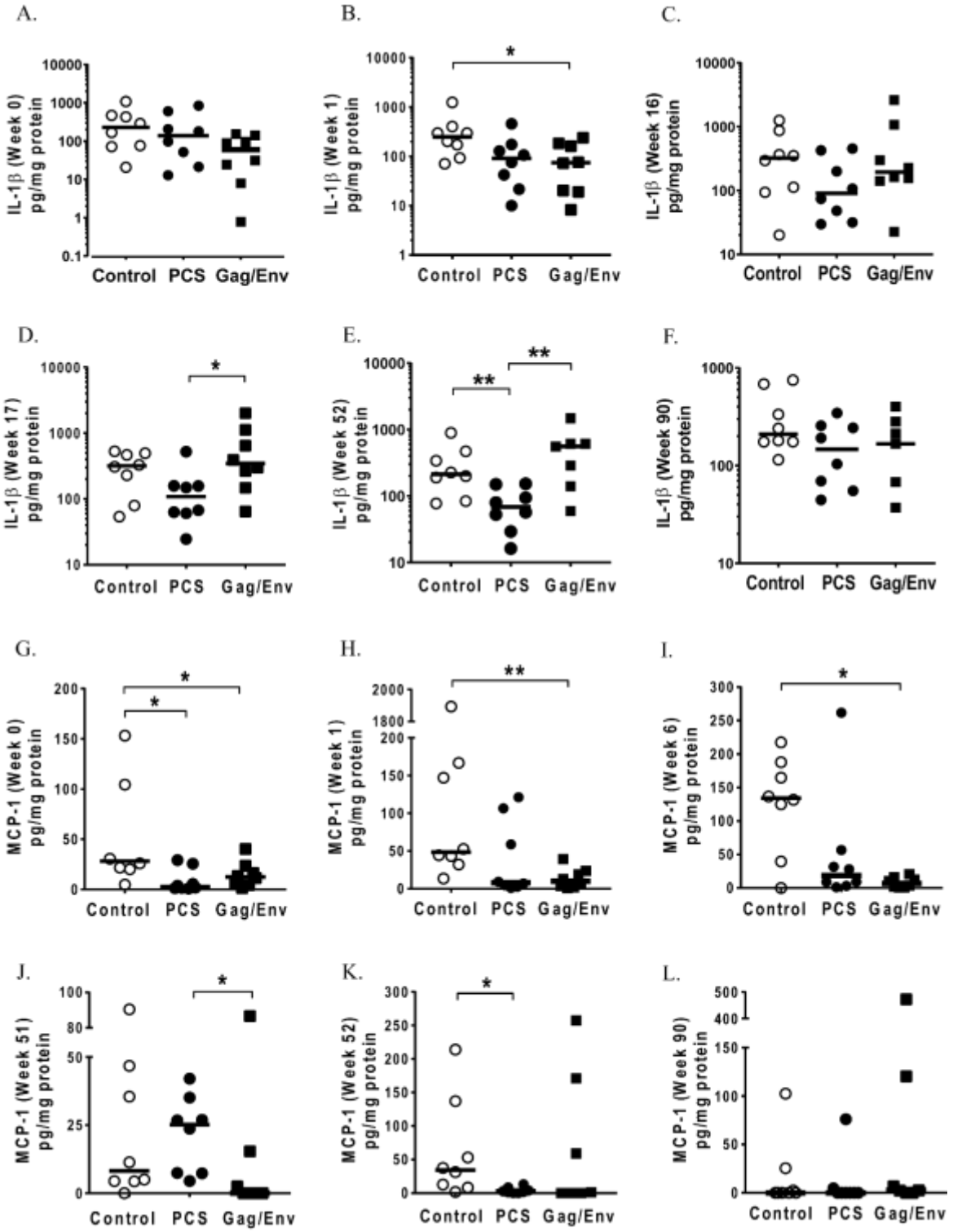

Fig 7. Significant differences in CVL levels of IL-1β and MCP-1 among the 3 vaccine groups at different time points including Week 0 and Week 90. Data presented as values from individual monkeys with median. (A-F) IL-1β (G-L) MCP-1. *p<p0.05, **p<0.01, and ***P<0.001. Non-significant differences between groups were not included.

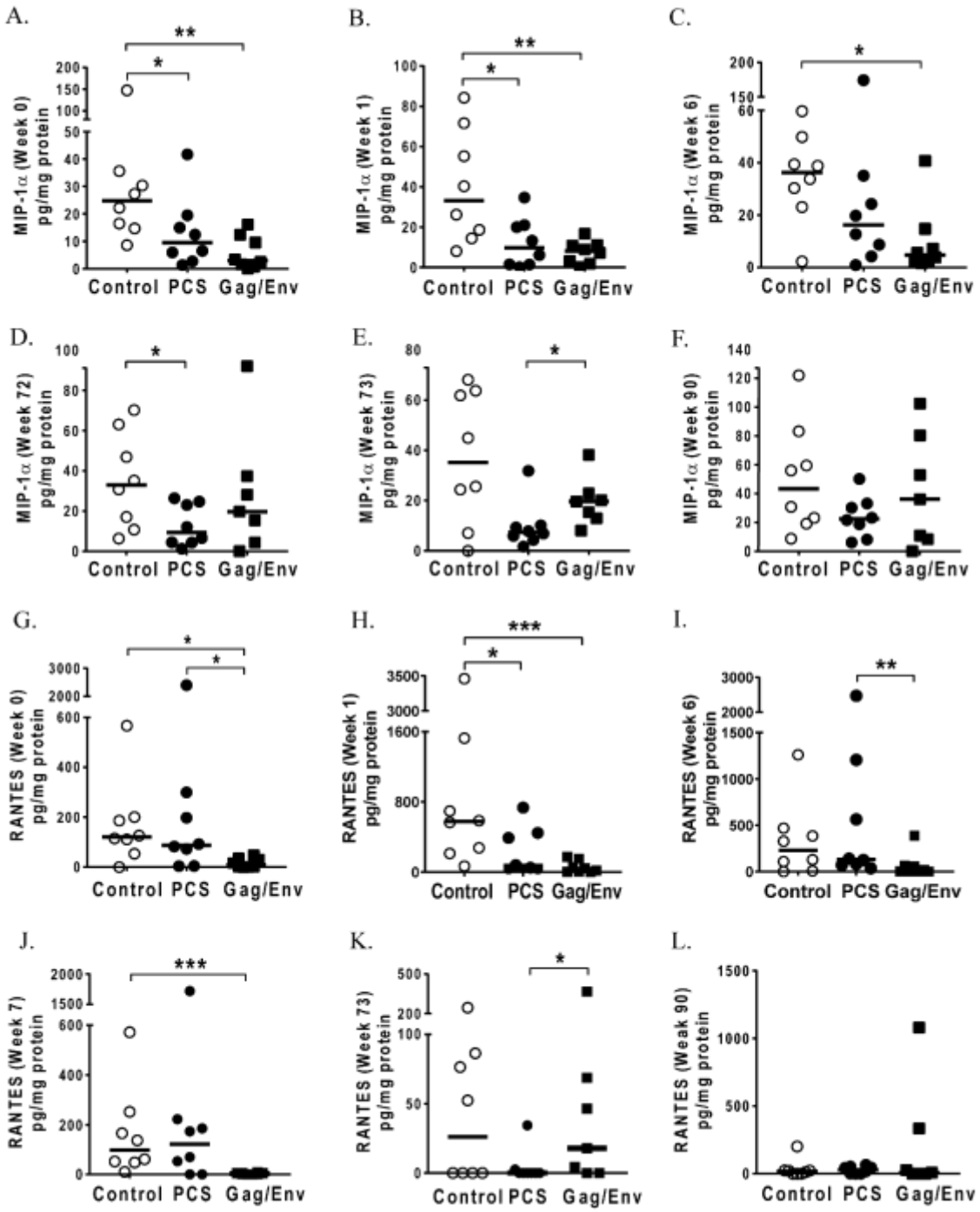

Fig 8. Significant differences in CVL levels of MIP-1α, and RANTES among the 3 vaccine groups at different time points including Week 0 and Week 90. Data presented as values from individual monkeys with median. (A-F) MIP-1α (G-L) RANTES *p<p0.05, **p<0.01, and ***P<0.001. Non-significant differences between groups were not included.

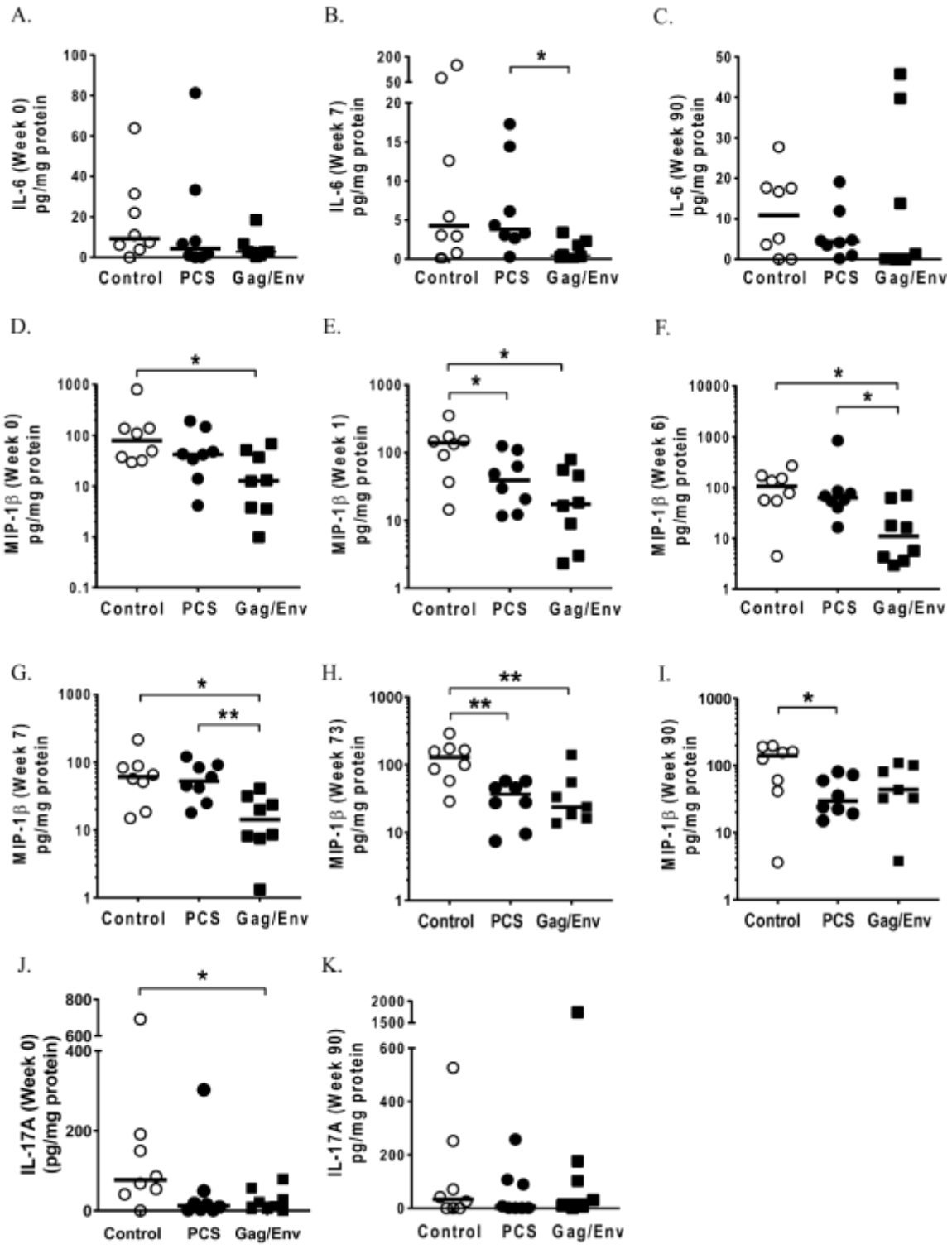

Fig 9. Significant differences in CVL levels of IL-6, MIP-1β, and IL-17A among the 3 vaccine groups at different time points including Week 0 and Week 90. Data presented as values from individual monkeys with median. (A-C) IL-6 (D-I) MIP-1β (J-K) IL-17A. *p<p0.05, **p<0.01, and ***P<0.001. Non-significant differences between groups were not included.

6. DISCUSSION

T lymphocytes, particularly CD4+ T cells, are important mediators of the adaptive immune system and play significant role in coordinating the adaptive immune responses through secreting cytokines and chemokines. CD4+ T cells are also the very target for HIV infection [256]. Studies have demonstrated that infection activates CD4+ T cells and other immune cells, which in turn further facilitates HIV infection, and HIV-specific CD4+ T cells are preferentially targeted by HIV-1 [54, 257, 258]. The infection-activation-infection cycle sustains HIV infection [54] and is one of the great challenges in HIV vaccine development. Because immunization with candidate HIV-1 vaccines would inevitably activate the immune system, including CD4+ T cells, the cytokines secreted by these cells and other immune cells further activate and recruit more HIV target cells [259]. This increase in preferential target cells could diminish the anti-viral immune responses of candidate HIV-1 vaccines [259]. Thus, the balance between the vaccine-induced protective responses and the response contributing to susceptibility needs to be considered in HIV vaccine development [259].

Inflammation activates and attracts HIV target cells. Studies have shown that elevated cervico-vaginal mucosal (CVM) inflammation is associated with increased susceptibility to HIV/SIV infection and reduced efficacy of anti-HIV microbicides [201, 260, 261]. Studies have shown that vaccine vector-induced immune activation can have deleterious effects on vaccine efficacy [44, 60, 61]. However, the effect of different vaccine immunogens on mucosal inflammation has not been reported. The aim of this study is to evaluate the cervico-vaginal inflammatory responses to two vaccines delivering immunogens with different length and characteristics. Using a non-human primate model, our study is the first to demonstrate that vaccine immunogens may influence the mucosal inflammatory cytokines. This study showed that immunization with SIV immunogens

delivered with vesicular stomatitis virus vector (VSV) and nanoformulations influenced the cervico-vaginal mucosal inflammatory cytokines of Mauritian cynomolgus macaques. Immunization with the full Gag and full Env immunogens resulted in the increase of a broader spectrum of CVL inflammatory cytokines with higher magnitude than that by the short PCS peptides. The full Gag and Env immunogen-induced elevation of multiple pro-inflammatory cytokines in the cervico-vaginal mucosa persisted 18 weeks after the final boost. Whereas, repeated immunizations with the short PCS peptides had little persistent effect on the cervico-vaginal mucosal inflammatory cytokines.

Both vector and immunogens influenced CVL inflammatory cytokines

This study showed that prime immunization with the empty VSV vector, rVSVpcs or rVSVgag/env seem to increase the levels of multiple cervico-vaginal inflammatory cytokines. Both the VSV vector and the immunogens appear to contribute to the magnitude and spectrum of the increase of mucosal inflammatory cytokines. Although multiple cytokines increased in the control group after prime with the VSV vector, only the level of IL-6 was increased >3 fold. Whereas, greater than 3 fold increase of five cytokines [IFNγ (4.6), RANTES (4.8), GM-CSF (3.4, 3), MCP-1 (4.4), and IL-17A (3.3)] was observed in the monkeys immunized with rVSVpcs. The modest effect of rVSVgag/env on the mucosal inflammatory cytokines after prime could be due to the slower replication of rVSVgag and rVSVenv than the rVSV and rVSVpcs. The unique characteristic of VSV as vaccine vector to induce strong immunogen-directed humoral and cellular immune responses [237, 253, 262] largely depends on its fast replication [263]. However, it has been demonstrated that although flexible packaging capacity is a major advantage in using VSV as a vaccine vector, adding inserts often lead to slower viral replication and therefore, low viral

titres [262]. It is possible that due to carrying larger inserts, the rVSVgag/env initially induced a weaker and delayed innate immune response after prime, as seen in a previous study comparing CTL responses between VSV-GagEnv and VSV-Gag only immunization [237].

This study also revealed that the effect of VSV vector on mucosal inflammatory cytokines appears to be short lived, as the increase of cytokines after the prime did not last, and further boost with the VSV vector had little effect on the cytokine levels. Whereas, the effect of immunogens on mucosal inflammatory cytokines lasted much longer as demonstrated by the persistent elevation of multiple inflammatory cytokines 6-8 months after boosting with the Gag/Env vaccine. Although nanoformulated immunogens were used alone or together with VSV vectored immunogens to boost the immune responses, the effect on the cervico-vaginal mucosal inflammatory cytokines is likely due to the host response to the immunogens. As previous studies have shown that IgGs to VSV were rapidly induced after immunization with VSV vector and VSV vectored immunogens, and neutralizing antibodies against VSV are detectable even at 300 days post immunization [253, 264]. Thus, the short lived effect of VSV vector on the mucosal inflammatory cytokines could be due to the induced anti-VSV antibodies. However, the anti-VSV vector antibodies did not seem to affect the influence of immunogens on the cervico-vaginal mucosal inflammatory cytokines. As shown in Figure 2-5, over the course of immunization and boosts the PCS vaccine and Gag/Env vaccine differentially influenced cervico-vaginal mucosal inflammatory cytokines. Because only VSV vectored immunogens were used for prime and boost 4, and only nanoformulated immunogens were used for boost 2, the effect of vector and immunogens can be inferred.

The size of immunogens influence the spectrum, magnitude and persistence of mucosal inflammatory cytokines

This study demonstrated for the first time that immunization with immunogens of different sizes may have distinct effects on the magnitude, spectrum, as well as the persistence of multiple cervico-vaginal mucosal inflammatory cytokines. The full Gag and full Env immunogens induced higher and continued elevation of several inflammatory cytokines (RANTES, IL-1β, IL-8, MIP-1α, MIP-1β) over the course of immunization/boosts. Whereas, only the elevation of MCP-1 persisted in the PCS vaccine immunized monkeys despite the multiple boosts. The effect of immunogen size on mucosal inflammatory cytokines became more apparent after Boost 2 using only nanopackaged immunogens. Boosting with the nanoformulated PCS immunogens only led to meagre fluctuations of a few inflammatory cytokines. Whereas, boosting with the nanoformulated full length Gag and Env immunogens elicited a much higher fold increases in IP-10, IL-1β, IL-8, and MIP-1α.

The sustained elevation of mucosal inflammatory cytokines observed in the Gag/Env group after repeated immunogen exposure is likely the effect of immunogen size. Since full Gag and full Env proteins contains many more immunological epitopes than the 12 20-amino acid PCS peptides, it is possible to assume that subsequent repeated boosting with Gag/Env vaccine after the prime reactivated a much broader spectrum of T cells recognizing different epitopes. The activation of these T cells, as well as other immune and epithelial cells, may have resulted in the continued elevation of these pro-inflammatory cytokines. These differences in immune response between the two types of immunogens echo an earlier study that showed substantially higher magnitude and total breadth of HIV-1-specific CD4+ and CD8+ T lymphocyte responses to full length HIV-1 Gag/Pol/Env immunogens in comparison with the conserved HIV-1 regions [265].

The effect of nanoformulated immunogens on CVL inflammatory cytokines

The advantages of intranasal vaccine administration of peptide immunogens and its capacity to elicit both mucosal and systemic immune responses are widely documented [266-268]. Similarly, nanoparticles have been shown to enhance robust humoral and cellular immune responses against various immunogens when used as a carrier [269-272]. Therefore, the mucosal delivery and the intrinsic characteristics of the nanoparticles may activate APCs, and induce stronger host immune responses. In this study nanoformulated PCS peptides and Gag and Env DNA were used as intranasal boosts together with VSV vectored immunogens. Because only nanoformulated immunogens were used for boost 2 we may observe the effect of nanoformulated immunogens on cervico-vaginal inflammatory cytokines. The results showed that increased cervico-vaginal inflammatory cytokines were associated with intranasal boost of nanoformulated immunogens. Intranasal application with nanoformulated PCS peptides elevated MCP-1 for 34 weeks. Intranasal boost with nanoformulated Gag and Env DNA sustained the elevation of MIP1α and MIP1β for 34 weeks in addition to multiple inflammatory cytokines.

The effect of vaccine immunogens on relative inflammatory cytokine level among vaccine groups

Although monkeys were randomly assigned and MHC haplotypes of the different vaccine groups were balanced, significant differences exist in cytokine/chemokine levels at pre-immunization among the different vaccine groups. At Week 0 baseline, the levels of IP-10, MIP-1α, MIP-1β, MCP-1 IL-8, IL-17A, and RANTES in the Control group were higher than that of the Gag/Env vaccine and/or the PCS vaccine groups (Fig.6-9). Immunizations with the PCS vaccine and the

Gag/Env vaccine appear to change the relative differences of multiple CVL cytokines/chemokines among the vaccine groups during course of immunization and some of the effect can be seen 28 weeks after final boost. Significantly lower MIP-1β and IL-8 in the PCS vaccine group than that of controls were only observed 28 weeks after the final boost. The increase of IL-1β after the 2nd and 3rd boost with the Gag/Env vaccine changed its relative level among the three groups. Similar effects were also observed for MCP-1, IP-10, and IL-10.

Do the changes of mucosal inflammatory cytokines matter?

General increase in levels of pro-inflammatory cytokines such as IL-8, IL-1β, IP-10, MCP-1, and β-chemokines MIP-1α, MI-1β and RANTES in the Gag/Env group was observed after multiple boosting. As previously discussed, inflammatory cytokines and chemokines play a significant role in the pathogenesis of HIV-1, at the cellular level. These particular cytokines and chemokines are known to be produced by both innate immune cells such as monocytes, macrophages [53, 199], NK cells and dendritic cells [193, 194], as well as activated CD4+ and CD8+ T lymphocytes [51, 74], and epithelial cells [47]. Further, IL-8, IL-1β, IP-10 are pro-inflammatory cytokines that stimulate increased HIV-1 replication in macrophages and CD4+ T cells [70]. The chemotactic properties of these cytokines also contribute to the immune dysregulation that occurs HIV-1 infection. MCP-1, a chemokine mainly produced by macrophages, is a known regulator of monocyte, NK cells, and memory CD4+ T cells migration [73] while IL-8, IL-1β, IP-10 also recruit neutrophils, T cells, and macrophages to the site of infection [84]. Thus higher levels of these cytokines after immunizations may increase the number of viral target cells and possible induction of HIV replication. In addition, elevated levels of the very same cytokines/chemokines, as

biomarkers of genital inflammation, have also been associated with increase HIV-1 susceptibility [179].

The vaccine immunogen induced changes in mucosal inflammatory cytokines reflect host immune responses to pathogenic peptides. The increased mucosal inflammation may increase activated CD4+ T cells, the preferred targets of HIV-1. Previous observations have also shown that broader epitope binding and recognition, and the subsequently induced immune responses are not associated with viral control [134], but were instead associated with susceptibility to HIV-1 infection. Although multiple boosts (both intramuscular and intranasal) are not the same as the actual exposures to viral infection, they may reflect how the host immune system responds to the repeated exposure to the pathogenic peptides. The persistent elevation of multiple mucosal inflammatory cytokines primed and boosted by the full Gag and Env immunogens may result in the increase of HIV target cells that could eventually diminish the anti-viral immune responses of candidate HIV vaccines [259].

7. CONCLUSION

The study conducted in this thesis evaluated the effects of two vaccines delivering different SIV immunogens on the level of cytokines and chemokines in cervico-vaginal lavage samples from immunized female Mauritian cynomolgus macaques. As hypothesized, the PCS vaccine delivering short conserved peptides induced a lower magnitude and fewer mucosal inflammatory cytokines and chemokines than the vaccine delivering full-length Gag and Env proteins. This was evident from the results showing a general increase in several pro-inflammatory and chemotactic cytokines

in the Gag/Env group after multiple boosting. This increase eventually likely contributed to the relative differences in cervico-vaginal mucosal cytokines between the vaccine groups that persisted long after immunization. The persistent elevation of pro-inflammatory cytokines can have deleterious effects and affect the efficacy of the Gag/Env vaccine. The immunogen-induced mucosal inflammation may tip the balance between the vaccine-induced anti-viral responses and vaccine-induced susceptibility due to immune activation and the increase of target cells. In contrast, the PCS vaccine only induced transient increases of a few cytokines and chemokines after prime, and multiple boosts have shown no effect on mucosal inflammatory cytokines. The findings in this study demonstrated that a candidate HIV-1 vaccine may need to consider immunogen induced mucosal inflammation and immune activation. The immunogen induced mucosal inflammatory environment may tip the balance between vaccine induced anti-viral effect and vaccine induced susceptibility, thus influence the vaccine efficacy.

Analysis of plasma cytokine and chemokine levels might be important to compare systemic and mucosal inflammatory responses to the different vaccine immunogens. Evaluation of inflammatory responses to the immunogens in other mucosal sites could also be informative. In addition, it would also be helpful to determine which specific cell types are producing the detected cytokines and chemokines at each time point.

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9. APPENDIX (Acronyms and Abbreviations)

APC – Antigen Presenting Cell

ART – Antiretroviral Therapy

ARV – Anti-retroviral

CDC – Center for Disease Control and Prevention

CMV – Cytomegalovirus

CSW – Commercial Sex Workers

CTL – Cytotoxic T Lymphocyte

CVL – Cervicovaginal Lavage

DC – Dendritic Cells

EC – Elite Controllers

FGT – Female Genital Tract

GALT – Gut-Associated lymphoid tissue

GM-CSF – Granulocyte-Macrophage Colony-Stimulating Factor

HAART – Highly Activ Antiretroviral Therapy

HESN – HIV-exposed Seronegatives

HIV – Human Immunodeficiency Virus

HLA – Human Leukocyte Antigen

IDU – Intravenous Drug Users

IFN – Interferon

Ig - Immunoglobulin

IL – Interleukin

IN – Integrase

IP-10 – Interferon gamma-induced protein 10

KIR – Killer Immunoglobulin-like Receptor

LC – Langerhans Cells

LTNP – Long-term Non-progressors

MCM – Mauritian Cynomolgus Macaques

MCP-1 – Monocyte Chemoattractant Protein -1

MHC – Major Histocompatibility Complex

MIP – Macrophage Inflammatory Protein

MSM – Males having sex with males

Nef – Negative Regulatory Factor

NHP – Non-human Primate

NK cells – Natural Killer Cells

PAMPs – Pathogen Associated Molecular Pathways

PEP – Post-exposure Prophylaxis

PrEP – Pre-exposure Prophylaxis

PCS – Protease Cleavage Sites

PR – Protease

PRR – Pathogen-Recognition Receptor

RT – Reverse Transcriptase

rVSV – Recombinant Vesicular Stomatitis Virus

SIV – Simian Immunodeficiency Virus

ssRNA – Single Stranded RNA

Tat – Transactivator of Transcription

Th cell – T Helper cell

TLR – Toll-like Receptor

TNF – Tumor Necrosis Factor

Vif – Viral Infectivity Factor

VL – Viral Load

VMC – Voluntary Male Circumcision

Vpr – Viral Protein R

Vpu – Viral Protein Unique

VSV – Vesicular Stomatitis Virus

WHO – World Health Organization

WNPRC – Wisconsin National Primate Research Center