Regulation of Immune Activation in Models of Resistance to HIV Infection and Delayed Disease Progression

by

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Abstract

Understanding natural mechanisms of protection against HIV infection and disease progression are key priorities for informing vaccine and microbicide design. The research presented in this thesis aimed to characterize mechanisms of defence in HIV-exposed seronegative (HESN) individuals, who naturally resist infection by HIV, and HIV-controllers, who are HIV-infected, but suppress viral replication in the absence of treatment.

Previous studies have linked resistance to HIV infection with low basal levels of gene transcription and reduced production of inflammatory mediators, suggesting an overall state of immune quiescence in HESN. Immune quiescence may also be protective in HIV-infected individuals, as immune activation drives disease progression. The central hypothesis of this thesis is that immune quiescence protects against HIV infection and disease progression by limiting the pool of activated target CD4+ T cells susceptible to HIV infection. This hypothesis was addressed by evaluating immune function in HESN from the Pumwani commercial sex worker cohort and HIV-controllers from the Manitoba elite controller cohort.

In HESN, immune quiescence was marked by low levels of circulating activated T cells and low levels of the proinflammatory mediators IL-1α and IL-8 in the cervical mucosa. Regulatory T cells (Tregs), which suppress T cell activation, were elevated in HESN, and may represent a driver of immune quiescence. Low T cell activation and elevated Tregs

were associated with reduced cellular susceptibility to infection *in vitro*. These data suggest that immune quiescence protects against infection by limiting the activated target CD4+ T cell pool, in support of the central hypothesis.

HIV-controllers expressed low levels of the proinflammatory chemokines IP-10 and MCP-1 and low frequencies of activated T cells. These data demonstrate that immune quiescence is not only protective prior to exposure, but is also beneficial following infection. HIV-controllers also had elevated MIP-1α, reduced TGFβ and HIV-specific T cell proliferation responses, which contribute to protection by mechanisms other than immune quiescence.

Taken together, these data support a role for immune quiescence in protection from HIV infection and disease progression. Mechanisms of reducing inflammation and target cell activation should be considered during future HIV vaccine and microbicide development.

Dedication

This thesis is dedicated to my parents, Howie and Josie Card. Dad, your passion for learning inspired me to pursue a PhD, and I know you would be proud. Mum, your constant encouragement and unwavering confidence in me carried me through my education. Thank you both for everything.

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Chapter 1. Introduction

1.1 The HIV Pandemic

The human immunodeficiency virus type 1 (HIV-1) pandemic is undoubtedly one of the world's worst public health adversities in recent history. Its effects reach beyond health and disease, and have had devastating consequences on social and economic outcomes, particularly in high prevalence areas such as sub-Saharan Africa. HIV-1 (herein referred to as HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS). There are currently an estimated 33.3 million people infected with HIV worldwide, with an estimated 2.6 million new infections per year [1]. Moreover, women are disproportionately affected by HIV/AIDS and represent more than half of all HIV-infected individuals in sub-Saharan Africa. While current prevention strategies have helped to curb transmission, the rate of new infections remains high and additional prevention options are desperately needed. Understanding the mechanisms by which some individuals are able to naturally resist HIV infection, or control virus replication in the absence of therapy, have become crucial means to inform vaccine and microbicide design. The aim of this thesis is to characterize possible mechanisms of defence against HIV infection and disease progression using two observational cohorts based in Kenya and Canada.

1.1.1 Kenya

The 2007 Kenya AIDS indicator survey estimated HIV prevalence at 7.1% (approximately 2.9 million infections) [2], but prevalence reached 13.6% at the height of the epidemic

[3]. In sub-Saharan Africa, HIV transmission occurs primarily by heterosexual transmission, with sex workers and their clients representing 14.1% of incident infections [2]. The per-act rate of HIV transmission among Kenyan sex workers has fallen dramatically in recent years, however, likely due to increases in condom use and prevention of other sexually transmitted infections [4]. Chapters 3-6 of this thesis will focus on studies of an observational cohort of commercial sex workers (CSW) based in Nairobi, Kenya.

1.1.2 Canada

In contrast to Kenya, the burden of HIV is generally low in Canada. In 2008, there were an estimated 65,000 prevalent infections [5], which represents 0.19% of the population. HIV transmission in Canada occurs primarily by homosexual intercourse in men who have sex with men (MSM), but heterosexual intercourse and injection drug use also account for a large proportion of transmission events. The HIV epidemic in Canada is marked by diversity in demographic representation, with certain groups experiencing disproportionately high rates of infection. In particular, Canadian First Nations, Métis and people from HIV-endemic countries are overrepresented among the Canadian HIV-infected population [5]. This unique demographic is reflected in the HIV-infected population in Manitoba, where First Nations and Métis peoples account for approximately 15% of the population [6], but 38% of HIV diagnoses [7]. HIV cases in Manitoba are also characterized by relatively high rates of heterosexual transmission, late clinical presentation and rapid disease progression [7]. While high levels of viremia

and progressive CD4+ T cell decline mark the majority of untreated HIV cases, a small proportion of individuals demonstrate natural control of HIV replication. A cohort of HIV-controllers was established in Manitoba to evaluate mechanisms of viral control in this unique population. This cohort will be the focus of Chapter 7.

1.1.3 Origin and history of the HIV/AIDS pandemic

Several non-human primate (NHP) species are naturally infected with simian immunodeficiency virus (SIV). Separate simian-to-human transmission events gave rise to distinct groups of HIV. These cross-species transmissions include chimpanzee to human (HIV-1 groups M and N), gorilla to human (HIV-1 group O and P) and sooty mangabey to human (HIV-2) [8-11]. HIV-1 group M, or 'main', is the cause of the HIV pandemic. The spread of HIV was initially slow, but rapid development of infrastructure facilitated travel and spread of the virus throughout Africa and worldwide.

AIDS went undetected until 1981, when several cases of rare pneumonia were identified among gay men in Los Angeles and New York [12, 13]. HIV was cultured and identified as the causative agent of AIDS in 1983 [14, 15]. In 1984, HIV was found to be present in central Africa [16, 17]. A subsequent study described HIV seropositivity among two thirds of Nairobi prostitutes, demonstrating the presence and scale of the epidemic in East Africa [18]. In the 30 years since the discovery of AIDS, many treatment modalities have been developed, and there is an ongoing effort to develop effective prevention tools.

1.2 HIV Prevention and Treatment

1.2.1 HIV vaccine and microbicide development

HIV vaccine research has recently led to a number of efficacy trials. To date, there have been three HIV vaccines tested in clinical efficacy trials [19-21], the most recent of which demonstrated a 31.2% protective effect in vaccinees [21], although the mechanism of protection is unclear [22].

Until recently, clinical trials of microbicides in humans have had little success [23]. However, a microbicide candidate containing tenofovir, a nucleotide reverse transcriptase inhibitor, was recently tested in the CAPRISA 004 phase IIb trial. The gel reduced HIV acquisition by 39% overall, and by 54% in women with high (>80%) gel adherence [24], lending support for future development of similar formulations.

1.2.2 Antiretroviral therapy

Treatment of HIV infection with antiretroviral therapy (ART) has transformed what was once a fatal infection into a potentially manageable chronic disease. There are now a total of 23 approved drugs from six drug classes, which are most often prescribed in a combinational regimen known as highly active antiretroviral therapy (HAART). Timely initiation of combination therapy results in control of viral replication in the majority of cases. However, despite improvements and expansions of drug programs worldwide, many HIV patients are unable to access ART, especially in resource-poor settings [25].

Indeed, it is estimated that at the end of 2009, only 37% of HIV patients eligible for treatment in sub-Saharan Africa were able to access ART [1].

In addition to the beneficial effects of ART on HIV disease, the prospect of using treatment as a prevention tool shows promise [26]. Viral load is the primary determinant of transmission risk [27], and ART results in significant reductions in viral loads in blood and genital secretions, resulting in a 92% reduction in the risk of transmission to the uninfected partner [28].

1.3 HIV Virology

1.3.1 HIV classification and structure

HIV belongs to the viral family *Retroviridae* and the genus lentivirus. HIV group M can be subdivided into 11 major clades (A1, A2, B, C, D, F1, F2, G, H, J and K). Circulating recombinant forms (CRF) also contribute to the extensive diversity of HIV subtypes. Clades A1, D and regional CRFs are predominant in Kenya, whereas clade B circulates in North America [29, 30].

HIV is an enveloped virus containing two identical positive sense single-stranded RNA (ssRNA) genomes. The HIV genome encodes the envelope (Env) glycoproteins (gp120 and gp41), the internal structural core (Gag) proteins (p24, p55 and p17), the enzymatic polymerase (Pol) proteins (reverse transcriptase (RT), integrase (IN) and protease (PR)) and the accessory proteins Vif, Vpr, Rev, Vpu, Tat and Nef [31].

1.3.2 HIV life cycle

HIV enters target cells by binding of the viral envelope protein gp120 to CD4, which is expressed on the cell surface. A conformational change allows gp120 to bind either of the coreceptors (CCR5 or CXCR4), depending on tropism [32]. Transmission generally occurs in a CCR5-dependent manner, as has been demonstrated by viral tropism determination of transmitted isolates [33]. Protection from HIV infection occurs in individuals homozygous for a 32-base pair deletion in the CCR5 gene (CCR5Δ32), which results in a truncated CCR5 protein that is not expressed on the cell surface [34-36].

Following receptor binding, HIV enters the cell by fusion between the viral envelope and the cell membrane. Reverse transcription results in a single stranded complementary DNA copy, which is replicated to produce the double stranded DNA proviral genome. The process of reverse transcription is error-prone, leading to extensive diversity of progeny genomes. The proviral genome is transported to the nucleus and a linear form of the double-stranded DNA viral genome is inserted into the host chromosome. The integrated viral genome can remain latent for the lifetime of the host cell. However, binding of host factors to the 5' long terminal repeat (LTR) region can lead to transcription of the viral genome [32]. Such host factors are upregulated in activated cells, so efficient viral replication is dependent on host cell activation [32, 37]. Translation of HIV transcripts produces polyproteins that are subsequently cleaved by viral and cellular proteases into individual viral proteins. Assembly occurs at the plasma

membrane and is followed by budding of progeny virions out of the host cell [32].

1.4 HIV Pathogenesis

1.4.1 HIV transmission

HIV transmission occurs through contact with infected body fluids. This may occur through sexual transmission (heterosexual or homosexual), parenteral transmission (injection drug use, receiving contaminated blood products), or vertical transmission (transmission of HIV from mother to child *in utero* or through breast feeding). Although transmission dynamics vary by population, heterosexual transmission accounts for the majority of incident HIV cases worldwide [38].

1.4.2 Disease progression

The typical course of HIV disease progression in the absence of ART is shown in Figure 1. Following transmission, acute HIV infection typically lasts up to three months. Viremia peaks within a few weeks, and is followed by a sharp drop in viral load that coincides with the emergence of HIV-specific CD8+ T cells. This low point in viral load is termed the viral set point [37, 39] and is an indicator of HIV disease progression, such that individuals with low viral set points tend to exhibit slower progression to AIDS [40]. During acute infection, CD4+ T cell levels decline in the periphery but rebound as viral replication is contained. CD4+ T cells in the gut-associated lymphoid tissue (GALT) are extensively depleted early in infection, leading to early dysregulation of gut immunity.

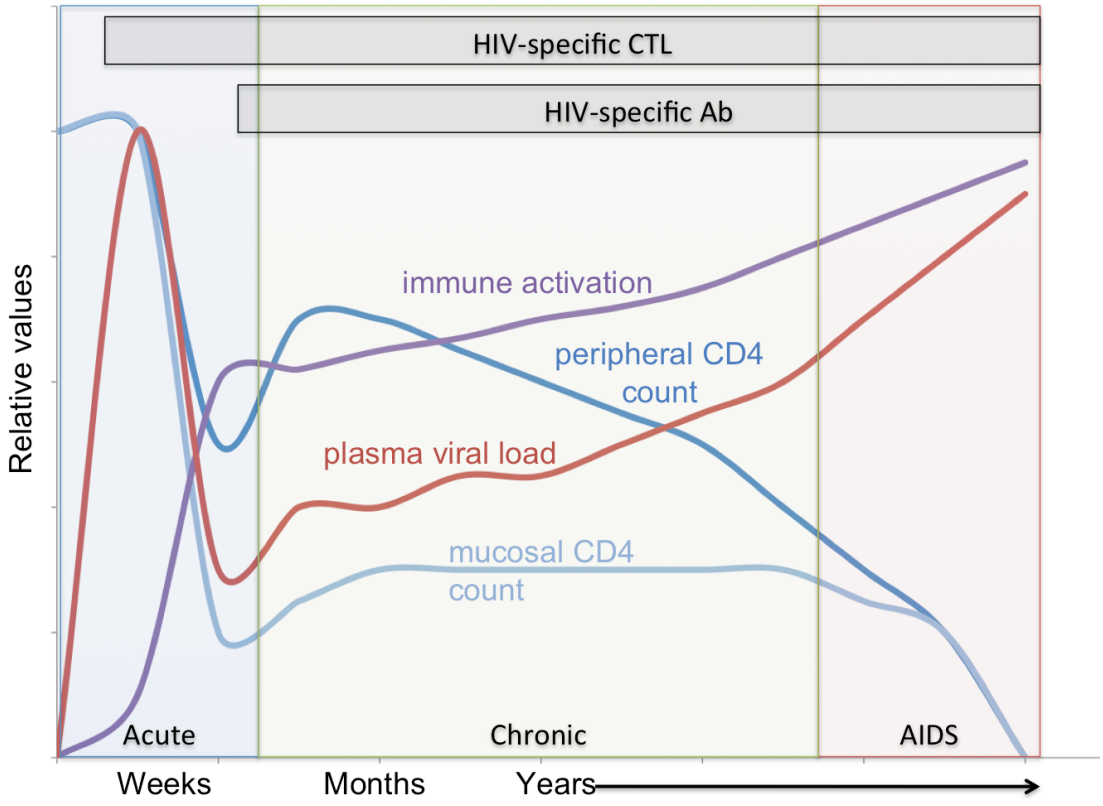


Figure 1. Clinical and immunologic events associated with disease progression in untreated HIV infection.

Acute infection is followed by a much longer chronic phase, in which there is excessive CD4+ T cell turnover and a progressive decline of CD4+ T cells in the periphery. The onset of AIDS occurs when the CD4+ T cell count falls below 200 cells/μl of blood [37, 39]. At this point, the immune system is unable to cope with infectious challenges, and the host eventually succumbs to opportunistic infections.

1.4.3 Models of natural protection

1.4.3.1 HIV-exposed seronegative

There are several documented examples of individuals who remain uninfected by HIV, despite multiple exposures to the virus. Examples of HIV-exposed seronegative (HESN) individuals include commercial sex workers (CSW), HIV-uninfected partners of HIV positive individuals, infants of HIV-infected mothers, parenterally-exposed health care workers, haemophiliacs exposed to contaminated blood products, and injection drug users [41, 42]. There are strengths and weaknesses associated with each model of HESN, but CSW are perhaps the best model of natural protection against HIV infection, as they are continually exposed to a diverse range of viral variants.

Investigators at the Universities of Manitoba and Nairobi have described a group of HESN CSW located in the Pumwani area of Nairobi, Kenya [43]. The Pumwani CSW Cohort has been followed since 1984 with the aim of elucidating the natural correlates of protection possessed by these women. The majority of cohort participants are HIV-infected at enrolment, and of those who were initially uninfected, most seroconvert

within a few years of follow-up [43]. HIV-uninfected participants who remain HIV-negative by both serology and PCR, are still active in commercial sex work and are followed in the cohort for at least seven years are considered to be relatively resistant to HIV infection, and represent an extreme phenotype of HESN. The results presented in Chapters 3-6 of this thesis are based on samples obtained from participants of the Pumwani CSW cohort.

1.4.3.2 HIV-controllers

HIV Elite Controllers and Viremic Controllers (collectively termed HIV-controllers) are HIV-infected individuals who, in the absence of antiretroviral therapy, maintain undetectable (<50 copies/ml) or low viral load measurements (<2000 copies/ml) during chronic infection [44]. The Manitoba Elite Controller Cohort (MECC) was established to evaluate mechanisms of viral control in this unique population. The results presented in Chapter 7 of this thesis are based on samples obtained from participants of the MECC study.

1.5 Basic Immunology

The human immune system can be broadly divided into innate and adaptive arms. The innate immune system provides protection against invading pathogens non-specifically, and lacks immunological memory. In contrast, the adaptive immune system responds to immunological challenges in an antigen-specific manner and has a memory component, which allows rapid and robust responses to an antigen upon repeated exposure.

1.5.1 Innate immune system

The innate arm of the immune system includes all aspects that lack immunological memory, and thus respond similarly to a pathogen each time it is encountered. Immune responses to pathogens are largely determined by cells of the innate immune system, including neutrophils, monocytes, macrophages, dendritic cells (DC), basophils, mast cells, eosoinophils, natural killer cells (NK) and natural killer T (NKT) cells. Innate cells recognize common pathogen-associated molecular patterns (PAMP) such as lipopolysaccharide (LPS) or double-stranded RNA (dsRNA) through pattern-recognition receptors (PRR) such as Toll-like receptors (TLR). Pathogen recognition triggers activation and maturation of innate cells, which then secrete soluble mediators such as cytokines and chemokines that signal the presence of infection and influence migration and function of other innate or adaptive cell types [45, 46]. The cytokine and chemokine microenvironment has a direct effect on the cells recruited to the site of infection and the polarization of the immune response.

Natural killer cells play important role in infection by destroying infected or malignant cells. Their function is modulated by the combined signals of inhibitory and activating receptors on the cell surface, such as killer immunoglobulin-like receptors (KIRs). If an activating KIR is engaged, it issues a “kill” signal to the NK cell. However, inhibitory KIRs can override this signal by binding to MHC class I molecules on target cells [45, 47].

An important link between innate and adaptive immunity is mediated through professional antigen-presenting cells (APC), such as macrophages and DCs. Macrophages (derived from blood-borne monocytes) patrol inflammatory sites and phagocytose pathogens, which are processed and presented to T cells [45, 46]. DCs are heterogeneous migratory innate immune cells that can be broadly subdivided into myeloid DCs (mDC) and plasmacytoid DCs (pDC), depending on their origin and function. They actively sample their resident environment through endoytosis. Upon activation, they mature and migrate to lymph nodes. Activated DCs (and other APCs) express high levels of MHC-I and -II and costimulatory molecules (CD40, CD80, CD86), allowing them to activate T cells.

1.5.2 Adaptive immune system

In contrast to innate immune cells, adaptive immune cells are antigen-specific. This specificity is due to the T and B cell receptors, the genes for which are recombined in such a way that each cell expresses a unique version of the mature protein, creating enormous diversity in the receptor repertoire. A second cardinal feature of adaptive immunity is the capacity for immunological memory, which leads to rapid and robust antigen-specific responses upon secondary exposure to an infectious agent.

1.5.2.1 B cells and humoral immunity

The humoral arm of the adaptive immune system is comprised of antibodies produced by B cells. When a B cell is activated through its B cell receptor (i.e. membrane-anchored

antibody), it proliferates and undergoes a process called affinity maturation, in which the genes of the B cell receptor are mutated in order to select for antibodies with optimal binding affinity for the cognate antigen. There are several classes and subclasses of antibodies (IgG1-IgG4, IgA1, IgA2, IgM, IgD and IgE), which dictate the antibody localization and function. In addition to affinity maturation, B cell activation stimulates antibody class-switching and maturation of immature B cells into antibody-secreting plasma cells and memory B cells [45, 48].

1.5.2.2 T cells

When T cells recognize peptide-MHC complexes through their T cell receptors (TCR), signal transduction cascades lead to transcriptional activation of genes involved in T cell cytokine production, proliferation and differentiation [48, 49]. There are two major categories of T cells. CD4+ T "helper" (T_H) cells, recognize peptide bound to MHC class II molecules on the surface of professional APCs. CD8+ cytotoxic T lymphocytes (CTL) recognize peptide bound to MHC class I molecules expressed by all nucleated cells, and kill target cells infected by viruses or intracellular bacteria. This may occur through the release of cytotoxic granules containing effectors such as perforin and granzyme B into target cells or through induction of apoptosis via the Fas-FasL pathway [48].

1.5.2.2.1 CD4+ T helper cell subsets

Since the initial description of dichotomous T_H1/T_H2 polarization [50], several subsets of T_H cells have been identified, prompting revision of the original T_H paradigm. There are

currently four main TH subsets, including TH1, TH2, TH17 and Treg cells, but additional subsets are continually being identified and characterized [51].

The cytokine environment in which a naïve CD4+ T cell becomes activated influences the polarization of the TH response through distinct signalling pathways and transcription factors. Specific transcription factors act as master regulators of the lineage (T-bet, GATA-3, RORc/RORγt and FOXP3 for TH1, TH2, TH17 and Treg lineages, respectively). The traditional view of TH cell polarization was that initiation of a particular developmental program would lead to differentiation of a committed lineage. However, recent evidence suggests that flexibility and plasticity of effector TH cells exists, such that CD4+ T cells can switch between expression of different master regulator transcription factors and change their cytokine expression profiles [51, 52]. Although TH1 and TH2 cells demonstrate relative lineage stability, Tregs and TH17 cells show effector differentiation plasticity. Murine studies have demonstrated reciprocal development of Tregs and TH17 cells, such that the conditions that lead to induction of one cell type suppress development of the other. For example, TGFβ can induce naïve TH cells to differentiate into Treg or TH17, depending on the cytokine microenvironment. In the presence of commensal microbiota or food antigens, DCs produce retinoic acid, favouring Treg differentiation. However, in the presence of pathogenic bacteria, DCs produce IL-6, which promotes TH17 differentiation [53-55].

1.5.2.2.2 Regulatory T cells

The immune system includes a network of regulatory components that allows appropriate responses against foreign pathogens, but tolerance of innocuous environmental or self antigens. The first line of defence against autoreactive T cells occurs during T cell development in the thymus through central tolerance, which involves deletion of autoreactive T cells [45]. When potentially harmful T cells evade central tolerance, they can be removed by peripheral tolerance mechanisms, including regulatory T cells (Tregs). When an immune response has been effectively mounted against a foreign antigen, Tregs also serve to return the immune system to a homeostatic resting state and prevent collateral damage to self-tissues.

Tregs are a heterogeneous subset of CD4+ T cells with immunosuppressive capacity, and constitute a major player in the regulatory network of the immune system. The idea that T cells may be involved in suppression of immune responses was first introduced in the 1970s, but an inability to positively identify these cells led to the dismissal of this idea. Interest in this concept was rekindled when mouse models demonstrated that suppression of effector T cell responses was mediated by T cells coexpressing CD4 and CD25 (IL-2 receptor α-chain) [56]. The importance of this CD4+ T cell subset in immune tolerance is illustrated by the development of autoimmune disease and allergy upon depletion of Tregs in mice [57]. Disease in Scurfy mice, which spontaneously develop severe autoimmune inflammation, is due to a mutation in the *Foxp3* gene [58]. Similarly, mutations in *FOXP3*, the human equivalent of murine *Foxp3*, lead to immune

dysregulation polyendocrinopathy entropathy X-linked (IPEX) syndrome, which is manifested by severe autoimmunity and allergy [59]. These findings indicate that FOXP3, a member of the forkhead/winged helix family of transcription factors, is the master regulator controlling the regulatory phenotype.

FOXP3 expression initiates differentiation into the Treg lineage, as illustrated by induction of the Treg phenotype in conventional CD4+ T cells by ectopic expression of FOXP3 [60-63]. FOXP3 acts to enhance and repress multiple pathways, resulting in the establishment of a transcriptional network that effectively programs the suppressive phenotype [64]. FOXP3 also enhances its own expression, while suppressing expression of transcription factors required for the differentiation of other TH lineages [64-67].

Various subsets of Tregs have been described on the basis of their origin and function. Tregs can be broadly subdivided into two populations. Natural Tregs arise during thymic selection and primarily function to regulate self-reactive T cells. Inducible Tregs arise from conventional CD4+ T cells in the periphery in response to signals from the environment. Inducible Tregs include T regulatory 1 (TR1) cells, which secrete IL-10, and TH3 cells, which secrete TGFβ [68]. While natural Tregs constitutively express high levels of CD25 and FOXP3, these markers are upregulated *de novo* in inducible Tregs, rendering the distinction between Treg subsets unclear. Tregs express numerous cell surface markers, including cytotoxic T lymphocyte antigen (CTLA)-4, glucocorticoid-induced TNF-receptor-related protein (GITR), OX40, CD39 and CD73 [69]. However,

these proteins are not Treg-specific, as they can also be expressed by activated T cells. In addition, subsets of Tregs demonstrate differential expression of these proteins. Thus, use of multiple markers allows for characterization of Treg subphenotypes, but identification of FOXP3-expressing CD4+ cells or anti-inflammatory cytokine-secreting cells currently is the standard for classification of cells with regulatory properties.

The heterogeneity of Tregs is underscored by the multitude of mechanisms by which these cells suppress immune responses. Some populations of Tregs suppress T cell activation by secreting anti-inflammatory cytokines, such as IL-10 and TGFβ [70]. CTLA-4 expressed on Tregs can suppress effector T cell activation by directly blocking CD28 binding to CD80 and CD86 on APCs, or by downregulating CD80/CD86 expression, thereby interfering with costimulatory signaling between DC and responder T cells [71]. CD80 and CD86 are also expressed on activated T cells, and can interact with CTLA-4 on Tregs directly, leading to suppression of T cell activation [72]. CTLA-4+ Tregs can also induce APCs to express indoleamine 2,3-dioxygenase (IDO), which acts to degrade the essential amino acid tryptophan needed for T cell activation and further promotes development of Tregs [73, 74]. Direct interactions between Treg and effector T cells can result in suppression of T cell activation in a manner dependent on adenosine and cyclic adenosine monophosphate (cAMP), which blocks T cell activation [75, 76].

Tregs specific for foreign antigens may develop in the periphery upon infection, but there are multiple lines of evidence suggesting that natural Tregs also play a role in

modulating responses to foreign antigens. The mechanism by which natural Tregs recognize pathogen-associated peptides is unclear. Studies comparing the TCR repertoires of Tregs to conventional T cells suggest that the two repertoires are only partially overlapping, with Tregs exhibiting a diverse TCR repertoire. This may account for some of the foreign antigen-specific nature observed in Tregs [77]. There is also evidence that Treg suppressive function can be stimulated by weakly cross-reactive variants of the index peptide, which may allow pathogen-derived antigens to stimulate these cells [78]. Additionally, Tregs may be non-specifically activated through TLR signalling, leading to suppression of responses to non-self antigens [79]. Alternatively, tissue damage resulting from infection may result in release of self-peptides, leading to activation of Tregs and non-specific suppression of effector T cells [80].

1.5.2.2.3 T cell memory

Memory T cells are the progeny of antigen-specific naïve cells that have undergone clonal expansion and contraction in response to infection. Upon secondary exposure to a pathogen, memory cells respond faster and with greater strength than naïve cells stimulated during the primary exposure [81]. These properties of immunological memory provide the basis for vaccination. However, memory T cell populations are heterogeneous, and vary in their expression of phenotypic markers, their functional capacities and homing properties. A conceptual framework for distinguishing between various memory T cell subsets is built upon these differences.

Memory cells can be divided into distinct subsets on the basis of their expression of CD45 isoforms and CCR7, a chemokine receptor that controls homing to lymph nodes [82]. Naïve T cells express CCR7 and CD45RA, but upon conversion to memory cells, CD45RA expression is lost. Central memory T cells (T_{CM}) express CCR7, and home to lymphoid tissues where they rapidly proliferate and produce IL-2 in response to antigen. In contrast, effector memory T cells (T_{EM}) lack CCR7 expression and patrol the periphery where they display immediate effector functions in response to pathogen recognition. Effector memory cells can sometimes regain expression of CD45RA. These cells are terminally differentiated and lack proliferative capacity but retain some effector capability. As such, they are referred to as terminally differentiated effectors (T_{EFF}). Although this paradigm provides a convenient structure for classifying memory T cells, it should be noted that a great deal of heterogeneity exists within subsets, particularly during chronic infection [83-85]. Memory subsets can be further characterized using CD27 and CD28, which are progressively lost with differentiation [86, 87]. A schematic representation of memory T cell subsets is shown in Figure 2.

1.5.2.2.4 Biomarkers of T cell activation

In addition to a wide array of soluble cytokines and chemokines that are upregulated during infection, changes in T cell phenotype occur, which are marked by upregulation of various proteins on the surface of cells. These biomarkers facilitate identification of activated T cells and act as a proxy measure for T cell function and generalized immune activation.

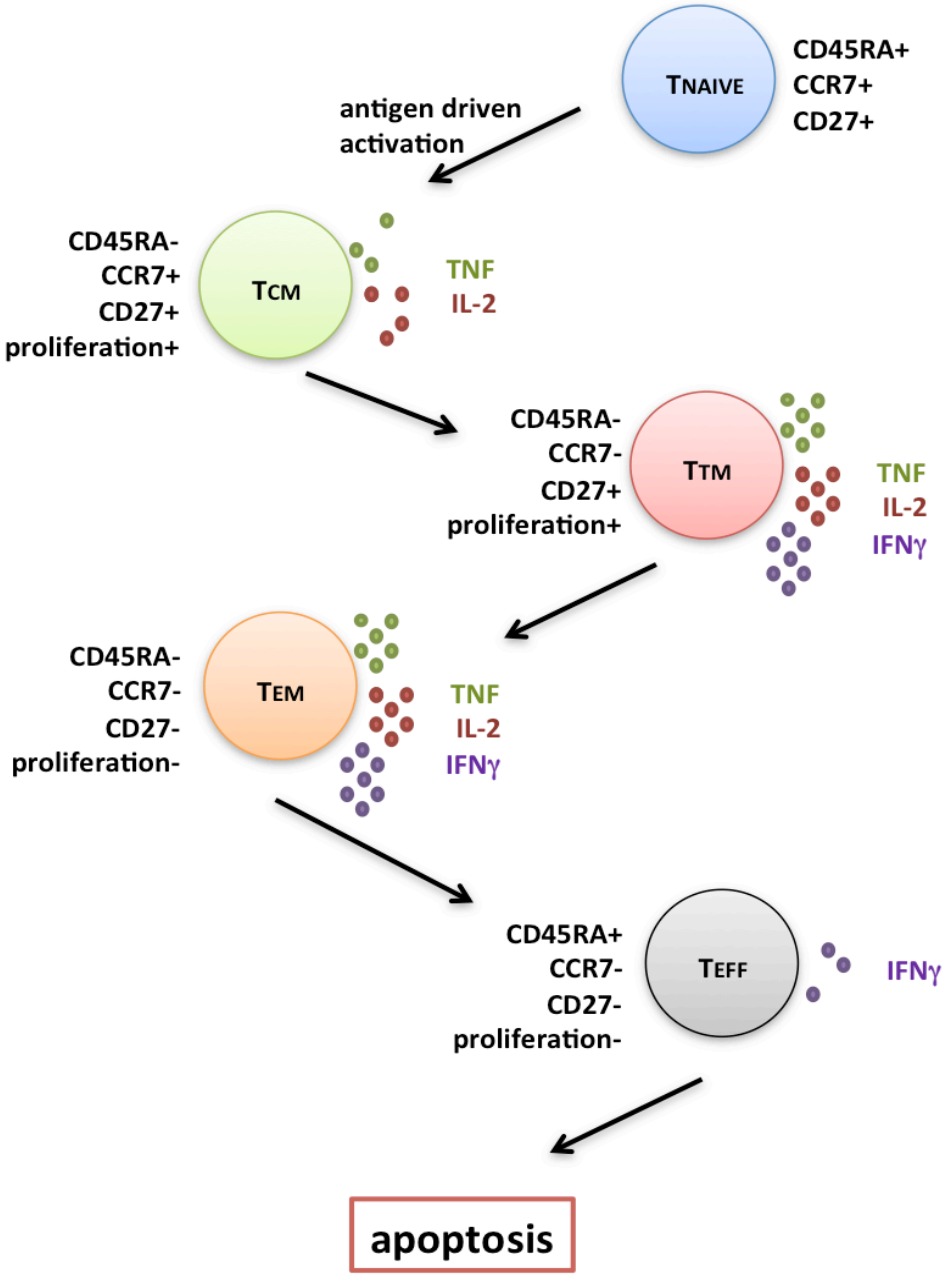


Figure 2. Model of T cell memory development. Following antigen-induced activation, naïve T cells differentiate into memory cells. Memory T cells demonstrate optimal functional capacity as central memory (TCM), transitional memory (TTM) or effector memory (TEM) cells. Persistent antigen stimulation drives memory T cells to a terminally differentiated effector state (TEFF), which is associated with low functional capacity, exhaustion, anergy and apoptosis.

In the early stages following activation, CD69 is upregulated on T cells. This protein plays an important role in preventing egress of activated T cells from lymph nodes and polarizes differentiation of naïve T cells toward a Treg phenotype by blocking TH17 differentiation [88-90]. As such, CD69 is an immunoregulatory molecule. However, since it is expressed upon cellular activation along with other key proteins involved in the cell cycle, CD69 is commonly considered to be a marker of acute T cell activation [91].

Markers expressed later in the T cell cycle include HLA DR and CD38. HLA DR is constitutively expressed by APCs, but is only expressed on T cells during activation. In contrast, CD38 is expressed at high levels on resting naïve T cells, but is downregulated on memory subsets. Activation of memory T cells stimulates expression of CD38 [92].

Other key indicators of T cell function include the capacity for proliferation (marked by Ki67), costimulation (CD28), inhibition and functional exhaustion (CTLA-4, PD-1, Tim-3), T cell senescence (CD57) and apoptosis (CD95/Fas or CD95L/FasL) [87]. These markers are not exclusive of the classical markers of T cell activation discussed above. For example, during chronic activation, CD95 is often coexpressed with other markers of activation [93] or senescence [94], and expression of various markers may vary based on the type of cell or memory subset studied. In this way, T cell phenotyping of multiple biomarkers is ideal for examining immune correlates of protection and disease.

1.5.3 Mucosal immunology

The majority of HIV transmissions occur at the genital mucosa. As such, mucosal immunology is a central issue in studies of HIV susceptibility. The mucosal surfaces of the human body include the gastrointestinal (GI) tract, the respiratory tract and the genital tract. These sites form a structural and immunological barrier, serving as the first line of defence against infectious agents. The cells of the mucosal immune system vary in phenotype and function compared to those observed in systemic circulation. For example, mucosal DCs promote retinoic acid biosynthesis, which promotes Treg differentiation and expression of $\alpha 4\beta 7$ integrin on T and B cells [95].

All mucosal surfaces are colonized by commensal bacteria, which cooperate with mucosal immune cells to induce tolerance, maintain homeostasis and fight infection. This is achieved through organization of polarized CD4+ T cell subsets in the mucosa. In particular, relationships between Tregs and T_H17 cells moderate the balance between tolerance and immunity, and their disruption in HIV disease has profound implications for disease pathogenesis [96]. Our own studies have linked disruptions in commensal microbiota in the female genital tract (FGT) with loss of Tregs in circulation [97].

1.5.3.1 HIV transmission and the genital mucosa

Although HIV can be transmitted at multiple sites including the blood, placenta and GI tract, the genital mucosa is the portal of entry for the majority of HIV transmissions. In the FGT, the multi-layered squamous epithelium of the vagina and ectocervix and the

single-layer columnar epithelium of the endocervix constitute the primary sites at which HIV gains access to target cells and establishes primary infection. The epithelium is penetrated by HIV virions through transcytosis or by traversing the gaps between cells [98]. In addition, the presence of concurrent sexually transmitted infections (STI) such as genital herpes, gonorrhoea and Chlamydia or perturbations in the normal flora of the FGT due to bacterial vaginosis, can lead to increased risk of transmission through introduction of breaches in the epithelial layer or inflammatory immune responses that recruit target cells and fuel virus propagation [99].

Penetration of the epithelial layer leads to uptake of HIV virions by various cell types. HIV readily infects intraepithelial CD4+ T cells as the virus passes through the epithelium. Subepithelial DCs can also be infected by CD4/CCR5-dependent infection, or through binding of HIV to C-type lectin receptors (CLR), although the outcome of CLR binding varies by DC subset [100]. At early stages of infection, a small focal founder population of infected cells is established, which most often arises from a single HIV transmission event [101]. Although HIV and SIV preferentially establish productive infection in activated CD4+ T cells, SIV infection models demonstrate that initial infection events often occur within resting CD4+ T cells, likely due to the relative abundance of these target cells available for infection [102]. In addition, residual activation of these cells and expression of the $\alpha4\beta7$ integrin on resting CD4+ T cells may facilitate infection in the mucosa [103, 104]. The $\alpha4\beta7$ integrin directly binds HIV gp120 and enhances infection [101, 105]. Viral transmission across the mucosal barrier triggers

signalling events in epithelial cells, resulting in production of proinflammatory chemokines, which in turn recruit activated CD4+ T cells and other inflammatory cells to the mucosa. This influx of activated target cells drives expansion of the local founder population and dissemination of infection by migration to lymph nodes and GALT, where activated CD4+ CCR5+ target cells are available in high numbers [103].

Studies of HIV susceptibility in relation to inflammation and T cell phenotypes in the FGT of HESN from the Pumwani CSW cohort are the focus of Chapter 5 of this thesis.

1.6 HIV Immunology

1.6.1 Innate response to HIV infection

As discussed in the previous section, DCs play an important role in transmission of HIV at mucosal surfaces and subsequent systemic dissemination of infection. Viral uptake and stimulation of TLR7 by viral RNA results in DC activation and production of type I interferons (IFNs) and proinflammatory cytokines. Longitudinal studies of plasma donors have demonstrated that the inflammatory responses induced in early infection persist and contribute to the pathology observed throughout progressive disease [106]. The type I IFN response has antiviral properties but also contributes to generalized CD4+ T cell loss through induction of apoptotic pathways [47]. Macrophages are also susceptible to HIV infection due to CD4 and CCR5 expression. During chronic disease, tissue-resident macrophages constitute a major reservoir for latent virus [107]. Lamina

propria-resident macrophages are also impaired as the gut mucosal barrier sustains damage, resulting in reduced microbial processing [108].

HIV infection results in dysregulation of multiple cell types, including DCs [109, 110]. Exposure to HIV has been shown to stimulate pDCs to produce the immunosuppressive enzyme IDO, which stimulates Treg differentiation [111], and mature mDCs from SIV-infected macaques stimulate differentiation of Tregs [112]. Although Tregs may limit inflammation induced by HIV infection, their expansion may disrupt the Treg-TH17 balance, exacerbating the loss of TH17 cells in the GALT that occurs early in disease [96].

1.6.2 HIV-specific B cells and antibodies

The role of B cells in a protective immune response to HIV is contentious. Neutralizing antibodies are not correlated with control of viremia [113]. However, non-human primate (NHP) studies have demonstrated passive protection of neutralizing monoclonal antibodies against virus challenge [114, 115]. In addition, recent studies have identified several highly potent antibodies with broad neutralization coverage of HIV [116-118], lending support for vaccine strategies aimed at inducing antibody antibodies.

1.6.3 HIV-specific T cells

HIV-specific CD8+ T cells arise within the first few weeks of infection as peak viremia declines, and correspond with the emergence of viral escape mutations [119], implicating CD8+ T cells in initial control of viral replication. In line with this, CD8+ T cells

isolated from HIV-infected individuals can inhibit viral replication *in vitro* [120], and depletion of CD8+ T cells from SIV-infected macaques results in a loss of viral control and rapid disease progression [121]. The earliest CD8+ T cell responses are often specific for HIV Env and Nef proteins [122], although responses directed at Gag are associated with viral control later in infection [123]. There are a large number of studies that convincingly demonstrate a requirement for HIV-specific CD8+ T cells in control of viral replication [120, 124-131], but effective CD4+ T cell help is also required [132, 133].

HIV-specific CD4+ T cell responses arise during acute infection, but contract rapidly [134]. HIV infects and progressively depletes CD4+ T cells, and HIV-specific memory cells are particularly susceptible to HIV infection [135]. However, early administration of ART can rescue strong HIV-specific CD4+ T cell responses [134, 136]. Robust HIV-specific CD4+ T cell responses are associated with better control of viral replication [131, 137-140] and HIV undergoes mutational escape from CD4+ T cell epitopes [141].

The induction of HIV-specific T cell responses in acute infection may help curb viremia, but the virus nevertheless disseminates and causes massive destruction to CD4+ T cells in the GALT. Animal models demonstrate that the timing and magnitude of T cell responses elicited in the FGT, the lymph nodes and the GALT are 'too late and too little' to effectively eliminate infected cells and prevent viral dissemination [103, 142]. However, mucosal HIV-specific T cells present at the time of exposure have been shown to be protective against simian-human immunodeficiency virus (SHIV) challenge [143].

During chronic infection, exposure of HIV-specific T cells to persistent viremia leads to functional exhaustion. T cell exhaustion is characterized by loss of proliferation capacity, polyfunctionality and cytotoxic ability, followed by apoptosis. Functional exhaustion is reflected by the up-regulation of different inhibitory molecules on the cell surface, including programmed death 1 (PD-1), CTLA-4, lymphocyte activation gene (LAG)-3, T cell immunoglobulin domain and mucin domain (Tim)-3, 2B4 (CD244) and CD160 [144]. PD-1 has been identified as a critical negative regulator in HIV infection [145-148], and Tim-3 has been linked to elevated sensitivity to Treg-mediated suppression [149].

1.6.4 Immune activation in HIV disease

HIV disease is characterized by aberrant chronic immune activation, the manifestations of which include polyclonal B cell activation [150], increased T cell turnover [151], elevated frequencies of activated T cells [152-154], high levels of activation-induced apoptosis among uninfected T cells [155-157] and elevated proinflammatory cytokine expression [158]. Immune activation is detrimental to the HIV-infected host, and is thought to drive disease progression. Indeed, T cell activation is a better predictor of disease progression than CD4 count or viral load [159-163], and high levels of immune activation are associated with impaired immune reconstitution in patients on ART [164, 165]. Perhaps one of the most important consequences of immune activation is the generation of activated target cells, which can further intensify HIV replication.

Non-human primate (NHP) models of SIV infection support a role for immune activation in driving disease progression. SIV infection of natural hosts does not generally result in AIDS, despite high levels of virus replication. This is in stark contrast to SIV infection of non-natural hosts, which develop similar disease manifestations as HIV-infected humans. Analysis of the immunopathology of SIV in non-natural hosts has demonstrated that establishment of chronic, generalized immune activation distinguishes pathogenic SIV infection from non-pathogenic SIV infection of natural hosts, in which low levels of immune activation are maintained [166-168].

There are numerous consequences of elevated immune activation in HIV infection. High T cell turnover [169, 170] and apoptosis [171] ultimately drain memory T cell pools, which are key mediators of immunity. Indeed, NHP models demonstrate that depletion of CD4+ T_{CM} is a hallmark of disease progression, and results in insufficient levels of T_{EM} cells to target infection [172]. This effect is further exacerbated by the loss of CD127/IL-7Rα expression in HIV disease [173-176], which is central in maintaining homeostasis of memory T cells. Loss of CD127 correlates with T cell activation, and results in increased apoptosis of memory cells [176]. Our lab has previously proposed that disturbance of CD127-mediated homeostasis of memory T cells is a crucial facet of immune activation leading to CD4+ T cell decline in progressive HIV disease [177].

Until recently, the source of systemic immune activation in HIV-infected individuals was unclear. HIV can directly activate DC and NK cells through stimulation of TLR7/8 with

ssRNA [178], leading to the establishment of a proinflammatory environment. However, the extent of immune activation cannot be explained solely by this mechanism, as activation can remain elevated in the absence of high viral loads [162, 164] and high SIV viral loads do not cause generalized systemic immune activation in natural hosts [166].

Recent studies have linked chronic immune activation in HIV disease with the destruction of mucosal barriers. In the early stages of HIV infection, there is a profound depletion of CD4+ T cells in the gut as a consequence of direct viral infection [179-181], an effect that is also observed in pathogenic SIV infection [182-184]. This is in contrast to the peripheral blood, where CD4+ T cells gradually decline over time. Importantly, TH17 cells are preferentially targeted during gut depletion of CD4+ T cells [185, 186]. Due to their role in defence against bacteria and fungi, the loss of TH17 cells renders the gut susceptible to attack by luminal microbes and promotes gut permeability, resulting in microbial translocation from the lumen into the systemic circulation [187]. The resulting increase in circulating microbial products drives immune activation through stimulation of PRRs. An example of this is the increased levels of LPS that have been observed in chronically HIV-infected individuals [187], which stimulates inflammation by binding through TLR4.

Collectively, these findings implicate immune activation in driving HIV disease pathogenesis through induction of tissue damage, inhibition of T cell function, impairment of immune reconstitution and propagation of viral replication.

1.6.5 Tregs in HIV disease

Due to their role in maintaining immune homeostasis and controlling lymphocyte activation and responses to antigen [188], Tregs have been intensely studied in the field of HIV immunology. However, their fate and function in HIV infection remains unclear. Treg levels among HIV-infected individuals is highly variable. Some reports point to a relative expansion of Tregs during progressive HIV disease, likely in response to increases in inflammatory signals [189-198], although conflicting studies have reported decreased Tregs in HIV-infected patients [199-204]. Tregs are susceptible to HIV infection [199, 205, 206], and absolute numbers of Tregs have consistently been reported to decline throughout progressive disease [195-197, 207-211]. This decline may contribute to aberrant T cell activation and accelerated disease progression [203, 207, 209, 210, 212]. In addition, Tregs directly suppress HIV replication in target cells, so loss of this subset could enhance viral replication [213]. Specific subsets of Tregs may also be preferentially affected by HIV infection. In particular, CD39+ Tregs are expanded in HIV-infected subjects [197, 214], and blocking the function of CD39 has been shown to relieve the strong suppressive effect of this Treg subset on effector cells [214]. In addition to direct variations in Treg subsets during infection, relative proportions of Tregs in the periphery may be affected by recruitment of these cells to sites of HIV infection and replication, such as lymph nodes [200, 215, 216] and the gastrointestinal [217, 218] and rectal [219] mucosa, although redistribution of tissue-resident Tregs to the peripheral blood has also been described [220].

Effective HAART has been shown to normalize Treg levels [197, 210, 221, 222] and reduce high rates of Treg turnover [223]. However, individuals with suppressed viral loads but incomplete CD4+ T cell recovery have elevated Tregs relative to individuals with normal CD4+ T cell counts after treatment [224].

Robust CD4+ and CD8+ T cell responses are crucial for control of HIV replication, yet as in other viral systems, these responses are subject to modulation by Tregs. Depletion of Tregs *in vitro* has been shown to lead to increased HIV-specific effector T cell responses [190, 199, 202, 207, 216, 225-227]. The majority of these studies examined the effect of Treg depletion on CD4+ or CD8+ T cell proliferation or cytokine production following stimulation with HIV antigens, but it was also shown that Tregs suppress the HIV-specific cytolytic and nonlytic antiviral response of CD8+ T cells [227]. The effect of Treg depletion may vary between HIV-infected subjects, such that Treg depletion does not rescue proliferative capacity of HIV-specific T cells in progressive disease [226, 227]. However, evaluation of the suppressive effects of Tregs in the lymph nodes of HIV-infected individuals demonstrated that unlike Tregs in peripheral blood, Tregs isolated from lymph nodes of viremic progressors maintained suppressive activity [216], supporting a model whereby functional Tregs accumulate in lymphoid tissue during advanced HIV disease. In addition, HIV-infected individuals demonstrate differences in effector cell sensitivity to Treg-mediated suppression, which complicates analysis of Treg suppressive capacity [149, 211, 228].

NHP models help to clarify some of the discrepancies associated with the role of Tregs in HIV. Consistent with HIV infection of humans [195-197, 207-211], pathogenic SIV infection of rhesus macaques is associated with a loss in Treg numbers, although the relative proportion of Tregs among peripheral CD4+ T cells is maintained [229]. Acute infection is accompanied by a relative increase in Tregs, which may prematurely blunt the development of HIV-specific T cell responses [229, 230]. In chronic infection of SIV-infected macaques, Treg activity correlates inversely with viral load and immune activation [229, 231], although a relative expansion of mucosal Tregs in the gut may be responsible for viral persistence due to suppressed antiviral responses [232]. Conflicting studies have reported a loss of mucosal Tregs in pathogenic SIV infection [233], possibly due to differences in virus strains and animal models. Non-pathogenic SIV infection of African green monkeys is associated with maintenance of Tregs and low immune activation, suggesting a beneficial role for Tregs in SIV disease [229].

Fluctuations in Treg subsets also have implications for HIV pathogenesis due to the reciprocal interactions between Treg and TH17 cells [96]. In pathogenic SIV infection, expansion of the Treg population is accompanied by a loss of TH17 cells [234], further exacerbating the effects of TH17 depletion on GALT integrity that occurs during acute infection. In line with this, elite controllers maintain a Treg-TH17 balance comparable to uninfected controls, whereas individuals with progressive disease experience an elevated Treg:TH17 ratio [235]. This homeostatic disruption may be a consequence of

enhanced production of IDO [111], which induces Treg differentiation and blocks CD4+ T cell conversion into T_H17 cells [236].

The role of Tregs in HIV susceptibility and disease were addressed by evaluating the frequency, phenotype and function of Tregs in HESN and HIV-controllers. The results are presented in Chapters 3-7 of this thesis.

1.7 Models of Natural Protection

1.7.1 HESN

The phenomenon of resistance to HIV infection, observed in multiple cohorts of HESN individuals across the world [41, 42], provides a unique opportunity to study the factors that mediate protection against infection. Studies on HESN have defined genetic associations and both innate and adaptive immune mechanisms that correlate with protection. It is clear that protection is complex, as no single factor accounts for all cases of resistance to HIV infection. Elucidating the natural correlates of protection and identifying common themes between cohorts of HESN will provide valuable information for informing vaccine and microbicide design [42, 237, 238].

1.7.1.1 Genetic correlates of protection

The most definitive correlate of protection against HIV infection described to date is the CCR5Δ32 polymorphism, which results in the generation of a non-functional CCR5 receptor that does not permit HIV to enter the cell. Individuals homozygous for this

deletion are protected against infection [34-36]. However, this mutation is limited to Caucasian populations, and does not contribute to the resistance observed in Asian or African cohorts, including the Pumwani CSW cohort. Polymorphisms in the CCR5 promotor region and CCR2, DC-SIGN and regulated upon activation normal T cell expressed and secreted (RANTES) genes have also been associated with protection in some individuals, but these polymorphisms all occur at low frequencies and do not explain the majority of HESN cases [239-241].

Polymorphisms in the interferon regulatory factor 1 (IRF-1) gene have been described in the Pumwani cohort. IRF-1 is involved in many biological processes, including regulation of immune responses and apoptosis, and can activate HIV transcription. Individuals with protective alleles demonstrate reduced baseline and IFNγ-stimulated IRF-1 protein expression in PBMC [242]. HESN also demonstrate differential kinetics of IRF-1 protein expression following IFNγ stimulation as a result of epigenetic regulation [243].

Molecular epidemiological studies have demonstrated a strong association between resistance to HIV infection and certain HLA Class I and Class II alleles [244-248], which function to present antigen to cognate T cells. The strongest association has been demonstrated in the Pumwani cohort, where the HLA A2/6802 supertype is enriched among HESN [249]. In addition, the expression of specific KIR alleles, such as KIR3DS1, by NK cells has been associated with resistance to HIV infection [250-252].

1.7.1.2 Immune correlates of protection

1.7.1.2.1 Innate immunity in HESN

Innate immunity is likely to contribute to protection upon primary exposure to HIV in HESN, before adaptive responses have developed. Subsequent exposures to HIV may also be limited by innate mechanisms, which block tissue infiltration entirely or attenuate cellular infection until adaptive HIV-specific T cells can target low-level infection [253, 254].

Accumulating functional evidence supports a role for NK activation in resistance to HIV infection [255-260]. Additionally, TLR stimulation of PBMC with TLR agonists resulted in higher cell responsiveness in HESN as demonstrated by elevated production of proinflammatory cytokines and chemokines [261].

Secreted factors have also been associated with reducing susceptibility to mucosal transmission of HIV [254, 262, 263]. The β-chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β and RANTES bind the HIV co-receptor CCR5, thereby reducing HIV infection of target cells by outcompeting the virus for receptor usage. Peripheral production of β-chemokines was associated with resistance to HIV infection [254], and elevated salivary β-chemokines were associated with oral sexual behaviour in HESN MSM [264]. In the Pumwani CSW cohort, expression of RANTES was found to be elevated in the FGT of HESN women [265], but peripheral β-chemokines were comparable between groups [266]. Elevated IL-22 has also been associated with

resistance to infection in HESN, and is thought to act by induction of acute-phase serum amyloid A, which downregulates CCR5 expression on target cells [267].

Cationic proteins such as secretory leukocyte protease inhibior (SLPI), lactoferrin and α- and β-defensins are expressed in vaginal secretions and have anti-HIV activity [268]. Elevated levels of α-defensins were associated with resistance to infection among uninfected female partners in serodiscordant relationships [269]. However, an evaluation of lactoferrin, SLPI and RANTES levels in HESN found these proteins to associate with bacterial vaginosis and inflammation rather than protection [270].

Proteomic evaluation of cervicovaginal lavage (CVL) samples from the FGT of HESN women from the Pumwani cohort have led to the identification of other soluble factors potentially contributing to resistance to HIV infection in these women. Mucosal expression of the anti-protease Trappin-2/elafin was identified as elevated in HESN, and shown to have anti-HIV inhibitory properties *in vitro* [271]. In addition, anti-proteases including members of the serpin B family, a-2 macroglobulin-like 1 (A2ML1) and cystatin A were found to be upregulated in HESN from the Pumwani cohort, and have been proposed to mediate resistance to infection through direct antiviral activity, modulation of immune responses and maintenance of mucosal integrity [272].

1.7.1.2.2 Adaptive immunity

Adaptive immune responses to HIV have been described in multiple cohorts of HESN. These responses likely arise in response to small focal infections that are cleared before dissemination or through immune processing of nonfunctional viral particles or viral fragments, and may be important in clearing low-level infections that occur during subsequent exposure to HIV. This model is consistent with studies demonstrating protection of rhesus macaques from virus challenge following exposure to subinfectious doses of SIV [273].

1.7.1.2.2.1 T cell responses

Multiple lines of evidence suggest that HIV-specific T cell responses are associated with resistance against HIV infection. HIV-specific T cells have been detected in peripheral blood and mucosal samples from HESN from Pumwani [274-285] and other CSW cohorts [276, 285-288], men who have sex with men (MSM) [289-292], discordant couples [290, 291, 293-309], occupationally exposed health care workers [290, 310-312], intravenous drug users [291, 313] and perinatally exposed infants [314-321]. Both CD4+ [277, 283, 289, 294-296, 302, 303, 308, 309, 311] and CD8+ [274, 277, 286, 290, 293, 296, 298, 311, 312, 314, 316-318] T cell responses have been linked with resistance to HIV infection in HESN.

The detection of HIV-specific T cell responses in HESN does not imply that these responses are necessarily involved in protection against infection. Indeed, there are

multiple examples of seroconversion in the presence of natural [280-282, 288] or vaccine-induced HIV-specific T cell responses [322]. In addition, the majority of HIV-infected individuals mount HIV-specific T cell responses, but still experience high levels of viral replication and disease progression. In contrast, studies of HIV-infected individuals with non-progressive disease have demonstrated that the quality of the HIV-specific T cell responses is important in discriminating between protective and non-protective responses [323]. Similar studies have demonstrated qualitative differences between responses in HESN and HIV-infected patients. Proliferation was found to be a protective qualitative aspect of CD4+ T cell responses in HESN from the Pumwani cohort [283], and this finding was confirmed in a comparable cohort [288]. In addition, secretion of IL-2, but not IL-10, by HIV-specific CD4+ T cells characterized responses in uninfected partners in serodiscordant relationships [295]. HIV-specific T cells from HESN have also been shown to secrete β-chemokines such as MIP-1α, MIP-1β and RANTES [294, 299, 302, 307, 308], which suppress HIV replication *in vitro* due to competitive binding to the HIV coreceptor CCR5 [294]. Polyfunctionality, which is the simultaneous production of multiple cytokines from the same cell, has also been associated with HIV-specific T cell responses in HESN [292, 307, 308, 320, 324], paralleling studies implicating similar responses in control of virus replication in HIV-infected subjects [323]. However, further studies are needed to determine if polyfunctional T cells provide similar protection against infection as they do against disease progression.

An assessment of how Tregs affect HIV-specific T cell response magnitude and quality in HESN from the Pumwani CSW cohort is presented in Chapter 4 of this thesis.

1.7.1.2.2.2 Humoral immunity and IgA

HIV-specific IgA has been isolated from the FGT of HESN CSW from the Pumwani cohort [325-328], as well as uninfected partners of discordant couples [326, 328]. However, similar antibodies were not detected in a Gambian HESN CSW cohort [329], and a recent study from Pumwani correlated HIV-specific IgA with increased HIV exposure, but found no association between HIV-specific IgA and virus neutralization or shedding [330].

1.7.1.2.3 Immune quiescence and activation

HIV preferentially establishes productive infection in activated T cells [102, 331]. This preferential infection is due to the large number of host dependency factors required for efficient HIV replication [332-334], which are primarily expressed in activated cells. Low levels of T cell activation may therefore protect against infection by limiting substrates available for HIV replication. Consistent with this hypothesis, mucosal administration of the anti-inflammatory compound glycerol monolaurate (GML) prior to SIV challenge protected monkeys from infection by limiting inflammation at the site of SIV exposure and reducing infiltration of activated target cells [335].

Interrogation of gene expression using microarray technology was used to investigate CD4+ T cell function in HESN from the Pumwani cohort [336]. These analyses revealed

that unstimulated CD4+ T cells from HESN had a lower level of generalized gene expression than controls, suggesting a resting or quiescent cellular state. A subsequent study confirmed these observations by examining gene expression in whole blood [337]. Genes involved in T cell receptor signalling and host factors required for HIV replication were among the genes expressed at low levels in both studies [336, 337]. Unstimulated cells from HESN were also found to secrete lower levels of cytokines *ex vivo* compared to control groups, but this difference in cytokine secretion was not observed following stimulation. These data suggest that HESN have a low baseline level of cellular activation, but respond normally to stimulation and are not immunosuppressed. This phenotype has been termed **immune quiescence** [336]. The overexpression of serpin B anti-proteases in CVL from HESN are supportive of the immune quiescence hypothesis, as serpins have anti-inflammatory activity and their presence in the mucosa may limit inflammation [272, 338].

Evidence for immune quiescence as a protective mechanism in HESN is not limited to the Pumwani cohort. Independent studies of uninfected hemophiliacs who were exposed to HIV-contaminated blood products [339] and of high-risk MSM [340] both found less spontaneous or mitogen-induced lymphoproliferation in HESN compared to healthy controls. In addition, reduced frequencies of activated T cells have been found in several cohorts of HESN [339-343]. Reduced T cell activation and proinflammatory cytokine production was associated with higher levels of unprotected sex in a cohort of

HESN CSW from Abidjan, Côte d'Ivoire [343], suggesting that frequent sexual encounters may induce regulation of inflammation in HESN.

T cell activation will be evaluated in peripheral blood and mucosal samples from HESN in Chapters 3 and 5, respectively. The relationship between T cell activation and cellular susceptibility to infection will be addressed in Chapter 6.

1.7.2 HIV-controllers

Although studies of HESN provide valuable information about how HIV infection may be prevented, it is also important to evaluate mechanisms of protection in HIV-infected individuals. Studies of HIV-controllers have identified both genetic and immune correlates of viral control, which may be translated into vaccine strategies that aim to reduce viral loads in HIV-infected individuals, thereby delaying disease progression and preventing transmission to uninfected partners.

1.7.2.1 T cells and HIV control

The weight of the evidence from multiple studies on HIV control implicates HIV-specific CD8+ T cells in suppression of viral replication [120, 124-131]. CD8+ T cells restricted by certain HLA Class I types are commonly associated with protective responses. In particular, HLA B*57 and B*27, among other alleles, are enriched in HIV-controllers [131, 344], and specific residues that affect the MHC peptide binding groove have been identified that mediate the protective function of these alleles [345]. CD8+ T cells

expressing HLA B*57 exert selective pressure on HIV, forcing the emergence of escape mutations that render the virus less fit for replication [123, 346, 347].

CD4+ T cells are crucial for establishing effective adaptive immune responses to HIV and have been associated with protection in several studies on HIV-controllers [131, 137-140]. Despite multiple examples of T cell control of viral replication, not all HIV-controllers express protective HLA alleles and there are cases of HIV control in the absence of HIV-specific T cells [131, 348], suggesting alternate means of protection.

The quality of HIV-specific responses has implications for HIV control [85, 323]. HIV-specific CD8+ T cells characterized by polyfunctionality [126] or perforin production [129] distinguish protective responses in long-term non-progressors (LTNP) and HIV-controllers, and proliferation responses have been identified as a correlate of slow disease progression in multiple cohorts [126, 349-352], including Pumwani [353, 354].

An evaluation of HIV-specific T cell responses in HIV-contollers from the MECC study will be presented in Chapter 7.

1.7.2.2 Immune quiescence and activation

As discussed, immune activation is a critical factor driving disease progression in HIV-infected individuals. As such, a potential mechanism of HIV control involves a limitation of the pool of activated target CD4+ T cells. Indeed, although HIV-controllers tend to

demonstrate elevated levels of inflammation and T cell activation relative to uninfected individuals [162], this level is drastically reduced in comparison to non-controllers [138, 196, 208, 212, 355].

In contrast to HIV-infected individuals with progressive disease, studies of HIV-controllers and LTNP have demonstrated maintenance of normal Treg levels in the blood [197, 210, 212] and rectal mucosa [219]. The maintenance of normal Treg levels is also reflected in a normal Treg-TH17 balance, in contrast to individuals with progressive disease [235]. Maintenance of this balance may help prevent TH17 losses in the gut and prevent excessive systemic immune activation arising from microbial translocation.

Assessments of T cell activation, Tregs and inflammation in HIV-contollers from the MECC study will be presented in Chapter 7.

1.8 Study rationale, hypothesis and outline

At the time that this project was initiated, the first indications of immune quiescence as a mechanism of protection from infection were arising from studies conducted in the Fowke lab, which demonstrated that cells from HESN had lower levels of baseline gene expression and cytokine production [336]. These observations led to several new directions aimed at characterizing immune quiescence and its role in protection. Specifically, we aimed to examine the activation state of T cells in HESN, the role of Tregs in controlling T cell activation in HESN, the state of inflammation and T cell

activation in the FGT of HESN and the effects of immune quiescence on susceptibility to infection at the cellular level. We were further interested in determining whether immune quiescence would be protective against disease progression in HIV-infected individuals.

The central hypothesis of this thesis is that immune quiescence protects against HIV infection and disease progression by limiting the pool of activated target CD4+ T cells susceptible to HIV infection.

The results of experiments aimed at testing this hypothesis are presented in Chapters 3-7 of this thesis. Chapters 3-6 focus on studies conducted in the Pumwani CSW cohort. In Chapter 3, markers of T cell activation and Tregs are compared between HESN and HIV-uninfected, but susceptible (HIV-N) women. In Chapter 4, the functional capacity of Tregs is characterized in HIV-N, HESN and HIV-infected women. In Chapter 5, immune quiescence is evaluated in the FGT of HIV-N and HESN. In Chapter 6, the relationship between immune quiescence and cellular susceptibility to infection is investigated in HIV-N and HESN. In Chapter 7, the effects of inflammation, immune activation and Tregs on disease progression are evaluated in HIV-controllers and non-controllers from the MECC study.

Chapter 2. Materials and Methods

2.1 General Reagents

2.1.1 Solutions

Phosphate-buffered saline (PBS): 48.5g PBS powder: 137.93mM NaCl, 2.67mM KCl, 8.1mM Na_2HPO_4, 1.47mM KH_2PO_4 (Gibco), dissolved in 1L of ddH20.

FACS wash: PBS with 2% fetal calf serum (FCS), heat inactivated at 56°C for 1 hour (HyClone).

10% Paraformaldehyde (PFA): 48ml ddH20, 2ml 5M NaCl, 5g PFA (Sigma). Solution was heated for 1 minute, then 20μl of 10N NaOH was added to drive PFA into solution. PFA was made fresh weekly.

R-10 Cell culture media: RPMI-1640 (HyClone) supplemented with 10% heat-inactivated FCS and 1% Penicillin/Streptomycin (Gibco).

Complete media: RPMI-1640 supplemented with 10% heat-inactivated FCS and 1% Penicillin/Streptomycin/Fungizone (PSF, Gibco) and 10 international units (IU) of IL-2 (NIH AIDS Research and Reference Reagent Program).

Freezing media: 10% dimethyl sulfoxide (DMSO, Sigma) and 90% FCS.

ELISA coupling buffer: 1.4g Na2CO3 (Sigma), 2.93g NaHCO3 (Sigma) dissolved in 1L of ddH2O, pH 9.6.

ELISA blocking buffer: 2% Goat Serum (Sigma), 0.01% Tween-20 (BDH Chemicals) in PBS.

2.1.2 Antigens used for stimulation

Dynabeads CD3/CD28 T cell expander: Obtained from Invitrogen. Used as a positive control in T cell stimulation assays.

HIV gag peptide pool: Obtained from Sigma. Peptides were generated from consensus sequences based on HIV isolates from the Pumwani CSW cohort and ancestral sequences from the Los Alamos database. HIV peptide pool was used to stimulate HIV-specific T cells. See Appendix (Section 10.1.2) for complete list of peptides included in pool.

Phytohemagglutinin (PHA): Obtained from Sigma. Used to stimulate peripheral blood mononuclear cells (PBMC) prior to *in vitro* infection with HIV isolates.

2.2 General Methods

2.2.1 Cohorts

2.2.1.1 Pumwani Commercial Sex Worker Cohort

HIV status was determined by serology and confirmed by HIV-1 PCR as described below. Study subjects were classified as highly-exposed seronegative (HESN) if they were HIV-uninfected at the time of enrolment into the cohort, remained HIV-uninfected for seven years of follow-up and continued sex work during this time. HIV-uninfected women who were enrolled in the cohort for less than seven years were classified as HIV-negative (HIV-N). The women in the HIV-N population are exposed to the same virus strains and other immunological challenges as HESN, but the majority of them are expected to seroconvert.

2.2.1.2 Manitoba Elite Controller Cohort (MECC).

HIV-infected subjects were enrolled into the Manitoba Elite Controller Cohort (MECC) study during clinic visits at the Winnipeg Health Sciences Centre and Nine Circles Community Health Clinic. Elite Controllers were defined as antiretroviral-naïve individuals with undetectable plasma viral load measurements (< 50 HIV RNA copies/ml) on at least 3 consecutive visits. Viremic Controllers were defined as antiretroviral-naïve individuals with plasma viral load measurements between 50 and 2000 HIV RNA copies/ml on at least 3 consecutive visits. HIV-infected non-controllers were antiretroviral-naïve and were defined by viral load measurements > 5000 HIV RNA copies/ml on at least 3 consecutive visits. Healthy HIV-uninfected control individuals

were also included as a comparison group in the MECC study. These subjects were healthy lab volunteers recruited from The University of Manitoba.

2.2.1.3 Ethics

Written informed consent was obtained from all study participants of the Pumwani CSW and MECC cohorts. The Research Ethics Boards at the University of Manitoba and Kenyatta National Hospital approved the study protocols.

2.2.2 Samples

2.2.2.1 Blood sample collection and processing

Plasma samples were separated from heparinized whole blood by centrifugation at 1600rpm (585 x g) for 7 minutes, and stored at -80°C. Following separation of blood plasma, PBMC were isolated from whole blood by ficoll density gradient centrifugation. Whole blood was layered onto ficoll (Lymphoprep, MJS BIoLynx Inc.) then centrifuged at 1400rpm (448 x g) for 25 minutes. The PBMC layer was extracted, diluted with FACS wash, and centrifuged at 1600rpm (585 x g) for 10 minutes. The cell pellet was resuspended in R-10 media and centrifuged at 1400rpm (448 x g) for 10 minutes. Cells were resuspended in R-10 media and counted using trypan blue (HyClone). Samples from the Pumwani CSW cohort were used immediately. Samples from the MECC cohort were suspended in freezing media (10-20x10^6 cells/ml) and aliquoted into cryovials (Nalgene) then preserved in liquid nitrogen. Prior to use in phenotyping and functional T cell assays, cryopreserved PBMC samples were thawed in a 37°C water bath, washed

twice in R-10 media by centrifugation at 1400rpm (448 x g) for 10 minutes, and rested for 6 hours at 37°C. Cell counts and viability were then determined by trypan blue staining. Samples were characterized if more than 80% of cells were viable.

2.2.2.2 Cervicovaginal lavage (CVL) collection and processing

CVL samples were collected from a 2ml wash of the ectocervix using PBS. Lavages were collected from the posterior fornix into a 15 ml conical tube and transported to the laboratory on ice. CVL samples were centrifuged at 1400rpm (448 x g) for 7 minutes to remove cells and debris, then were frozen into 1ml aliquots and stored at -70°C for subsequent analysis.

2.2.2.3 Cervical mononuclear cell (CMC) collection and processing

CMC were obtained using a standard cervical cytobrush and scraping, which were transferred into 5 ml of PBS in a 50ml conical tube (BD Falcon) and transported to the laboratory on ice. The tube containing the cytobrush was vortexed vigorously for 30 seconds. The cytobrush was agitated in the PBS to dissociate cell clumps and mucus. Extra cells were flushed from the cytobrush. The cervical cell suspension was then filtered through a 100 micron nylon cell strainer (BD Falcon) fitted onto a 50ml conical tube. The specimen collection tube and filter were washed with 5ml of R-10 media to collect remaining cells. Filtrate was centrifuged at 1600rpm (585 x g) for 10 minutes. Supernatant was discarded and pellet was reconstituted in 3ml of PBS and transferred into a 5ml FACS tube (BD Falcon) for flow cytometry.

2.2.2.2 HIV testing and confirmation

Plasma samples from the Pumwani and MECC cohorts were tested for HIV seropositivity. HIV testing was performed using the Recombigen (Trinity Biotech) enzyme-linked immunosorbent assay (ELISA). All samples testing positive in the first assay were confirmed by Detect HIV1/2 immunoassay (Adaltis). Only those samples giving positive results in both assays are considered HIV-1 positive.

2.2.2.3 CD4 T cell enumeration

CD4+ T cell counts were assessed for all HIV-infected participants in the Pumwani CSW and MECC cohorts. Whole blood collected in EDTA tubes was assessed using the Tritest CD3/CD4/CD8 flow cytometry assay (BD Biosciences). To calculate CD4+ T cell count, lymphocyte counts were multiplied by the percentage of CD4+ T cells.

2.2.2.4 Plasma viral load determination

Plasma viral loads were determined for samples from the MECC cohort. HIV RNA in EDTA plasma was extracted and quantified using the automated Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 Test (Roche Diagnostics). The lower limit of detection of this assay is 40 HIV RNA copies/ml with a dynamic range of: 40-10^7 HIV RNA copies/ml.

2.2.3 Flow cytometry

2.2.3.1 Surface staining

Staining of PBMC was performed in 96 well v-bottom plates (Sarstedt), whereas CMCs were stained in 5ml FACS tubes (BD Falcon). Cells were aliquoted into plates (0.5-1x10^6 cells/well) or tubes and pelleted by centrifugation at 1600rpm (585 x g for tubes or 487 x g for plates) for 5 minutes. The supernatant was dumped from the plate or tube and pellets were resuspended by vortex. Cells were then washed with FACS wash by centrifugation at 1600rpm (585 x g for tubes or 487 x g for plates) for 5 minutes, followed by removal of supernatant and resuspension of the cell pellet. Master mixes of antibodies used for staining cell surface markers were prepared according to pre-determined optimal volumes and added to cells. Cells were incubated with antibodies for 30 minutes at 4°C in the dark. Cells were then washed with FACS wash by centrifugation at 1600rpm (585 x g for tubes or 487 x g for plates) for 5 minutes and either stained for intracellular markers (section 2.2.3.2) or fixed with 1% PFA or Fixation Buffer (BD Biosciences). PBMC were transferred to 5ml FACS tubes for data acquisition.

2.2.3.2 Intracellular cytokine staining

Following surface staining, cells were washed with 200μl of FACS wash then 100μl of Fixation/Permeabilization solution (included in the BD Cytofix/Cytoperm Fixation/Permeabilization Kit, BD Biosciences) was added to each well to permeabilize the cell membrane, allowing penetration of antibodies for intracellular markers. Cells were incubated for 20 minutes at 4°C in the dark then washed with 150μl of Perm/Wash

solution (included in the BD Cytofix/Cytoperm Fixation/Permeabilization Kit, BD Biosciences). Use of this buffer maintains permeabilization of cells throughout ICS procedure. Master mixes of antibodies used for staining intracellular markers (e.g. cytokines) were prepared according to pre-determined optimal volumes and added to cells. Cells were incubated with antibodies for 30 minutes at 4°C in the dark. Cells were then washed with 200μl of Perm/Wash and resuspended in 1% PFA or fixation buffer, then transferred to 5ml FACS tubes for data acquisition.

2.2.3.3 FOXP3 staining

FOXP3 is a nuclear transcription factor, and must be stained intracellularly. Following surface staining, cells were transferred to 5ml FACS tubes and washed with 1ml of FACS wash. FOXP3 Fixation/Permeabilization solution was prepared by diluting one part Fixation/Permeabilization Concentrate in three parts Fixation/Permebilization Diluent (both included in the FOXP3 Staining Buffer Set, eBioscience). Diluted FOXP3 Fixation/Permeabilization solution was added to each tube (1ml/tube) to permeabilize the cell membrane, allowing penetration of antibodies for FOXP3 staining. Cells were incubated in FOXP3 Fixation/Permeabilization solution for 60 minutes at 4°C in the dark. Cells were then washed with 2ml of FOXP3 Permeabilization buffer (included in the FOXP3 Staining Buffer Set, eBioscience), which was first diluted from 10X to 1X using distilled water. FOXP3 antibody (clone PCH101, eBioscience) was added to cells, which were then incubated for 60 minutes at 4°C in the dark. Cells were then washed with 2ml

of FOXP3 Permeabilization buffer and resuspended in 1% PFA or fixation buffer for data acquisition.

2.2.3.4 CFSE proliferation assay

Proliferation assays were performed by carboxyfluorescein diacetate succinimidyl ester (CFSE) dye dilution. CFSE passively diffuses into cells, where it reacts with intracellular amines, forming fluorescent conjugates that can be detected at the cellular level by flow cytometry. Cells must be stained with CFSE prior to antigen stimulation. As cells divide, the cellular fluorescence intensity decreases, allowing tracing of proliferated cells [356]. Lyophilized CellTrace CFSE (Invitrogen) was reconstituted in DMSO (Sigma) at a concentration of 10mM and stored at -20°C. Immediately prior to staining, 10mM CFSE was diluted in 1ml PBS (0.5-2μl CFSE/ml PBS). Cells were washed twice in PBS to remove FCS from media then were resuspended at a concentration of $1x10^7$ cells/ml in PBS. An equal volume of diluted CFSE stain was added to cells, followed by incubation for 8 minutes at 37°C in the dark. Following incubation, an equal volume of cold FCS was added to cells for one minute, quenching the staining reaction. Cells were then washed twice in FACS wash and resuspended in R-10 media for stimulations (described in section 2.2.4). Following six days of stimulation, cells were washed with FACS wash and stained for surface and intracellular markers, as described above (sections 2.2.3.1 and 2.2.3.2). Data were acquired by flow cytometry. The excitation and emission peaks of CFSE are 492nm and 517nm, respectively, resembling those of fluorescein

isothiocyanate (FITC). Thus, CFSE was detected by flow cytometry using the channel normally reserved for FITC detection on the blue (488nm) laser.

2.2.3.5 Viability assay

The viability assay employs an amine-reactive fluorescent dye that reacts with free amines, resulting in fluorescent staining. The dye is restricted to surface amines on viable cells, resulting in dim fluorescence. In contrast, dead cells have compromised membranes and stain brightly due to reaction of the dye with amines in the cell interior in addition to those on the cell surface [357]. Lyophilized LIVE/DEAD Aqua dye (Invitrogen) was reconstituted in DMSO at a concentration of 0.5μg/ul, aliquoted and stored at -20°C. Prior to staining, 0.5μg/μl LIVE/DEAD Aqua dye was diluted to 0.005μg/μl (1μl of LIVE/DEAD Aqua in 99μl PBS). Staining of PBMC was carried out in 96 well v-bottom plates, whereas CMC were stained in 5ml FACS tubes. Cells were centrifuged at 1600rpm (585 x g for tubes or 487 x g for plates) for 5 minutes, and supernatant was removed. Diluted LIVE/DEAD Aqua dye was added to cells at a volume of 6.25μl/test. The total staining volume was brought up to 100μl with FACS wash. Plates or tubes were pulse vortexed to mix cells with dye, followed by 20 minutes of incubation at room temperature in the dark. Cells were washed with 200μl FACS wash, then staining of surface and intracellular markers was performed as described above. Data were acquired by flow cytometry. The excitation and emission peaks of LIVE/DEAD Aqua dye are 367nm and 526nm, respectively, resembling those of AmCyan. Thus,

LIVE/DEAD Aqua dye was detected by flow cytometry using the channel normally reserved for AmCyan detection on the violet (405nm) laser.

2.2.3.6 Compensations

Compensation is a process by which spillover fluorescence is removed so that fluorescence values for a parameter reflect only the fluorescence of the antibody or dye of interest. This is done by measuring the fluorescence intensity of each dye individually, then applying a computer-derived algorithm to remove spectral overlap between channels. Compensations were performed for individual antibodies using anti-mouse or anti-rat CompBeads (BD Biosciences), which are nanoparticles that bind any rat or mouse κ light-chain bearing antibodies, respectively. Anti-mouse or anti-rat CompBeads and negative control CompBeads (naked beads with no binding capacity) were vortexed then diluted in PBS (one drop of beads per 300μl of PBS). A single 5ml FACS tube was prepared for each antibody in a multicolour experiment. A volume of 100μl of diluted beads was added to each tube, followed by addition of 1μl of the corresponding antibody, such that mouse-anti-human and rat-anti-human antibodies were mixed with anti-mouse and anti-rat CompBeads, respectively. Tubes were incubated for 20 minutes at room temperature in the dark to allow for beads to bind antibody.

CompBeads could not be used to prepare compensation tubes for CFSE or LIVE/DEAD Aqua dyes. Thus, cells were single-stained with each of these dyes to evaluate spectral overlap. In experiments involving CFSE staining, compensation tubes were prepared with other cells prior to stimulations, and acquired six days later. However, LIVE/DEAD

Aqua dyes stain dead cells with the greatest intensity. Therefore, cells used for compensation were treated with 100% DMSO for 30 minutes then washed with FACS wash prior to staining with LIVE/DEAD Aqua dye.

2.2.3.7 Data acquisition and analysis

Stained samples were acquired on a LSRII flow cytometer (BD Biosciences). Samples were all suspended in at least 200μl of 1% PFA or fixation buffer (BD Biosciences) prior to acquisition. CMC samples were suspended in 750μl of fixation buffer. When acquiring data, 50,000-100,000 lymphocyte events were acquired for each PBMC sample. When acquiring CMC samples, all events in the sample were acquired.

Positive events were defined using fluorescence minus one (FMO) controls. An FMO control is a sample stained with all antibodies in a panel, except for one. The background fluorescence in the unstained channel is measured using the FMO sample then positive gates are set accordingly. Flow cytometry data were analyzed using FlowJo software, version 9.3.1 (Tree Star Inc.). Coexpression of multiple markers was determined using the boolean gating function in FlowJo. Visualization of polyfunctional cells was perfomed using SPICE software, version 5.2 (provided by Mario Roederer, National Institute of Allergy and Infectious Diseases, National Institutes of Health and available at http://exon.niaid.nih.gov/spice) [358].

2.2.4 Stimulations

Fresh PBMC from the Pumwani cohort and thawed PBMC from the MECC were stimulated in cell culture to assess T cell function. Results of PBMC stimulation experiments will be reported in Chapter 4 (Pumwani cohort) and Chapter 7 (MECC).

PBMC were suspended at $1x10^6$/ml in R-10 media, and aliquoted into 5ml snap-capped FACS tubes (BD Falcon) at 1ml/tube or 24 well cell culture plates (Costar) at 1ml/well. PBMC were stimulated with Dynabeads CD3/CD28 (bead to cell ratio 1:1), HIV gag peptides (2μg/ml/peptide), or left unstimulated (media containing DMSO vehicle control). Costimulatory antibodies (1μl/ml each of anti-CD28 and anti-CD49d, BD Biosciences) were added to each tube or well.

For intracellular cytokine staining (ICS) assays, secretion of cytokines must be inhibited using golgi transport inhbitors. After one hour of stimulation at 37°C, 1μl/ml of GolgiPlug containing brefeldin A (BD Biosciences) and 1μl/ml of GolgiStop containing monensin (BD Biosciences) was added to each well, followed by an additional 13 hours of incubation. For proliferation assays, cells were loaded with CFSE dye (Invitrogen) prior to stimulation then cells were incubated for 6 days.

Unstimulated controls were used to calculate background-subtracted responses. In addition, cytokine and proliferation responses to HIV gag peptide stimulation were measured in four HIV-uninfected low-risk volunteers from the University of Manitoba.

The threshold for positive responses was set as two standard deviations above the mean response detected.

2.2.5 Cytokine and chemokine bead arrays

Cytokine and chemokine concentrations were determined in CVL samples from the Pumwani CSW cohort and plasma samples from the MECC study using a custom multiplex bead array kit (Millipore Corporation). This approach allowed for simultaneous detection of 21 analytes: fractalkine (CX3CL1), interferon (IFN)-α2, IFNγ, interleukin (IL)-1α, IL-1β, IL-1 receptor agonist (ra), IL-2, soluble IL-2 receptor (sIL-2R)-α, IL-6, IL-7, IL-8 (CXCL8), IL-10, IL-15, IL-17, IFNγ-induced protein (IP)-10 (CXCL10), monocyte chemotactic protein (MCP)-1 (CCL2), MCP-3 (CCL7), macrophage-derived chemokine (MDC, CCL22), macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4) and tumor necrosis factor (TNF)-α.

The filter plate was pre-wet with 200μl of assay buffer/well (both provided in Millipore kit) then incubated on a plate shaker for 10 minutes at room temperature. Assay buffer was removed from the plate by vacuum filtration then 25μl of assay buffer and 25μl of sample was added to all sample wells. Lyophilized cytokine standards (provided in Millipore kit) were reconstituted with ddH_2O and serially diluted to make the standard curve, which ranged from 3.2 to 2000 pg/ml. A volume of 25μl of each standard was applied to the appropriate standard curve wells in the filter plate. Lyophilized quality control samples (provided in Millipore kit) were reconstituted with ddH_2O then added to

quality control wells along with 25μl of assay buffer. Negative control wells contained 25μl of assay buffer. For MECC plasma samples, lyophilized serum matrix (provided in Millipore kit) was reconstituted in 1.0ml of ddH2O then 25μl was added to standard and control wells. Antibody-coated beads (provided in Millipore kit) were sonicated for 30 seconds and vortexed for one minute to break up aggregates. Mixed beads were added to all wells at 25μl/well.

The filter plate was then sealed and incubated on a plate shaker overnight at 4°C (CVL samples) or one hour at room temperature (plasma samples). Following incubation, buffer was removed by vacuum filtration then the plate was washed twice with 200μl of wash buffer/well (provided in Millipore kit). Detection antibody (provided in Millipore kit) was added at 25μl/well then the plate was sealed and incubated with shaking for one hour (CVL samples) or 30 minutes (plasma samples) at room temperature. Following incubation, 25μl of Streptavidin-phycoerythrin (SA-PE) was added to each well then the plate was sealed and incubated on a plate shaker for 30 minutes at room temperature. Following incubation, buffer was removed by vacuum filtration and plate was washed twice with 200μl wash buffer/well (provided in Millipore kit). Beads were resuspended in 150μl sheath fluid/well then the plate was sealed and incubated on a plate shaker for 5 minutes at room temperature.

Transforming growth factor beta (TGFβ) was analyzed in CVL and plasma samples separately using a single-plex kit (Millipore Corporation). The TGFβ assay required that

the MECC plasma samples be acidified and diluted prior to analysis. Samples were diluted in a 96 well round-bottom plate (Sarstedt) by adding 15μl of plasma to 60μl of sample diluent (provided in Millipore kit). Acidification of diluted samples was done by adding 6μl of 1.0N HCl to each well. The plate was incubated on a plate shaker for 15 minutes at room temperature. Following incubation, 20μl of each treated sample was further diluted in 100μl of assay buffer for a final 1:30 dilution, which was added to the filter plate. The remainder of the assay was conducted as described above for multiplex assay. The TGFβ standard curve ranged from 9.8 to 2500 pg/ml.

Data were acquired on a Bioplex 200 System (BioRad Corporation) and analyzed using Bioplex Manager software, version 5.0 (BioRad Corporation). The sensitivity ranged between 0.2 and 10 pg/ml, depending on the analyte measured. Analyte concentrations below the lower limit of detection were assigned a value of 0 pg/ml. Several analytes were below the limit of detection in CVL samples. As such, data were analyzed if analyte was detectable in >20% of samples.

2.3 Section Specific Methods

2.3.1 T cell phenotypes in HIV susceptibility

2.3.1.1 Flow cytometry panels for ex vivo T cell phenotyping study

Freshly-isolated PBMC were immunophenotyped using multi-colour flow cytometry to characterize T cell memory, activation and Treg frequency. Panels used to assess T cell phenotypes are shown in Table 1.

Table 1. Flow cytometry panels used to assess T cell phenotypes in HIV susceptibility and disease.

Fluorochrome	Panels			
	T cell Activation & Memory		Regulatory T cells	
	Marker	Source	Marker	Source
FITC	CD69	BD	CD127	BD
PE	CD127	BD	CD25	BD
PE-CY5	CD95	BD	--	--
PE-CY7	CCR7	BD	CCR7	BD
ECD	CD45RA	Invitrogen	CD45RA	Invitrogen
APC	CD38	BD	FOXP3	eBioscience
AmCyan	CD3	BD	CD3	BD
AlexaFluor 700	CD4	BD	CD4	BD
Pacific Blue	CD8	BD	CD8	BD
APC-Cy7	HLA DR	BD	--	--

2.3.1.2 Statistical analysis of ex vivo T cell phenotyping study

Continuous variables (i.e. demographic data and T cell phenotypes) were analyzed by non-parametric Mann-Whitney *U* tests. Relationships between continuous variables were assessed using Spearman nonparametric correlation. Differences were considered to be statistically significant if $p<0.05$. All statistical analyses were performed using GraphPad Prism for Mac OS X, version 5.0a (GraphPad software).

2.3.2 Regulatory T cell function

2.3.2.1 Treg depletions

Tregs were depleted from PBMC using an EasySep CD25 positive selection kit (StemCell Technologies). PBMC were washed twice in FACS wash by centrfugation at 1400rpm (448 x g) for 10 minutes then resuspended at 10^7-20^7 cells in 100μl of FACS wash in 5ml snap-capped tissue culture tubes (BD Falcon). Cells were then treated with 50μl of EasySep positive selection cocktail containing CD25-specific antibody and incubated for 15 minutes at room temperature. Following the incubation, 25μl of magnetic

nanoparticles were added to the cell suspension, which was then incubated for 10 minutes at room temperature. The cell suspension was then brought to a total volume of 2.5ml with FACS wash, and the tube was placed in an EasySep magnet for 10 minutes to remove cells bound by magnetic CD25 antibody. The supernatant containing PBMC depleted of CD25+ cells was decanted into a collection tube. Cells were washed in R-10 media by centrifugation at 1400rpm (448 x g) for 10 minutes then resuspended in R-10 media and counted using trypan blue. Negative control (mock-depleted) samples were treated the same as CD25-depleted samples but were not incubated with the CD25 positive selection cocktail. The purity of CD25-depleted cells was assessed by flow cytometry. Mock-depleted and CD25-depleted PBMC were stimulated in snap-capped FACS tubes as described in Section 2.2.4 above.

2.3.2.2 Flow cytometry panels for Treg function study

Flow cytometry was used to characterize T cells at baseline, following Treg depletion and following stimulation. The CD25 antibody clone 2A3 (BD Biosciences) was used to assess purity of CD25-depleted cells, as it binds to a different epitope on the CD25 molecule than the M-A251 clone, which is included in the CD25 positive selection cocktail. Panels used to assess T cells phenotypes are shown in Table 2.

Table 2. Flow cytometry panels used to assess Treg phenotype and function.

Fluorochrome	Panels							
	Baseline Treg		Treg Depletions		Day 1 Cytokine		Day 6 Proliferation	
	Marker	Source	Marker	Source	Marker	Source	Marker	Source
FITC	CD103	Invitrogen	--	--	TNFα	BD	CFSE	Invitrogen
PE	CD25	BD	CD25	BD	IFNγ	BD	--	--
PE-CY5	CTLA4	BD	--	--	CD45RA	Invitrogen	CD45RA	Invitrogen
PE-CY7	CCR7	BD	--	--	CCR7	BD	CCR7	BD
ECD	CD45RA	Invitrogen	--	--	CD8	Invitrogen	CD8	Invitrogen
APC	FOXP3	eBioscience	FOXP3	eBioscience	IL-2	BD	--	--
AmCyan	--	--	--	--	Live/Dead	Invitrogen	Live/Dead	Invitrogen
AlexaFluor700	CD4	BD	CD4	BD	CD4	BD	CD4	BD
Pacific Blue	CD3	BD	CD3	BD	CD3	BD	CD3	BD
APC-Cy7	--	--	--	--	--	--	--	--

2.3.2.3 Statistical analysis of regulatory T cell function study

Demographic and clinical characteristics of the study groups were compared by Kruskal-Wallis non-parametric ANOVA. Comparisons of Treg frequency and T cell responses in mock- and CD25- depleted cultures were assessed by two-way repeated measures ANOVAs. Comparisons of dichotomous variables (i.e. presence of response) between groups were performed with Fisher's exact test. Relationships between continuous variables (i.e. Treg phenotype and magnitude of change in response) were assessed using Spearman non-parametric correlations. Comparisons of cytokine polyfunctionality between mock- and CD25-depleted cultures were done using non-parametric permutation tests (10,000 permutations). All statistical comparisons were performed in GraphPad Prism for Mac OS X, version 5.0a (GraphPad software), with the exception of the permutation tests, which were performed in SPICE software, version 5.2 (provided by Mario Roederer, National Institute of Allergy and Infectious Diseases, National Institutes of Health, and available at http://exon.niaid.nih.gov/spice) [358].

2.3.3 Immune quiescence in the female genital tract mucosa

2.3.3.1 Flow cytometry panels for ex vivo phenotyping of PBMC and CMC

Freshly-isolated PBMC and CMC were immunophenotyped to assess T cells phenotypes using panels shown in Table 3. CMC samples were included in analysis if they had discernable CD4+ and CD8+ T cells populations as indicated by flow cytometry.

Table 3. Flow cytometry panels used to assess T cell phenotypes in the mucosal immunology study.

Fluorochrome	Panels			
	T cell activation		Regulatory T cells	
	Marker	Source	Marker	Source
FITC	CD69	BD	CD103	Invitrogen
PE	CCR5	BD	CD25	BD
PE-CY5	CD38	BD	CTLA4	BD
PE-CY7	CXCR4	BD	CCR7	BD
ECD	CD8	Invitrogen	CD45RA	Invitrogen
APC	--	--	FOXP3	eBioscience
AmCyan	Live/Dead	Invitrogen	Live/Dead	Invitrogen
AlexaFluor 700	CD4	BD	CD4	BD
Pacific Blue	CD3	BD	CD3	BD
APC-Cy7	HLA DR	BD	--	--

2.3.3.2 Statistical analysis of mucosal immunology study

Continuous variables (i.e. levels of cytokines and chemokines, T cell phenotypes) were compared between groups by Mann-Whitney *U* tests. Dichotomous variables (i.e. presence of cytokines and chemokines) were compared between groups using Fisher's exact test. Comparisons of continuous variables between matched samples (i.e. CMC vs. PBMC) were performed using Wilcoxon signed-rank tests. Relationships between continuous variables were assessed using Spearman nonparametric correlation. Differences were considered to be statistically significant if $p<0.05$. All statistical

analyses were performed using GraphPad Prism for Mac OS X, version 5.0a (GraphPad software).

2.3.4 Immune quiescence and susceptibility to HIV infection in vitro

2.3.4.1 Viruses

The HIV strain used for *in vitro* infections was a primary isolate from ML1956, a participant of the Pumwani CSW cohort. We chose this strain over lab-adapted strains because primary isolates are more representative of the viruses encountered by participants of the Pumwani cohort during sex work. HIV_{ML1956} was originally derived by primary co-culture with PHA-stimulated PBMCs, as described by Lane *et al*. [359]. Briefly, PBMCs from HIV-uninfected donors were stimulated with PHA for 3 days then co-cultured with PHA-stimulated PBMCs from the HIV-infected patient. Co-cultures were maintained in complete media (R-10 media containing IL-2), and were supplemented periodically with fresh PHA-stimulated cells from the HIV-uninfected donor to provide the virus with new substrates for replication. Supernatant was removed at regular intervals and virus production was monitored by HIV p24 ELISA. The culture supernatant was harvested at peak p24 production, and the virus was collected and stored at -135°C for future studies.

The sample used to isolate virus from ML1956 was collected in 2001. Full-length HIV proviral sequencing was performed on the isolate, and it was found to be a recombinant of clades A2, C and D, consistent with a high proportion of CRFs in the cohort [30].

2.3.4.2 Virus amplification

The virus stock used for *in vitro* infection experiments was amplified from archived master stocks. Fresh PBMC from an HIV-uninfected donor were suspended in T75 culture flasks (BD Falcon) at $4x10^6$ cells/ml in complete media and stimulated with 5μg/ml of PHA for 3 days at 37°C. The PHA blasts were washed twice in complete media and counted using trypan blue. Cells were then resuspended at $20x10^6$ cells/ml in complete media and taken into the containment level 3 lab for infections.

In the containment level 3 lab, virus stocks were added to PHA blasts to give a multiplicity of infection (MOI) of at least 2, and then cells and virus were incubated for two hours at 37°C. After incubation, cultures were diluted with complete media to bring cells to a concentration of $4x10^6$/ml. Culture flasks were then incubated overnight at 37°C. The following day, 75% of the media was removed and replaced with fresh complete media. Culture flasks were incubated for three days then supplemented with fresh complete media to dilute cells to $2x10^6$/ml. Aliquots of supernatant were removed daily and p24 production in virus cultures was monitored by p24 ELISA. At peak p24 production, supernatant was collected into conical tubes and centrifuged at 1500rpm (514 x g) for 10 minutes. Supernatant was aliquoted into cryovials at 1ml/vial and stored at -80°C for use in infection assays described below.

Complete media and fresh PHA-stimulated 'feeder' cells from the same donor were added back to the cells remaining in the culture flask, and infection cultures were incubated for an additional 7 days, during which time virus production was monitored by p24 ELISA. An additional virus harvest was collected and stored at -80°C for use in infection assays.

2.3.4.3 $TCID_{50}$ virus titration assay

Virus stocks were titrated using a 50%-tissue culture infective dose ($TCID_{50}$) virus titration assay, which calculates the amount of virus required to infect 50% of inoculated wells. Fresh PBMC from an HIV-uninfected donor were suspended in T75 culture flasks at $4x10^6$ cells/ml in complete media and stimulated with 5μg/ml of PHA for 3 days at 37°C. Cells were washed twice in complete media by centrifugation at 1400rpm (448 x g) for 10 minutes, and counted using trypan blue. Cells were resuspended at $2x10^6$ cells/ml in complete media.

Complete media was added to wells of a 96-well round-bottom tissue culture microtiter plate (Costar) at 150μl/well, except for six wells that were to be inoculated with undiluted virus stock, which only received 135μl of complete media well. Plates were taken into the containment level 3 lab for infections.

In the containment level 3 lab, frozen viral stocks were thawed and 65μl of virus was added to the six wells containing 135μl of complete media. Virus was mixed by pipetting

and four-fold dilutions were carried across the plate by transferring 50μl of diluted virus into wells containing 150μl of complete media (1:4, 1:16, 1:64, 1:256, etc.). Six replicates were plated for each dilution of virus. Cells suspended at $2x10^6$ cells/ml were added to all wells of the plate at 50μl/well (equivalent to $1x10^5$ cells/well). Negative control wells consisted of 200μl of complete media alone, 50μl of virus in 150μl of complete media (1:4 dilution) and 50μl of cells in 150μl of complete media. Plates were incubated at 37°C for four hours to allow for virus uptake by cells.

After the incubation, plates were sealed with parafilm and centrifuged at 1200rpm (263 x g) for 5 minutes. The media was changed by removing 150μl of supernatant from each well and replacing it with 150μl of fresh complete media. Plates were sealed with parafilm and centrifuged again at 1200rpm (263 x g) for 5 minutes. The media was changed again by removing 150μl of supernatant from each well and replacing it with 150μl of fresh complete media. This step removed most of the initial virus inoculum, allowing more sensitive detection of initial replication of HIV in the well. Plates were incubated at 37°C for 3 days.

On day 3, 100μl of supernatant was removed and replaced with 110μl of fresh complete media. The additional 10μl of media were added to account for media lost to evaporation during the incubation. Culture plates were incubated at 37°C until day 7.

On day 7, 110μl of supernatant was harvested and transferred into a non-sterile round-bottom 96 well plate (Sarstedt) containing 12μl of 10% Triton-X100 (Sigma), which disrupts the viral envelope and inactivates the virus in the supernatant. The plates were then sealed with plate sealers (AlumaSeal CS Films for cold storage, Sigma) and frozen at -70°C for at least 24 hours. The wells in the infection culture plate were supplemented with 120μl of fresh complete media then were incubated until day 14. On day 14, supernatants were harvested in the same manner as day seven. Following viral harvest, infections were terminated and plates were sterilized and discarded.

Virus production in infection cultures was determined by p24 ELISA, as described below in section 2.3.4.5. The $TCID_{50}$ was calculated using the 50% endpoint dilution (i.e. dilution of virus stock which infects 50% of cell cultures) to determine the concentration of infectious virus in the stock using the algorithm of Reed and Muench [360].

2.3.4.4 in vitro infections of primary PBMC

PBMC isolated from HESN or HIV-N participants of the Pumwani CSW cohort were suspended at $2x10^6$ cells/ml in R-10 media and incubated overnight at 37°C in T25 flasks (non-tissue culture treated, BD Falcon). The following day, cells were washed in R-10 media and counted using trypan blue, then resuspended at $2x10^6$ cells/ml in complete media. Ten million cells were stimulated with 5μg/ml PHA for 72 hours for subsequent infection (see section 2.3.4.4.2). Ten million unstimulated cells were used immediately for unstimulated infections (section 2.3.4.4.1).

2.3.4.4.1 Unstimulated infections

In the containment level 3 facility, a sterile round-bottom 96 well culture microtiter plate (non-tissue culture treated, Corning) was prepared for each PBMC donor. A frozen aliquot of HIV_{ML1956} passage P5/D76/H8/02-02-10 (refers to passage 5, grown in donor 76, harvested 8 days post-infection on February 2, 2010) was thawed and diluted in complete media to achieve a concentration of final MOI of 0.1 at the highest dilution. Virus was mixed by pipetting and four-fold dilutions were carried across the plate to achieve final MOIs 0.1, 0.025, 0.0063, 0.0016 and 0.0004. Six replicates were plated for each dilution of virus. Cells suspended at $2x10^6$ cells/ml were added to wells containing virus at 50μl/well (equivalent to $1x10^5$ cells/well). Negative control wells consisted of 200μl of complete media alone, 50μl of virus in 150μl of complete media (1:4 dilution) and 50μl of cells in 150μl of complete media. The wells bordering the plate were filled with 200μl of PBS to minimize loss of volume due to evaporation over the course of the culture. PBS was topped up as required. Plates were incubated at 37°C for 4 hours to allow for virus uptake by cells.

After the incubation, plates were sealed with parafilm and centrifuged at 1200rpm (263 x g) for 5 minutes. The media was changed by removing 150μl of supernatant from each well and replacing it with 150μl of fresh complete media. Plates were sealed with and centrifuged again at 1200rpm (263 x g) for 5 minutes. The media was changed again by

removing 150μl of supernatant from each well and replacing it with 150μl of fresh complete media. Plates were incubated at 37°C for 3 days.

On day 3, 100μl of supernatant was removed and replaced with 110μl of fresh complete media. Culture plates were incubated at 37°C until day 9. Infections of PHA-stimulated PBMC from same donors as unstimulated infections were also set up on day 3, as described in section 2.3.4.4.2 below.

On day 9, 100μl of supernatant was harvested and transferred into a non-sterile round-bottom 96 well plate containing 11μl of 10% Triton-X100. The plates containing harvested treated supernatants were sealed with plate sealers and frozen at -70°C. The wells in the infection culture plate were supplemented with 110μl of fresh complete media then were incubated until day 16. On day 16, supernatants were harvested in the same manner as day 9. Following viral harvest, infected cells were collected for flow cytometry and plates were sterilized and discarded.

2.3.4.4.2 PHA-stimulated infections

PHA-stimulated PBMC were collected on day 3 for infection. Cells were washed in R-10 media and counted by trypan blue exclusion, then resuspended at $2x10^6$ cells/ml in complete media. Infection cultures were set up in the containment level 3 lab according to the same protocol as unstimulated infections (section 2.3.4.4.1). However, only three replicates of each dilution were prepared. Supernatants were harvested and frozen on

days 6 and 13 post-inoculation, which corresponded to days 9 and 16 of unstimulated infection cultures. Cells were collected for flow cytometry on day 13.

2.3.4.5 p24 ELISA

2.3.4.5.1 ELISA reagents

Coating antibody: HIV-1 p24 hybridoma cells (183-H12-5C, NIH AIDS Research and Reference Reagent Program) were cultured to produce an IgG1 monoclonal antibody reactive with HIV-1 p24. Antibody was purified from cell supernatant using Protein-G sepharose. Eluted antibody was dialyzed vs. PBS and stored at -70°C.

Detection antibody: Rabbit polyclonal anti-p24 (Advanced Biotechnologies) was diluted in blocking buffer and normal mouse sera (Sigma) (20μl antibody, 0.8ml buffer, 180μl mouse sera). An equal volume (1.0ml) of glycerol was added to make a final dilution of 1:100 then the diluted antibody was stored at -20°C until use.

Secondary antibody: Biotin anti-rabbit IgG goat antibody (Sigma) was diluted 1:100 using equal volumes of blocking buffer and glycerol then stored at -20°C until use.

2.3.4.5.2 ELISA protocol

A sandwich ELISA was used to detect the presence of HIV p24 in supernatants from infection cultures.

Coating antibody was thawed and diluted in coupling buffer to 2μg/ml prior to coating of plates. ELISA plates (96 well flat-bottom maxisorb, Nunc) were coated with 100μl of diluted monoclonal anti-p24 antibody per well overnight at 4°C. Buffer was then removed and coated plates were stored at -70°C until use.

Coated plates were thawed and 100μl of blocking buffer was added to each well. Plates were sealed and incubated for two hours at 37°C to block exposed areas of wells not coated by the monoclonal anti-p24 antibody. Supernatant was them removed and plates were washed three times with 150μl of ddH2O per well.

Frozen supernatants from infection cultures were thawed and 75μl was transferred to corresponding wells of ELISA plates. A standard curve was added to the plate using recombinant p24 diluted in blocking buffer. The standard curve ranged from 280 to 0.273ng/ml. The plate was then sealed and incubated overnight at 4°C.

Wells were washed six times with 300μl ELISA wash buffer per well (Amplicor) using a microplate washer (ELx405, Biotek). An aliquot of detection antibody (rabbit polyclonal anti-p24) was thawed and 150μl of antibody was diluted in 10ml of blocking buffer. Diluted detection antibody was added to the ELISA plate at 100μl/well then plate was sealed and incubated at 37°C for 90 minutes.

Wells were washed six times with 300μl ELISA wash buffer per well using the microplate washer. An aliquot of secondary antibody (biotin anti-rabbit goat IgG) was thawed and 100μl of antibody was diluted in 10ml of blocking buffer. Diluted secondary antibody was added to the ELISA plate at 100μl/well then the plate was sealed and incubated at 37°C for 90 minutes.

Wells were washed six times with 300μl ELISA wash buffer per well using the microplate washer. Streptavidin horseradish peroxidase (SA-HRP, Invitrogen) was diluted in blocking buffer (0.5μl SA-HRP in 10ml of buffer) and added to the ELISA plate at 100μl/well then the plate was sealed and incubated at room temperature for 30 minutes.

Wells were washed six times with 300μl ELISA wash buffer per well using the microplate washer. Tetramethyl benzidine (TMB) substrate solution (Invitrogen) was added to the ELISA plate at 100μl/well then plate was sealed. Plates were monitored for development of a blue colour from the reaction between the TMB substrate and p24-bound HRP enzyme. Development times were variable but ranged from 2-8 minutes. When plates were developed, 50μl of 3% HCl was added to each well to stop the reaction.

Optical density of wells was read at OD_{450nm} on a SpectraMax Plus spectrophotometer (Molecular Devices). Data was acquired and analyzed using SoftMax Pro software, version 3.1.2 (Molecular Devices).

2.3.4.6 Flow cytometry panels for in vitro infection study

Freshly-isolated PBMC were immunophenotyped to assess T cells phenotypes at baseline (i.e. prior to infection). Cells collected from infection cultures with MOI of 0.1 were also characterized to evaluate phenotypes of infected cells, which were identified by intracellular staining of HIV p24 (KC57 clone, Beckman Coulter). Panels used to evaluate ex vivo and post-infection T cell phenotypes are shown in Table 4.

Table 4. Flow cytometry panels used to assess T cell phenotypes in the *in vitro* infection study.

Fluorochrome	Panels					
	T cell activation		Regulatory T cells		Infected cells	
	Marker	Source	Marker	Source	Marker	Source
FITC	CD69	BD	CD103	Invitrogen	HIV p24	Beckman Coulter
PE	CCR5	BD	CD25	BD	CD127	BD
PE-CY5	CD38	BD	CTLA4	BD	HLA DR	BD
PE-CY7	CXCR4	BD	CCR7	BD	--	--
ECD	CD8	Invitrogen	CD45RA	Invitrogen	CD3	Invitrogen
APC	--	--	FOXP3	eBioscence	CD69	BD
AmCyan	Live/Dead	Invitrogen	Live/Dead	Invitrogen	Live/Dead	Invitrogen
AlexaFluor 700	CD4	BD	CD4	BD	--	--
Pacific Blue	CD3	BD	CD3	BD	--	--
APC-Cy7	HLA DR	BD	--	--	CD25	BD

2.3.4.7 Statistical analysis of in vitro infection study

Comparisons of continuous variables (i.e. infection frequency, HIV p24 production, T cell phenotypes) between study groups were performed using non-parametric Mann-Whitney *U* tests. Comparisons of T cell phenotypes between infected and uninfected cells or high and low MOIs were performed using the Wilcoxon signed-rank test for matched pairs. Relationships between continuous variables were assessed using Spearman nonparametric correlation. Differences were considered to be statistically

significant if $p<0.05$. All statistical analyses were performed using GraphPad Prism for Mac OS X, version 5.0a (GraphPad software).

2.3.5 Correlates of protection in HIV-controllers

2.3.5.1 ex vivo T cell phenotyping

Thawed PBMC were immunophenotyped using multi-colour flow cytometry to characterize T cell memory, activation, Treg frequency and HIV-specific T cell responses. Panels are shown in Table 5.

2.3.5.2 Stimulations and detection of responses

Thawed PBMC were stimulated in 48 well plates as described in section 2.2.4. Flow cytometry panels used in cytokine and proliferation assays are shown in Table 5.

Table 5. Flow cytometry panels used to assess T cell phenotypes and function in HIV-controllers.

Fluorochrome	Panels					
	Ex vivo T cell phenotypes		Day 1 Cytokine		Day 6 Proliferation	
	Marker	Source	Marker	Source	Marker	Source
FITC	--	--	IFNγ	BD	CFSE	Invitrogen
PE	CD25	BD	IL-2	BD	--	--
PE-Cy5	CD38	BD	CD38	BD	CD38	BD
PE-Cy7	CCR7	BD	CCR7	BD	CCR7	BD
ECD	CD45RA	Invitrogen	CD45RA	Invitrogen	CD45RA	Invitrogen
APC	FOXP3	eBioscience	CD8	BD	CD8	BD
V500	CD8	BD	Live/Dead	Invitrogen	Live/Dead	Invitrogen
AlexaFluor 700	CD4	BD	CD4	BD	CD4	BD
Pacific Blue	CD3	BD	--	--	--	--
APC-Cy7	HLA DR	BD	CD3	BD	CD3	BD

2.3.5.3 Statistical analysis for MECC study

Comparisons of continuous variables were done using Kruskal-Wallis non-parametric ANOVA, followed by Mann-Whitney *U* tests for pair-wise comparisons. Relationships between continuous variables were assessed using Spearman nonparametric correlation. Dichotomous variables were compared with Fisher's exact test or Chi-square test for trend. Differences were considered to be statistically significant if $p<0.05$. All statistical analyses were performed using GraphPad Prism for Mac OS X, version 5.0a (GraphPad software).

Chapter 3. T Cell Phenotypes and HIV Susceptibility

3.1 Rationale

Non-human primate models have shown that resting CD4+ T cells are susceptible to SIV infection, yet only produce low levels of virus. Infection of activated CD4+ T cells is required for dissemination of infection due to the dependency of SIV and HIV on host factors expressed in activated cells for viral replication [331-334, 361]. Emerging data from the Pumwani cohort [272, 336] and others [339-342] suggest that immune quiescence may play a role in resistance to HIV infection. The extent to which immune quiescence affects the T cell phenotype and the mechanism(s) by which immune quiescence is maintained in HESN are unclear. To address these gaps in knowledge, T cell activation was evaluated in PBMC from HESN and HIV-N CSW from the Pumwani cohort and regulatory T cells were investigated as a potential mediator of immune quiescence in this population.

3.2 Hypothesis

1. HESN will demonstrate lower levels of T cell activation and higher levels of regulatory T cells than HIV-N controls.

3.3 Objective

1. Determine expression of biomarkers of T cell activation on bulk T cells and memory subsets and evaluate Treg frequency and phenotype in HESN and HIV-N CSW.

3.4 Results

3.4.1 Study population

Fresh blood samples from a total of 54 participants from the Pumwani CSW cohort were analyzed for the T cell phenotyping study. The participants included newly enrolled HIV-uninfected controls (HIV-N, n=33) and HESN (n=21). Samples were also obtained from HIV-infected participants (n=46), but those results are beyond the scope of this thesis, and will be presented elsewhere. As shown in Table 6, self-reported duration of prostitution and duration of follow-up within the cohort differed significantly between groups (both $p<0.0001$), with HESN representing the longest duration of sex work and follow-up. HESN women were also older than HIV-N participants ($p<0.0001$). This is to be expected, as most participants are HIV-infected upon entry into the cohort, and of those who are seronegative, the majority are infected within a few years [43]. HIV-resistant individuals are healthy and can remain in sex work and, therefore, are older.

3.4.2 T cell activation and memory phenotyping

To determine levels of CD4+ and CD8+ T cell activation, expression of CD69, HLA DR, CD38 and CD95/Fas were measured by flow cytometry. Markers of T cell memory (CCR7, CD45RA and CD127/IL-7Rα) were also assessed. Representative staining of T cell activation and memory markers is shown in Figure 3. Frequencies of CD4+ and CD8+ T cells expressing each marker and median fluorescence intensities (MFI) are summarized in Table 7.

Table 6. Characteristics of participants included in study of T cell phenotypes and HIV susceptibility[a]			
	HIV-N (n=33)	**HESN** (n=21)	**p-value**[b]
Age, years	38.5 (31.75-44.0)	49.5 (42.5-53.75)	**<0.0001**
Duration of prostitution, years	7.5 (4.0-15.25)	19.0 (13.0-26.5)	**<0.0001**
Duration of follow-up, years	2.25 (0.5-4.5)	15.25 (13.5-18.8)	**<0.0001**
Clients/week	25.0 (20.0-35.0)	20.0 (10.0-35.0)	0.230

[a] Patient data are median (interquartile range) values.
[b] p-values were calculated using Mann Whitney *U* tests.

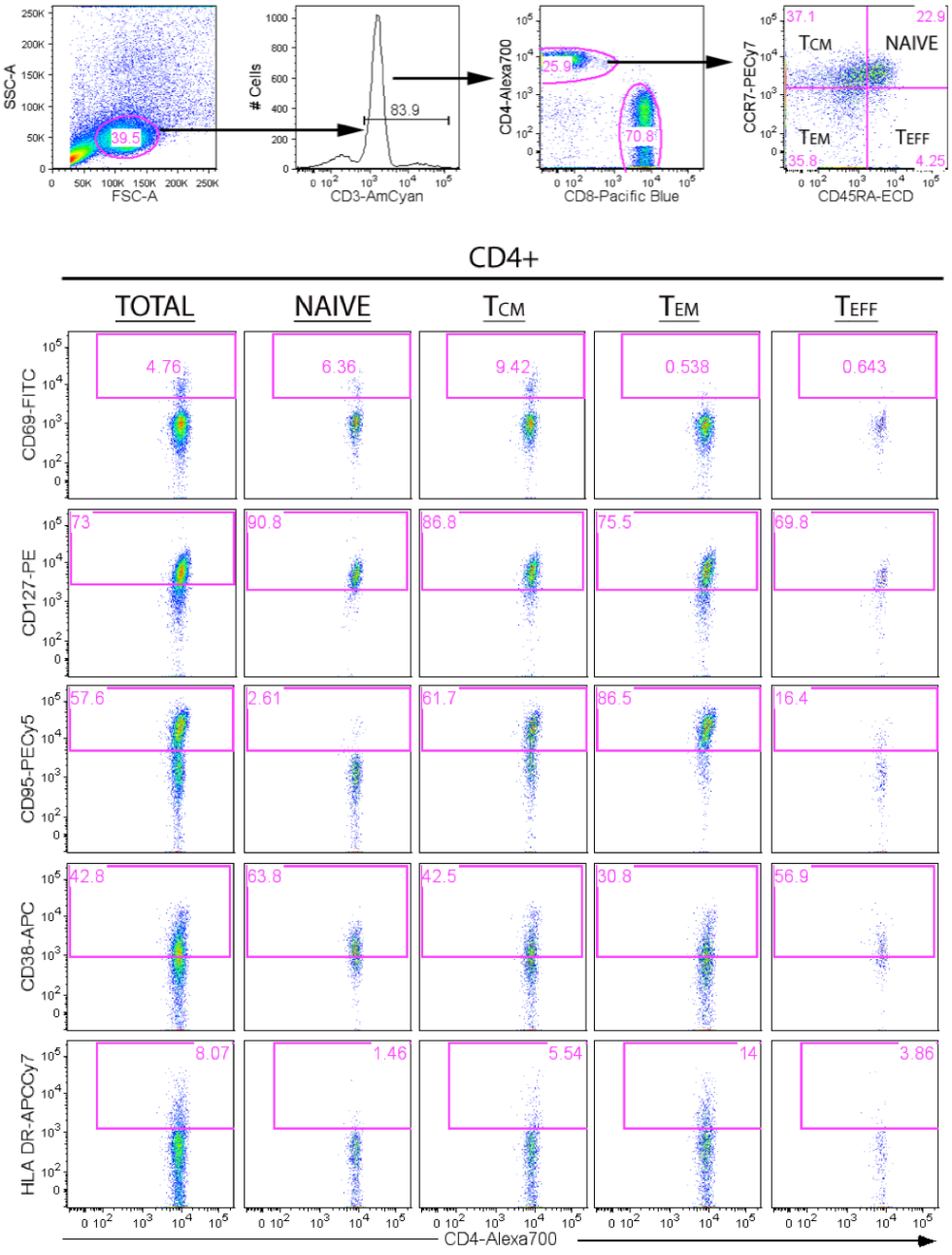


Figure 3. Representative staining of T cell memory and activation markers on PBMC samples from participants of the Pumwani CSW cohort. Samples were first gated on lymphocytes using FSC-A and SSC-A properties. CD3+ T cells were identified and further discriminated based on CD4 and CD8 expression. Memory subsets were identified by expression of CD45RA and CCR7. Expression of CD69, CD127, CD95, CD38 and HLA DR was evaluated for bulk CD4+ T cells and T cell memory subsets. Gating on CD4+ T cells is shown. A parallel approach was used to evaluate CD8+ T cell memory and activation. Data from ML 2747 (HIV-N) is used in this example.

Table 7. Expression of activation and memory markers on CD4+ and CD8+ T cells in HIV-N and HESN.

Subset	Parameter	Expression[a]		p-value[b]
		HIV-N (n=33)	HESN (n=21)	
Total CD4+ T cells	**CD69+ %**	**10.4 (7.05-13.3)**	**6.80 (5.00-10.2)**	**0.021**
	CD69 MFI	9880 (7336-11950)	8189 (7328-10810)	0.155
	CD127+ %	82.7 (77.6-85.0)	81.5 (78.4-86.3)	0.575
	CD127 MFI	7155 (6388-7878)	7522 (6017-8594)	0.545
	CD95+ %	46.5 (40.3-63.3)	55.0 (39.4-63.6)	0.651
	CD95 MFI	13434 (11812-14306)	12857 (10793-14189)	0.224
	CD38+ %	40.3 (28.1-46.6)	29.0 (20.9-48.2)	0.127
	CD38 MFI	1813 (1660-2055)	1758 (1536-1913)	0.142
	HLA DR+ %	3.90 (2.65-5.27)	3.49 (2.99-6.18)	0.674
	HLA DR MFI	2515 (2338-2908)	2882 (2619-3094)	0.118
	CD38+ HLA DR+ %	1.98 (1.52-2.61)	1.81 (1.24-3.33)	0.842
	TNAIVE: CD45RA+ CCR7+ %	**17.4 (12.4-26.5)**	**12.3 (7.08-20.5)**	**0.032**
	TCM: CD45RA- CCR7+ %	34.6 (29.9-39.8)	33.0 (24.6-40.3)	0.311
	TEM: CD45RA- CCR7- %	**39.8 (32.3-44.9)**	**47.9 (39.3-52.3)**	**0.014**
	TEFF: CD45RA+ CCR7- %	6.52 (3.46-10.1)	5.46 (2.62-11.6)	0.777
Total CD8+ T cells	**CD69+ %**	**12.6 (8.68-16.7)**	**8.30 (4.60-12.7)**	**0.008**
	CD69 MFI	8961 (7834-10131)	8492 (6500-9648)	0.202
	CD127+ %	60.8 (52.5-70.5)	56.1 (51.3-64.5)	0.189
	CD127 MFI	6085 (5300-6656)	6010 (4978-6500)	0.323
	CD95+ %	44.3 (32.9-56.4)	43.1 (22.4-58.0)	0.310
	CD95 MFI	**7588 (6807-8194)**	**6740 (5932-7957)**	**0.097**
	CD38+ %	25.0 (16.0-29.7)	19.1 (11.7-35.5)	0.275
	CD38 MFI	1467 (1362-1592)	1404 (1304-1619)	0.421
	HLA DR+ %	8.67 (5.89-10.6)	8.69 (6.21-16.0)	0.506
	HLA DR MFI	2555 (2343-2768)	2600 (2459-2850)	0.434
	CD38+ HLA DR+ %	3.81 (2.89-5.45)	4.34 (2.19-7.31)	0.319
	TNAIVE: CD45RA+ CCR7+ %	**32.1 (19.9-40.2)**	**23.7 (16.2-29.6)**	**0.024**
	TCM: CD45RA- CCR7+ %	9.33 (6.67-11.1)	7.69 (5.41-11.0)	0.349
	TEM: CD45RA- CCR7- %	31.0 (27.0-40.0)	34.0 (26.2-49.9)	0.445
	TEFF: CD45RA+ CCR7- %	**25.2 (21.0-32.1)**	**31.8 (22.3-38.0)**	**0.099**

[a] Expression of phenotypic markers is listed as median of percent positive (+) cells or median fluorescence intensity (MFI) units. Interquartile range is shown in parentheses.

[b] p-values were calculated using non-parametric Mann-Whitney U tests. Parameters that demonstrated statistically significant ($p<0.05$) or trending ($p<0.10$) p-values are highlighted in bold text.

3.4.3 T cell activation and memory in HESN and HIV-N

The aim of the study was to characterize biomarkers of T cell activation of HESN. Most notably, CD4+ and CD8+ T cells expressing CD69 were found at a lower frequency in HESN relative to HIV-N (p=0.021 and p=0.008, respectively) (Figure 4A, B).

In addition to differences in CD69 expression, HESN also demonstrated a unique distribution of memory T cell subsets (Figures 5A, B). HESN had significantly lower frequencies of CD4+ and CD8+ naïve T cells (T_{NAIVE}: CD45RA+ CCR7+), when compared to HIV-N (p=0.032 and p=0.024, respectively). In the CD4+ T cell compartment, this reduced T_{NAIVE} subset was accompanied by elevated frequencies of effector memory T cells (T_{EM}: CD45RA- CCR7-) in HESN relative to HIV-N (p=0.014). In the CD8+ T cell compartment, the reduced T_{NAIVE} subset was associated with a relative expansion of terminally-differentiated effector T cells (T_{EFF}: CD45RA+ CCR7-) in HESN (p=0.099).

3.4.4 Activation of memory T cell subsets

The levels of CD69, CD38, HLA DR, CD95 and CD127 were analyzed on each memory T cell subset. Frequencies of CD4+ and CD8+ naïve, T_{CM}, T_{EM} and T_{EFF} cells expressing each marker are summarized in Tables 8 and 9. The expression of activation markers on memory cell subsets tended to reflect the bulk CD4+ and CD8+ T cells. However, some differences are notable.

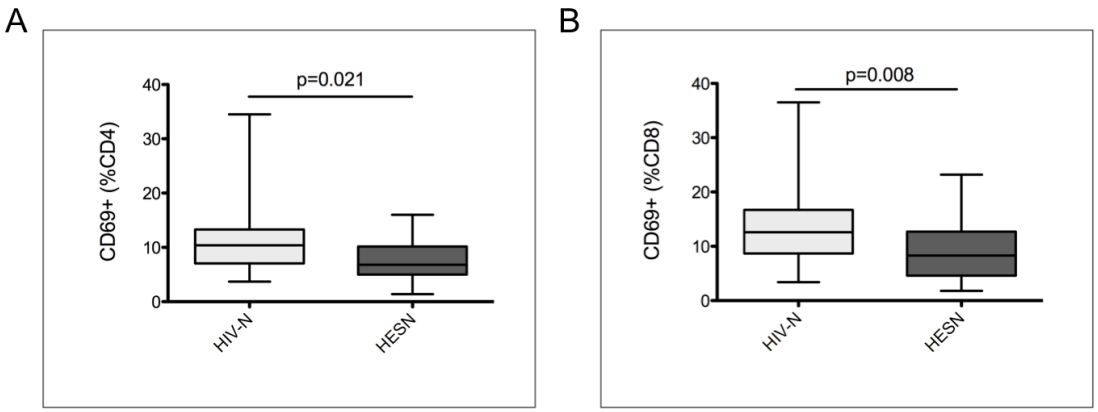


Figure 4. Comparison of CD69 expression on T cells between HIV-N and HESN. (A) The frequency of CD4+ T cells expressing CD69 is lower in HESN than HIV-N participants. (B) The frequency of CD8+ T cells expressing CD69 is lower in HESN than HIV-N participants. Box plots represent median frequencies of positive cells and respective interquartile ranges. Whiskers represent range in frequencies of positive cells. Statistical comparisons were done using Mann Whitney *U* tests.

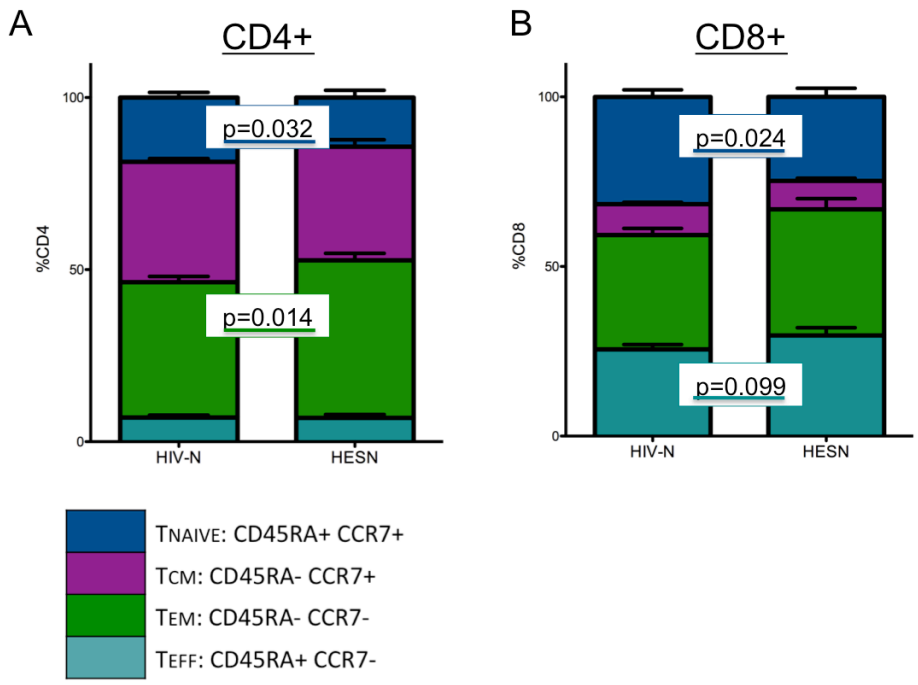


Figure 5. Comparison of T cell memory subsets between HIV-N and HESN. The contribution of memory cell subsets to the total T cell pool was analyzed in HIV-N (n=31) and HESN (n=20) using relative expression of CD45RA and CCR7 on CD4+ and CD8+ T cells. (A) HESN have fewer CD4+ TNAIVE and more CD4+ TEM cells than HIV-N. (B) HESN have fewer CD8+ TNAIVE and more CD8+ TEFF cells than HIV-N. Stacked bars and whiskers represent mean and SEM.

The frequency of CD69+ CD4+ and CD8+ T cells was lower in HESN, as described above. Analysis of CD69 expression on CD4+ memory subsets revealed that the differences observed in bulk T cells were largely reflected in memory subsets. These differences were most notable in the T_{NAIVE} (p=0.077) and T_{EFF} (p=0.013) subsets (Table 8, Figure 6). In the CD8+ T cell compartment, the major differences in CD69 expression were found in the T_{CM} (p=0.060), T_{EM} (p=0.088) and T_{EFF} (p=0.081) subsets (Table 9, Figure 7).

Analysis of activation markers on memory subsets revealed some trends that were not evident on bulk T cells. For example, CD38 expression on CD4+ T_{CM} cells was lower in HESN than HIV-N participants (p=0.032) (Table 8, Figure 6). Similarly, analysis of CD95 expression on memory CD8+ T cells revealed lower CD95+ T_{EM} in HESN when compared to HIV-N (p=0.049) (Table 9, Figure 7).

3.4.5 Regulatory T cells in HESN and HIV-N

The reduced levels of T cell activation in HESN led to the investigation of Tregs, which suppress T cell activation. Tregs were identified by coexpression of high levels of CD25 and FOXP3 on CD4+ T cells (Figure 8A). We also examined additional markers, demonstrating that CD4+ CD25+ FOXP3+ cells expressed low levels of CD127 and primarily exhibited a T_{CM}/T_{EM} phenotype, consistent with known characteristics of Tregs. A comparison of Treg frequency between HIV-N (n=33) and HESN (n=21) revealed that Tregs were significantly elevated in HESN women when compared to HIV-N controls (p = 0.016) (Figure 8B).

Table 8. Expression of activation markers on CD4+ T cell memory subsets in HiV-N and HESN.

Subset	Parameter	Expression[a]		p-value[b]
		HIV-N (n=31)	HESN (n=20)	
CD4+ Naïve (CD45RA+ CCR7+)	**CD69+**	**10.9 (6.9-16.2)**	**8.03 (5.69-12.2)**	**0.077**
	CD127+	92.6 (90.2-94.8)	92.6 (87.8-94.4)	0.519
	CD95+	3.01 (1.33-5.53)	3.00 (1.99-4.36)	0.670
	CD38+	68.5 (53.5-79.4)	54.7 (42.7-74.4)	0.170
	HLA DR+	2.72 (1.47-3.93)	2.11 (1.06-5.45)	0.706
	CD38+ HLA DR+	2.17 (1.31-3.44)	1.95 (0.80-5.36)	0.857
CD4+ TCM: Central Memory (CD45RA- CCR7+)	CD69+	10.5 (6.95-17.0)	8.88 (4.49-12.9)	0.139
	CD127+	89.5 (86.6-91.0)	87.7 (86.1-91.1)	0.588
	CD95+	52.0 (44.4-63.9)	58.8 (47.1-70.0)	0.220
	CD38+	**40.8 (27.7-46.5)**	**29.2 (18.4-40.0)**	**0.032**
	HLA DR+	2.85 (1.90-4.10)	3.50 (2.29-5.47)	0.236
	CD38+ HLA DR+	1.64 (0.88-2.35)	1.68 (1.08-3.27)	0.307
CD4+ TEM: Effector Memory (CD45RA- CCR7-)	CD69+	1.60 (1.05-4.61)	1.75 (1.44-3.84)	0.458
	CD127+	83.6 (78.7-87.7)	85.4 (81.7-88.7)	0.531
	CD95+	76.4 (65.6-81.7)	74.8 (67.1-84.6)	0.759
	CD38+	**23.0 (16.3-27.8)**	**17.0 (12.2-22.5)**	**0.074**
	HLA DR+	4.62 (3.93-6.59)	5.59 (3.71-9.13)	0.340
	CD38+ HLA DR+	2.04 (1.51-2.65)	1.81 (1.49-3.57)	0.797
CD4+ TEFF: Terminal effector (CD45RA+ CCR7-)	**CD69+**	**1.55 (1.09-3.05)**	**0.77 (0.01-3.38)**	**0.013**
	CD127+	85.1 (79.2-91.4)	84.6 (78.3-90.3)	0.857
	CD95+	8.60 (3.36-20.8)	9.65 (6.06-17.2)	0.829
	CD38+	60.9 (47.3-73.5)	53.8 (39.0-66.9)	0.226
	HLA DR+	2.54 (1.01-5.34)	1.60 (1.14-5.04)	0.631
	CD38+ HLA DR+	1.88 (0.80-3.07)	1.24 (0.68-3.99)	0.460

[a] Expression of phenotypic markers is listed as median of percent positive (+) cells. Interquartile range is shown in parentheses.

[b] p-values were calculated using non-parametric Mann-Whitney U tests. Parameters that demonstrated statistically significant ($p<0.05$) or trending ($p<0.10$) p-values are highlighted in bold text.

Table 9. Expression of activation markers on CD8+ T cell memory subsets in HIV-N and HESN.

Subset	Parameter	Expression[a]		p-value[b]
		HIV-N (n=31)	HESN (n=20)	
CD8+ Naïve (CD45RA+ CCR7+)	CD69+	10.3 (6.98-17.3)	9.05 (6.85-11.3)	0.180
	CD127+	88.5 (82.8-90.8)	86.7 (74.6-90.3)	0.319
	CD95+	7.86 (3.35-11.0)	5.34 (3.33-12.7)	0.992
	CD38+	35.1 (23.5-42.7)	31.7 (16.8-41.3)	0.323
	HLA DR+	1.75 (1.12-3.78)	1.85 (0.70-6.12)	0.738
	CD38+ HLA DR+	1.25 (0.51-2.34)	0.73 (0.37-3.64)	0.837
CD8+ TCM: Central Memory (CD45RA- CCR7+)	**CD69+**	**17.6 (12.6-26.8)**	**13.4 (8.68-18.2)**	**0.060**
	CD127+	71.8 (65.5-79.4)	71.5 (64.3-77.3)	0.911
	CD95+	55.2 (42.9-67.5)	56.6 (45.1-70.2)	0.656
	CD38+	24.6 (18.7-30.6)	18.4 (14.1-26.4)	0.112
	HLA DR+	10.2 (5.27-14.0)	11.7 (7.24-20.1)	0.363
	CD38+ HLA DR+	5.00 (3.47-7.84)	7.67 (3.57-12.2)	0.137
CD8+ TEM: Effector Memory (CD45RA- CCR7-)	**CD69+**	**4.86 (3.83-7.66)**	**3.90 (1.27-6.49)**	**0.088**
	CD127+	53.3 (38.3-59.1)	49.1 (46.1-60.3)	0.725
	CD95+	**78.0 (66.1-83.5)**	**70.4 (47.1-77.7)**	**0.049**
	CD38+	15.8 (10.4-23.0)	13.4 (9.7-21.6)	0.414
	HLA DR+	13.1 (10.7-19.4)	14.6 (10.5-19.6)	0.612
	CD38+ HLA DR+	5.53 (4.10-9.46)	6.24 (3.51-9.80)	0.674
CD8+ TEFF: Terminal effector (CD45RA+ CCR7-)	**CD69+**	**4.61 (2.80-8.23)**	**3.43 (1.41-6.14)**	**0.081**
	CD127+	43.4 (25.4-62.0)	42.2 (29.9-54.9)	0.553
	CD95+	45.9 (27.9-67.4)	38.5 (14.0-54.3)	0.157
	CD38+	24.6 (16.8-32.3)	20.5 (10.5-39.6)	0.303
	HLA DR+	7.65 (5.56-13.7)	7.99 (5.38-19.2)	0.680
	CD38+ HLA DR+	3.29 (1.72-7.45)	3.68 (1.59-9.74)	0.565

[a] Expression of phenotypic markers is listed as median of percent positive (+) cells. Interquartile range is shown in parentheses.

[b] p-values were calculated using non-parametric Mann-Whitney U tests. Parameters that demonstrated statistically significant ($p<0.05$) or trending ($p<0.10$) p-values are highlighted in bold text.

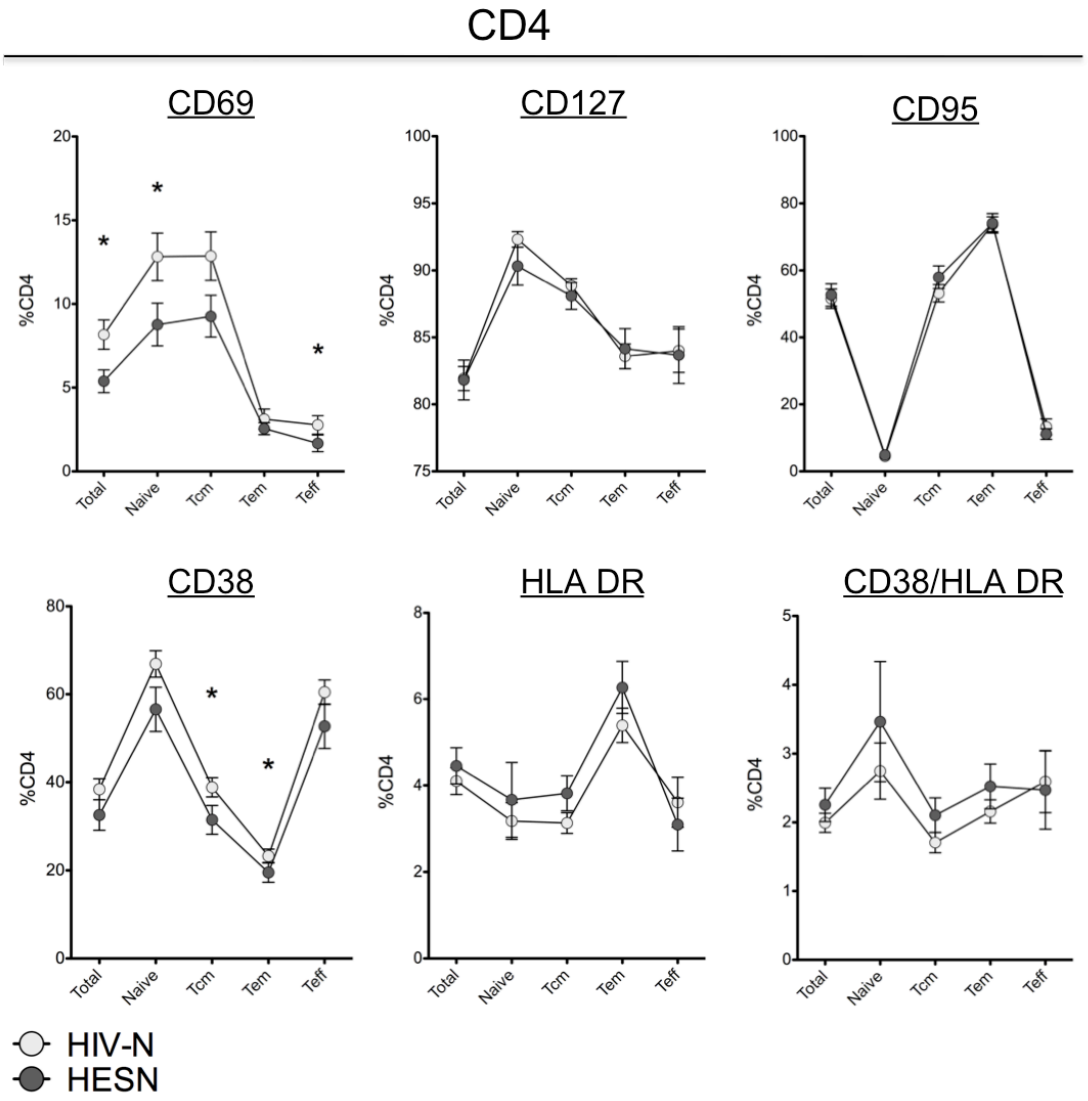


Figure 6. Activation of CD4+ T cell memory subsets in HIV-N and HESN. Expression of CD69, CD127, CD95, CD38, HLA DR and co-expression of CD38 and HLA DR were measured on bulk CD4+ T cells and individual memory CD4+ subsets and compared between HIV-N (n=31) and HESN (n=20). Mean and SEM are shown. Statistically significant ($p<0.05$) or trending ($p<0.10$) differences as determined by Mann-Whitney *U* tests are indicated by an asterisk (*). P-values for all comparisons are listed in Table 3.3.

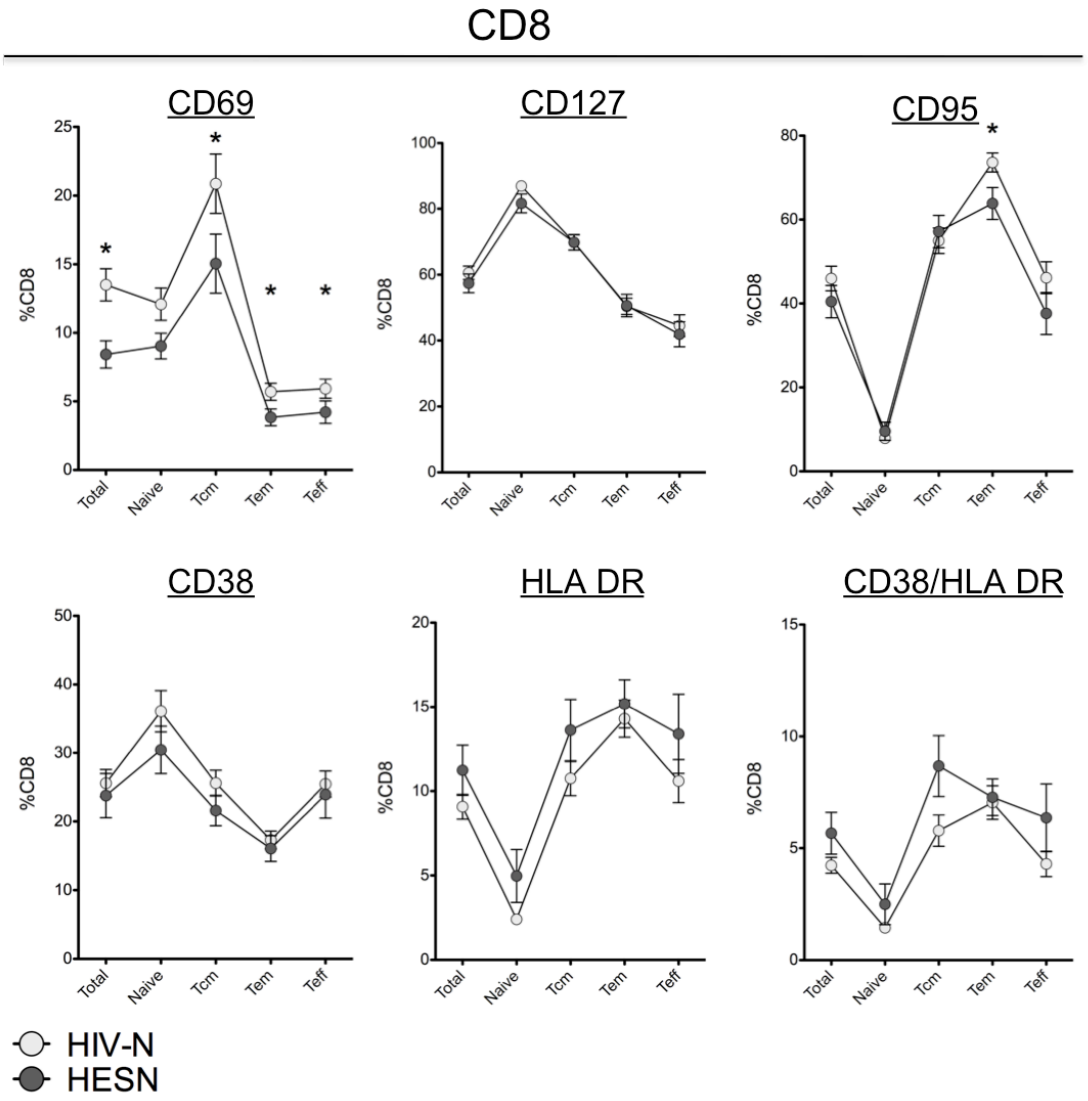


Figure 7. Activation of CD8+ T cell memory subsets in HIV-N and HESN. Expression of CD69, CD127, CD95, CD38, HLA DR and co-expression of CD38 and HLA DR were measured on bulk CD8+ T cells and individual memory CD8+ subsets and compared between HIV-N (n=31) and HESN (n=20). Mean and SEM are shown. Statistically significant ($p<0.05$) or trending ($p<0.10$) differences as determined by Mann-Whitney *U* tests are indicated by an asterisk (*). P-values for all comparisons are listed in Table 3.4.

3.4.6 Tregs and T cell activation

To evaluate the effects of Tregs on levels of T cell activation, Treg frequency was correlated with expression of CD69, CD38, HLA DR, CD127 and CD95 on CD4+ and CD8+ T cells (Table 10). Treg frequency correlated inversely with CD4+ CD38+ T cells ($p=0.032$, $r=-0.28$) and the MFIs of CD38 ($p=0.008$, $r=-0.35$) and CD69 ($p=0.026$, $r=-0.29$) on CD4+ T cells. Likewise, in the CD8+ T cell compartment, Treg frequency correlated inversely with the MFIs of CD38 ($p=0.021$, $r=-0.30$) and CD95 ($p=0.036$, $r=-0.29$). Treg frequency was positively correlated with the MFI of CD127 on both CD4+ ($p=0.002$, $r=0.41$) and CD8+ ($p=0.095$, $r=0.22$) T cells. Elevated levels of CD38, CD69 and loss of CD127 are associated with activated T cells. Thus, these data support the hypothesis that Tregs are involved in controlling baseline levels of T cell activation.

3.4.7 Effects of participant characteristics on phenotype

As discussed above, HESN were significantly older than HIV-N. Phenotype was therefore analyzed as a function of age within each study group (Table 11).

As shown in the previous sections, HESN had low levels of activation as indicated by CD69 expression on CD4+ and CD8+ T cells and elevated Treg frequencies. When the frequency of CD4+ or CD8+ CD69+ cells was correlated with age, no associations were found. Similarly, Treg frequency did not correlate with age in any study group, suggesting that these differences observed between HESN and HIV-N or HIV+ are due to their HESN status, rather than age (Table 11).

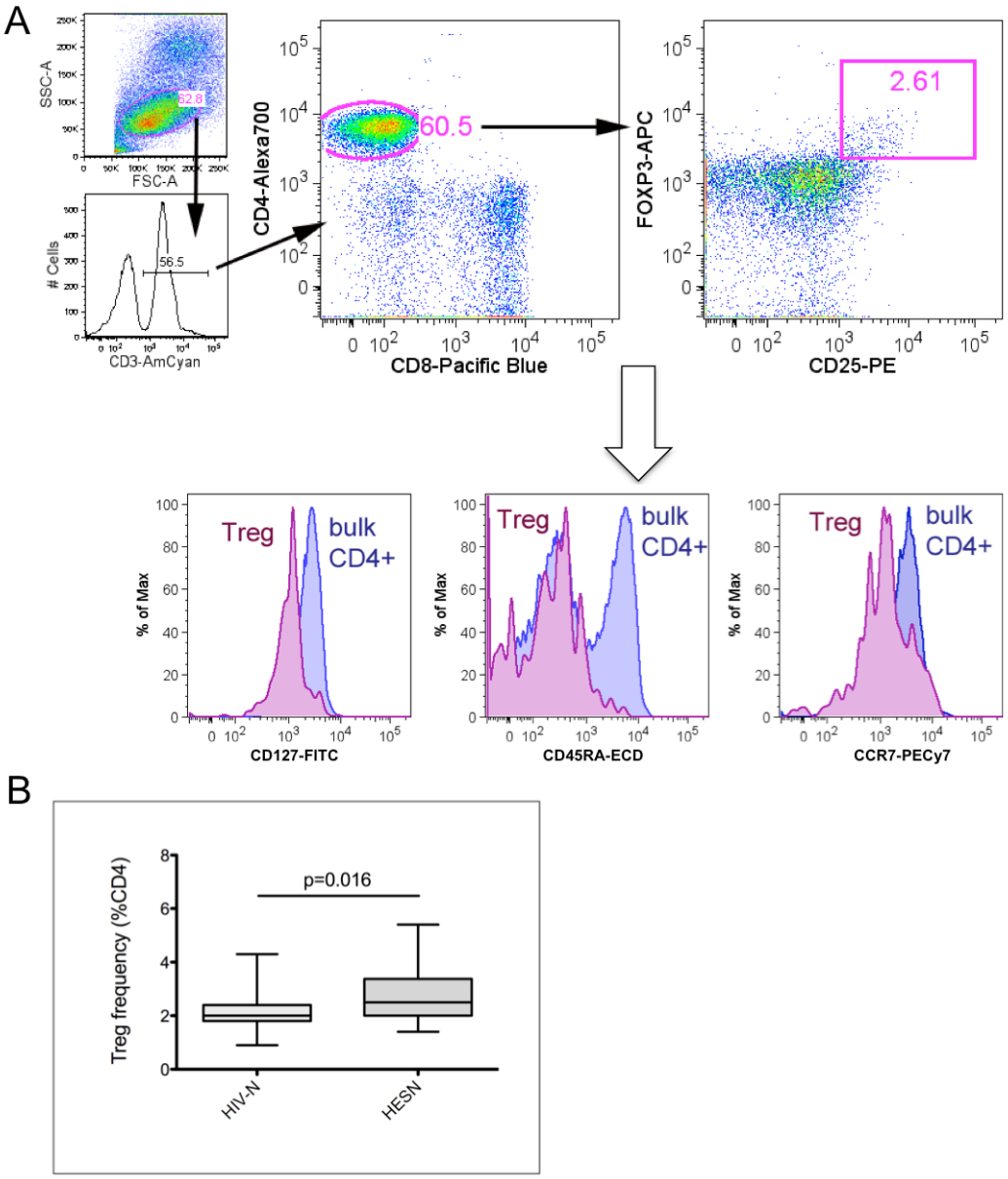


Figure 8. Regulatory T cells in HIV-N and HESN. (A) Representative staining of Tregs. Samples were gated on lymphocytes using FSC-A and SSC-A properties. CD3+ T cells were identified and further discriminated based on CD4 expression. CD4+ T cells co-expressing high levels of CD25 and FOXP3 were designated as Tregs. Tregs expressed low levels of CD127, were primarily CD45RA- and had intermediate expression of CCR7, consistent with a TCM/TEM phenotype. Data from ML2040 (HIV-N) is used in this example. (B) CD4+ CD25+ FOXP3+ Treg frequencies in HIV-N (n=33) and HESN (n=21). HESN have elevated Treg frequencies compared to HIV-N. Box plots represent median frequencies of positive cells and respective interquartile ranges. Whiskers represent range in frequencies of positive cells. P-values were calculated using the Mann-Whitney *U* test.

Table 10. Correlations of Treg frequency with markers of T cell activation.

Parameter	Treg/CD4+ T cell activation correlations		Treg/CD8+ T cell activation correlations	
	p-value[a]	r[b]	p-value	r
CD69+ %	0.461	--	0.951	--
CD69 MFI	**0.026**	-0.29	0.502	--
CD127+ %	0.103	--	0.914	--
CD127 MFI	**0.002**	0.41	0.095	0.22
CD95+ %	0.415	--	0.617	--
CD95 MFI	0.138	--	**0.036**	-0.29
CD38+ %	**0.032**	-0.28	0.112	--
CD38 MFI	**0.008**	-0.35	**0.021**	-0.30
HLA DR+ %	0.383	--	0.511	--
HLA DR MFI	0.161	--	0.987	--
CD38+ HLA DR+ %	0.967	--	0.737	--

[a] p-values calculated by Spearman rank correlation.
[b] r values are Spearman rho correlation coefficients. Spearman r values are only shown in the case of a significant ($p<0.05$) or trending ($p<0.1$) p-value. Negative r values indicate inverse correlations, positive r values indicate direct correlations.

Reduced CD4+ T_{CM} CD38+ and CD8+ T_{EM} CD95+ cells in HESN support the observation of low levels of T cell activation, as shown in section 3.4.4. CD38 expression did not associate with age in HIV-N or HESN groups. In the HIV-N group, age was positively correlated with CD95 expression on CD4+ and CD8+ T cells (p=0.027, r=0.40 and p=0.012, r=0.45, respectively) (Table 11). However, if the differences in CD8+ T_{EM} CD95+ cells in HESN were due to age, the HESN group would be expected to have elevated, rather than the observed reduced, frequencies of these cells.

Memory subset distribution also differed between HIV-N and HESN. In HIV-N, memory subsets were skewed with age, which correlated inversely with CD4+ and CD8+ naïve subsets (CD4: p=0.048, r=-0.37; CD8: p=0.004, r=-0.5) and positively with CD4+ T_{EM} (p=0.086, r=0.32) and CD8+ T_{EFF} (p=0.020, r=0.42), in accordance with previous studies, which showed that the naïve T cell compartment shrinks with age [362]. The inverse association between age and naïve T cell subsets may explain the observed difference between HESN and HIV-N.

In the HIV-N group, age was also positively correlated with CD4+ and CD8+ expression of HLA DR (CD4: p=0.006, r=0.47; CD8: p=0.025, r=0.39) and inversely correlated with CD8+ CD127 expression (p=0.003, r=-0.50). These results are consistent with previous studies on aging, which is marked by chronic inflammation, thymic involution and differentiation of T cells [362].

Behavioural characteristics were also investigated for their effects on phenotype. In HIV-N, duration of prostitution correlated with CD4+ and CD8+ expression of CD95 (CD4: p=0.020, r=0.43; CD8: p=0.010, r=0.46) and HLA DR (CD4: p=0.005, r=0.48; CD8: p=0.020, r=0.40) and inversely with CD4+ naïve T cells (p=0.054, r=-0.35). Older individuals tend to report longer duration of sex work, so these associations likely reflect the effects of age on the immune system.

In HIV-N participants, weak inverse correlations were found between clients/week and CD4+ or CD8+ expression of HLA DR (CD4+: p=0.097, r=-0.30; CD8+: p=0.082, r=-0.32). These results suggest that more client exposures may lead to lower T cell activation. Dampening of T cell activation could be an effect of Treg activity or other regulatory mechanisms fuelled by exposure to alloantigens or other inflammatory stimuli.

3.5 Summary

This study aimed to determine whether immune quiescence could be observed at the level of T cell activation in HESN women of the Pumwani cohort. Reduced CD69+ CD4+ and CD8+ T cells were observed in HESN, indicating low cellular activation. Furthermore, HESN had lower CD4+ CD38+ T_{CM} and CD8+ CD95+ T_{EM} cells. In addition, HESN had elevated Tregs relative to HIV-N, implicating Tregs as a potential driver of immune quiescence. Treg frequency correlated inversely with the frequency of activated T cells. Collectively, these data confirm the hypothesis that HESN would demonstrate lower levels of T cell activation and higher levels of regulatory T cells than HIV-N controls.

Table 11. Correlations of age and behavioural characteristics with T cell phenotypes

Variable	Phenotype	HIV-N (n=33)		HESN (n=21)	
		p-value[a]	r[b]	p-value	r
Age	CD4+ CD69	0.225		0.800	
	CD4+ CD127	0.860		0.236	
	CD4+ CD95	**0.027**	0.40	0.669	
	CD4+ CD38	0.156		0.739	
	CD4+ HLA DR	**0.006**	0.47	0.262	
	CD4+ naïve	**0.048**	-0.37	0.291	
	CD4+ TCM	0.615		0.833	
	CD4+ TEM	0.086	0.32	0.467	
	CD4+ TEFF	0.926		0.514	
	CD4+ Treg	0.247		0.847	
	CD8+ CD69	0.207		0.618	
	CD8+ CD127	**0.003**	-0.50	0.297	
	CD8+ CD95	**0.012**	0.45	0.437	
	CD8+ CD38	0.236		0.555	
	CD8+ HLA DR	**0.025**	0.39	0.189	
	CD8+ naïve	**0.004**	-0.50	0.246	
	CD8+ TCM	0.377		0.224	
	CD8+ TEM	0.180		0.251	
	CD8+ TEFF	**0.020**	0.42	0.149	
Duration of prostitution	CD4+ CD69	0.756		0.773	
	CD4+ CD127	0.382		0.883	
	CD4+ CD95	**0.020**	0.43	0.923	
	CD4+ CD38	0.369		0.262	
	CD4+ HLA DR	**0.005**	0.48	0.668	
	CD4+ naïve	0.054	-0.35	0.755	
	CD4+ TCM	0.545		0.472	
	CD4+ TEM	0.134		0.431	
	CD4+ TEFF	0.138		0.349	
	CD4+ Treg	0.563		0.119	
	CD8+ CD69	0.915		0.823	
	CD8+ CD127	0.339		0.475	
	CD8+ CD95	**0.010**	0.46	0.496	
	CD8+ CD38	0.921		0.620	
	CD8+ HLA DR	**0.020**	0.40	0.321	
	CD8+ naïve	0.129		0.751	
	CD8+ TCM	0.671		0.682	
	CD8+ TEM	0.301		0.474	
	CD8+ TEFF	0.623		0.183	
Clients/week	CD4+ CD69	0.980		0.696	
	CD4+ CD127	0.069	0.33	0.598	
	CD4+ CD95	0.661		0.653	
	CD4+ CD38	0.726		0.561	
	CD4+ HLA DR	0.097	-0.30	1.000	
	CD4+ naïve	0.196		0.107	
	CD4+ TCM	0.587		0.273	
	CD4+ TEM	0.376		0.427	
	CD4+ TEFF	0.397		0.263	
	CD4+ Treg	0.494		0.660	
	CD8+ CD69	0.782		0.970	
	CD8+ CD127	0.538		0.994	
	CD8+ CD95	0.223		0.387	
	CD8+ CD38	0.336		0.399	
	CD8+ HLA DR	0.082	-0.32	0.641	
	CD8+ naïve	0.342		0.157	
	CD8+ TCM	0.448		0.201	
	CD8+ TEM	0.127		0.228	
	CD8+ TEFF	0.140		0.357	

[a] p-values calculated by Spearman rank correlation.

[b] r values are Spearman rho correlation coefficients, shown in the case of a significant ($p<0.05$) or trending ($p<0.1$) p-value.

Chapter 4. Regulatory T cell Function

4.1 Rationale

The finding of elevated Tregs in HESN CSW prompted the question of their suppressive capacity. HIV-specific T cell responses have been detected in numerous cases of HESN, including HESN CSW from the Pumwani cohort [274-285], but the potential of *in vitro* depletion of Tregs to boost these responses, or reveal uncharacterized responses, in HESN needs to be addressed. Treg function was similarly examined in one study of exposed-uninfected neonates. Analogous to HESN CSW, the exposed-uninfected neonates had elevated Treg frequencies and detectable HIV-specific CD8+ T cell responses, and depletion of Tregs resulted in amplified cytokine production in response to HIV antigen stimulation [320].

4.2 Hypotheses

1. HIV-N, HESN and HIV+ will demonstrate comparable increases in T cell responses to CD3/CD28 stimulation following Treg depletion.
2. Depletion of Tregs from HESN and HIV+ CSW will boost pre-existing HIV-specific responses or reveal previously undetectable responses.
3. Amplification of T cell responses following Treg depletion will be strongest in individuals with highest Treg frequencies.

4.3 Objectives

1. Evaluate T cell cytokine and proliferation responses to stimulation with CD3/CD28 microbeads before and after depletion of Tregs from PBMC of HESN, HIV-N and HIV+ CSW.
2. Evaluate T cell cytokine and proliferation responses to HIV gag peptides before and after depletion of Tregs from PBMC of HESN, HIV-N and HIV+ CSW.
3. Correlate *ex vivo* Treg frequency and phenotype with suppressive capacity.

4.4 Results

4.4.1 Study population

Treg function was evaluated in a total of 17 participants of the Pumwani cohort, including 4 HIV-N, 6 HESN and 7 HIV+ women. The HIV-infected group consisted of 2 patients currently undergoing antiretroviral therapy at the time of sampling and 5 patients who were therapy-naïve. Consistent with the population sample discussed in Chapter 3, self-reported duration of prostitution and duration of follow-up within this population differed significantly between groups (p=0.031 and p-0.004, respectively), with HESN representing the longest duration of sex work and follow-up. HESN women were also significantly older than the other participants (p=0.036). These data are represented in Table 12.

Table 12. Characteristics of participants included in Treg function experiments[a]

	HIV-N (n=4)	**HESN** (n=6)	**HIV+** (n=7)	**p-value**[b]
Age, years	28.5 (24.8-33.8)	44.5 (32.0-52.8)	38.0 (30.0-40.0)	**0.036**
Duration of prostitution, years	7.5 (5.0-11.5)	20.0 (12.0-26.5)	7.0 (5.0-10.0)	**0.031**
Duration of follow-up, years	1.8 (1.5-4.3)	17.3 (14.0-18.0)	2.0 (1.0-3.5)	**0.004**
Clients/week	20.0 (10.5-31.0)	28.0 (8.8-35.0)	12.0 (5.0-18.0)	0.483
CD4 count			532.0 (487.0-689.0)	
Current ARV use, number of patients (%)			2 (28.6)	

[a]Patient data are median (interquartile range) values, unless otherwise indicated.
[b]p-values were calculated using Kruskal-Wallis non-parametric ANOVA.

4.4.2 Treg depletions

Tregs were depleted from bulk PBMC using immunomagnetic positive selection of CD25+ cells. Purity of CD25-depleted PBMC was assessed by flow cytometry (Figure 9A). As shown in Figure 9B, CD25 depletion effectively removed CD4+ CD25+ T cells ($p<0.0001$), including CD25+ FOXP3+ Tregs ($p<0.0001$).

4.4.3 Effect of Treg depletion on T cell proliferation

T cell proliferation was measured in mock- and CD25-depleted cell cultures six days post-stimulation with CD3/CD28 beads and HIV gag peptides using CFSE dye dilution in HIV-N (n=4), HESN (n=6) and HIV+ (n=5) participants (Figure 10). All individuals responded to CD3/CD28 stimulation. CD8+ T cell proliferation responses were significantly boosted following Treg depletion ($p=0.033$), regardless of HIV status, although Treg depletion failed to boost responses in some individuals (Figure 11A,B). A comparable trend was observed in the CD4+ T cell compartment, though this did not reach statistical significance ($p=0.086$).

Treg depletion similarly resulted in trends toward enhanced HIV gag-specific CD4+ ($p=0.071$) and CD8+ ($p=0.068$) T cell proliferation responses (Figure 11C,D). However, it is important to note that not all responses met the threshold of positive HIV gag-specific responses pre-determined in HIV-uninfected low-risk volunteers, as described in section 2.2.4 of Materials and Methods (indicated by dashed line in all panels of Figure 11).

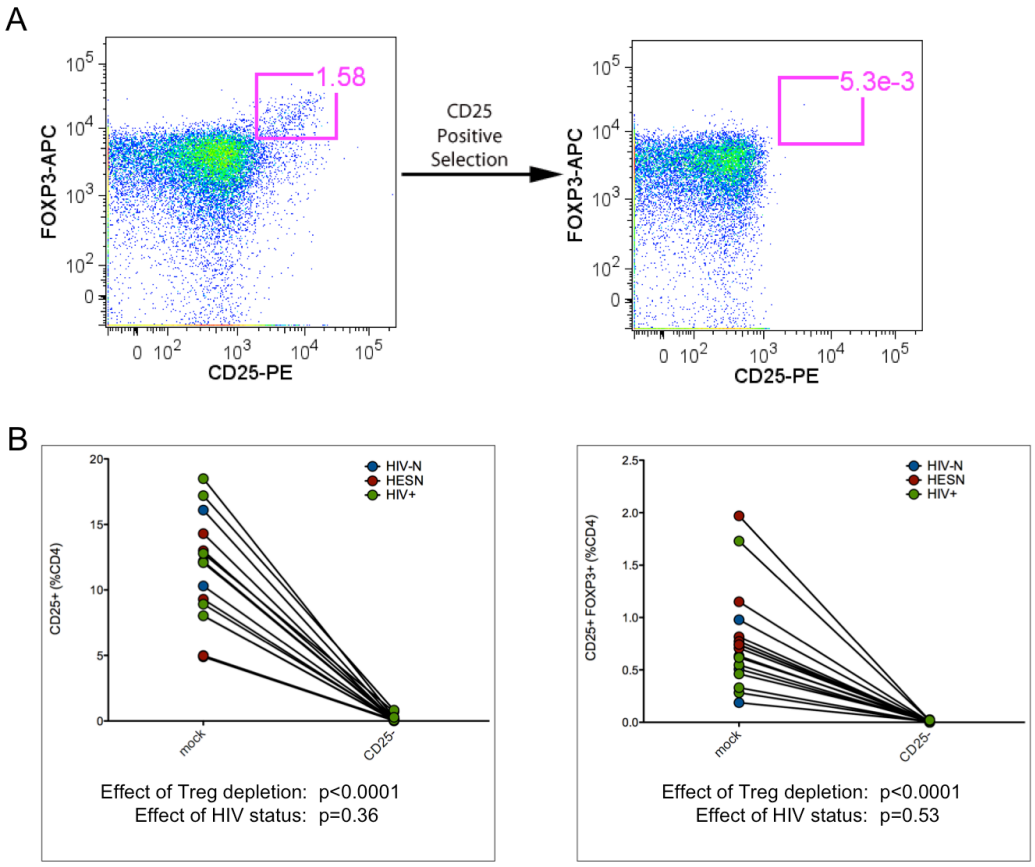


Figure 9. Purity of CD25-depleted PBMC. (A) Representative flow cytometry data of PBMC before and after depletion of CD25+ cells. Events displayed represent CD4+ T cells gated first on singlets, lymphocytes and CD3+ events. CD4+ T cells co-expressing high levels of CD25 (stained with detection antibody clone 2A3) and FOXP3 were designated as Tregs. Data from ML 1607 (HESN) is used in this example. (B) CD25 positive selection effectively depleted CD25+ (left panel) and CD25+ FOXP3+ Tregs (right panel) from PBMC of HIV-N (n=5), HESN (n=6) and HIV+ (n=6), regardless of HIV status. Negative control (mock-depleted) samples were treated the same as CD25-depleted samples but were not incubated with the CD25 positive selection cocktail. Results of two-way repeated measures ANOVA are indicated at the bottom of each panel.

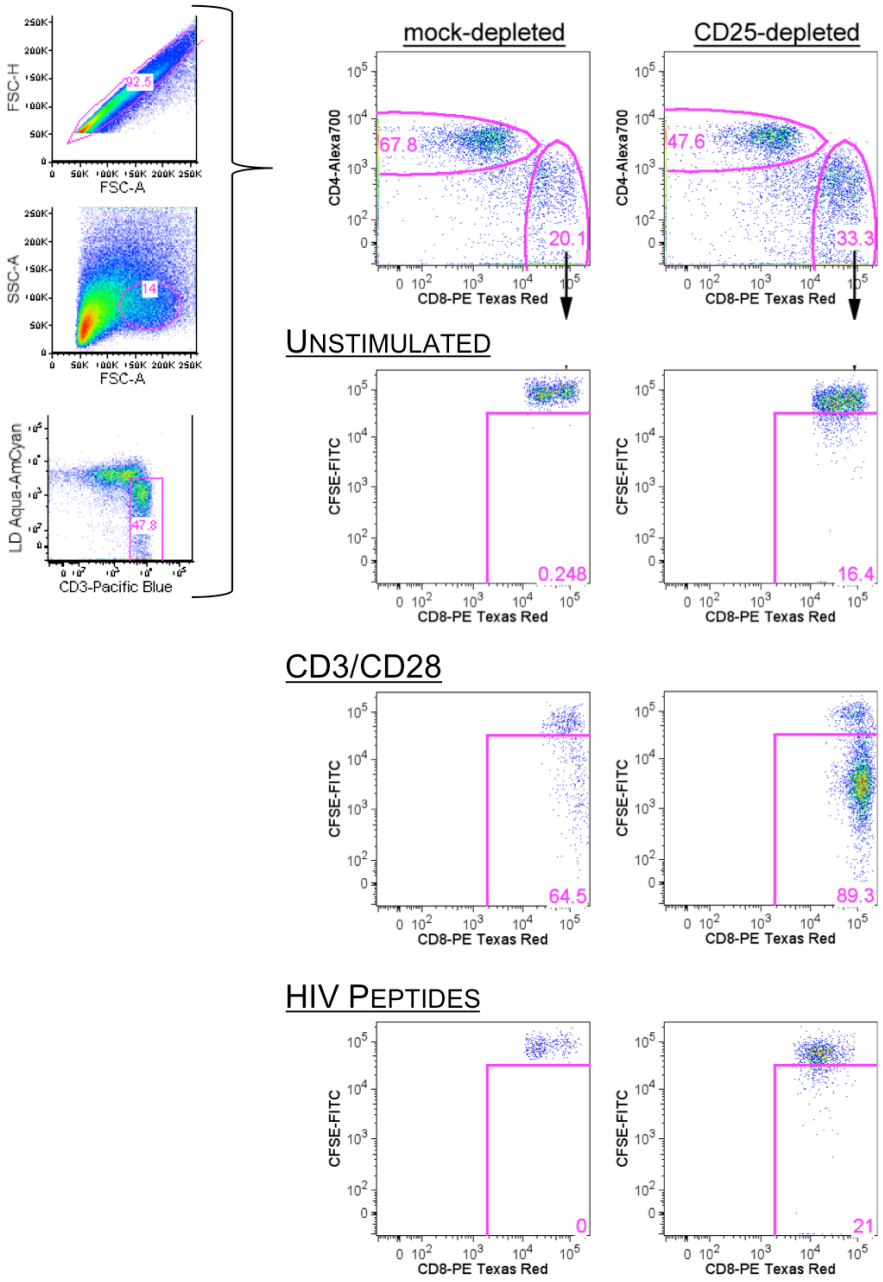


Figure 10. Representative staining of T proliferation responses. Samples were first gated on singlets using FSC-H and FSC-A properties, followed by lymphocyte identification using FSC-A and SSC-A. Live T cells were then identified by gating on CD3+ cells with dim staining of Live/Dead Aqua, and further discriminated based on CD4 and CD8 expression. CFSE dye dilution was used to detect proliferation 6 days post-stimulation with CD3/CD28 beads or HIV peptides in mock-depleted or CD25-depleted cultures. Gating on CD8+ T cells is shown. A parallel approach was used to evaluate CD8+ T cell proliferation. Representative data from ML1985 (HESN) is used in this example.

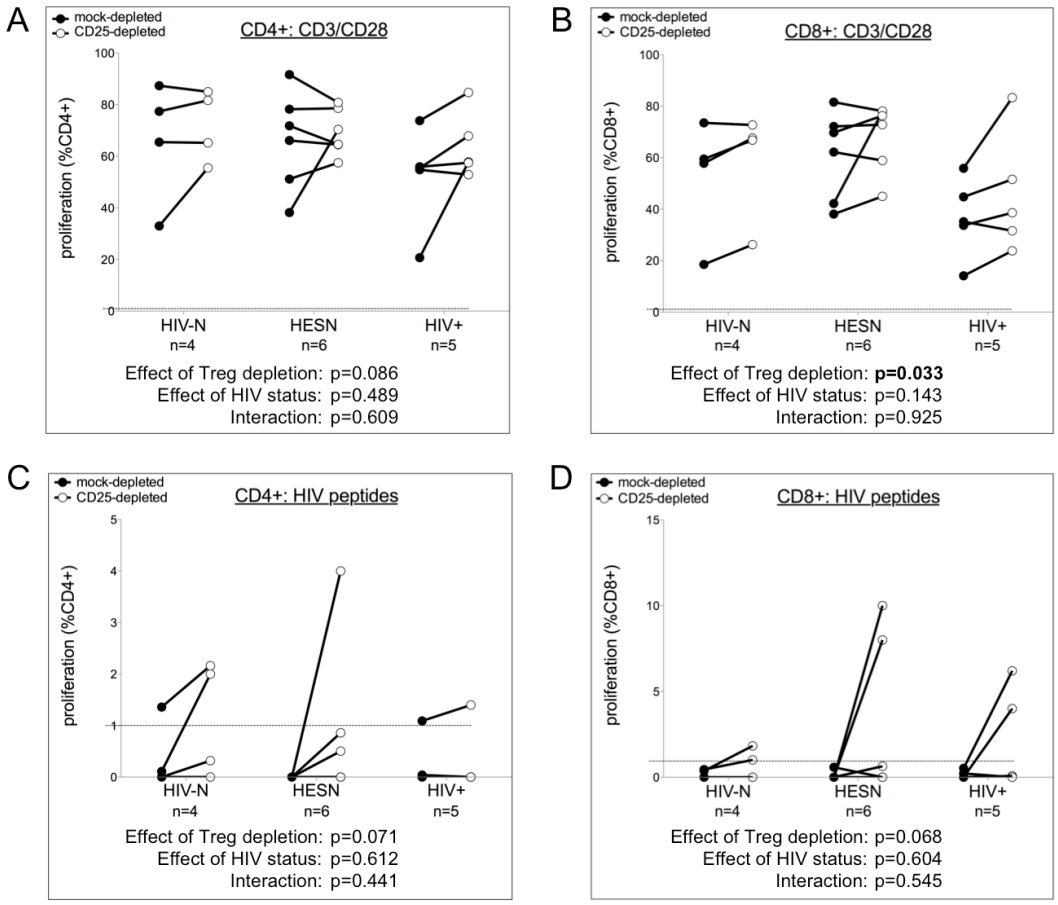


Figure 11. Effect of Treg depletion on T cell proliferation responses. (A-B) Treg depletion resulted in greater CD4+ (A) and CD8+ (B) T cell proliferation in response to stimulation with CD3/CD28 beads in the majority of individuals, though exceptions were observed. (C-D) Treg depletion augmented or revealed HIV-specific CD4+ (C) or CD8+ (D) T cell proliferative responses. Dotted line indicates background level of response as determined by T cell proliferation in response to HIV peptide stimulation in HIV-uninfected low-risk volunteers. Responses above dotted line are considered positive responses. Data includes responses from 4 HIV-N, 6 HESN and 5 HIV+. Results of two-way repeated measures ANOVA are indicated at the bottom of each panel.

We used this threshold to address whether Treg depletion would reveal responses that were previously undetectable. Before Treg depletion, 2 of 15 individuals (1 HIV-N and 1 HIV+) had positive HIV gag-specific CD4+ T cell proliferation responses (Figure 12A), and HIV gag-specific CD8+ T cell proliferation responses were undetectable in all individuals (Figure 12B). Following Treg depletion, HIV gag-specific CD4+ T cell proliferation responses were detectable in 4 individuals, including 2 individuals with previously undetectable responses (1 HIV-N and 1 HESN) (Figure 12A). In the CD8+ T cell compartment, Treg depletion revealed HIV gag-specific proliferation responses in 6 individuals with previously undetectable responses (2 HIV-N, 2 HESN and 2 HIV+) (Figure 12B). The effect of Treg depletion on unveiling CD8+ T cell responses was statistically significant ($p=0.017$).

4.4.4 Effect of Treg depletion on T cell cytokine production

Production of IFNγ, IL-2 and TNFα by CD4+ and CD8+ T cells was measured in mock- and CD25-depleted cell cultures 14 hours post-stimulation with CD3/CD28 beads and HIV gag peptides in HIV-N (n=4), HESN (n=3) and HIV+ (n=4) participants (Figure 13). CD4+ IFNγ, IL-2 and TNFα production in response to CD3/CD28 stimulation was enhanced following Treg depletion ($p=0.030$, $p=0.042$ and $p=0.080$, respectively) (Figure 14A). CD8+ TNFα production in response to CD3/CD28 stimulation was similarly enhanced following Treg depletion ($p=0.049$) (Figure 14B). While Treg depletion boosted CD3/CD28-stimulated CD8+ IFNγ and IL-2 production in some individuals, this effect was not observed in all cases and the effect was not statistically significant.

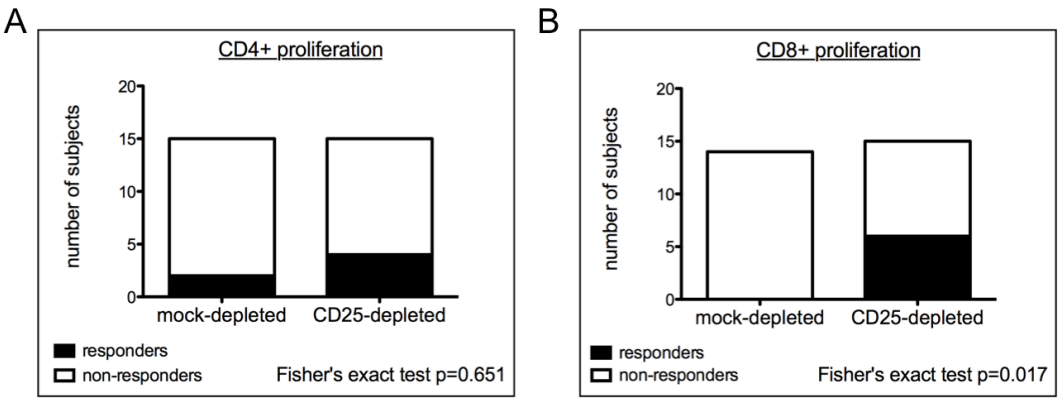


Figure 12. Effect of Treg depletion on detection of positive proliferation responses to HIV gag stimulation. (A) Positive CD4+ T cell proliferation responses were observed in 2/15 individuals in mock-depleted cultures. Following Treg depletion, positive CD4+ T cell proliferation responses were detectable in 4/15 individuals. (B) Positive CD8+ T cell proliferation responses were undetectable in mock-depleted cultures. Following Treg depletion, positive CD8+ T cell proliferation responses were detectable in 6/15 individuals, representing a statistically significant increase. Participants included 4 HIV-N, 6 HESN and 5 HIV+.

Treg depletion enhanced HIV gag-specific CD4+ TNFα production (p=0.064) (Figure 15A) and CD8+ IL-2 and TNFα production (p=0.044 and p=0.046, respectively) (Figure 15B). However, most responses fell below the threshold of positive HIV gag-specific responses pre-determined in HIV-uninfected low-risk volunteers, as described in section 2.2.4 (indicated by dotted line in all panels of Figure 15).

Analogous to the HIV gag-specific proliferation analysis, we used the threshold of positive HIV-specific responses to address whether Treg depletion revealed cytokine responses that were previously undetectable. Before Treg depletion, 2 of 11 individuals (1 HIV-N and 1 HIV+) had detectable HIV gag-specific CD4+ T cell cytokine responses. Following Treg depletion, HIV gag-specific CD4+ T cell cytokine responses were detectable in 6 individuals, including 5 individuals (1 HESN, 1 HIV-N and 3 HIV+) with previously undetectable responses (Figure 16A, top panel). The effects of Treg depletion on revealing individual cytokine responses were also analyzed to assess whether Tregs preferentially inhibit particular cytokines (Figure 16A, lower panels). Treg depletion had no effect on HIV gag-specific CD4+ IFNγ responses. On the contrary, 1 HIV+ individual had a CD4+ IFNγ response in the mock-depleted culture, but this response was lost following Treg depletion. In contrast, HIV gag-specific IL-2 and TNFα responses were revealed upon depletion of Tregs. Treg depletion exposed CD4+ IL-2 responses in 2 HESN and 3 HIV+ individuals, representing a notable increase in CD4+ IL-2 responses (p=0.064). CD4+ TNFα responses were absent in mock-depleted cultures. Following Treg depletion, CD4+ TNFα responses were revealed in 2 HIV+ patients.

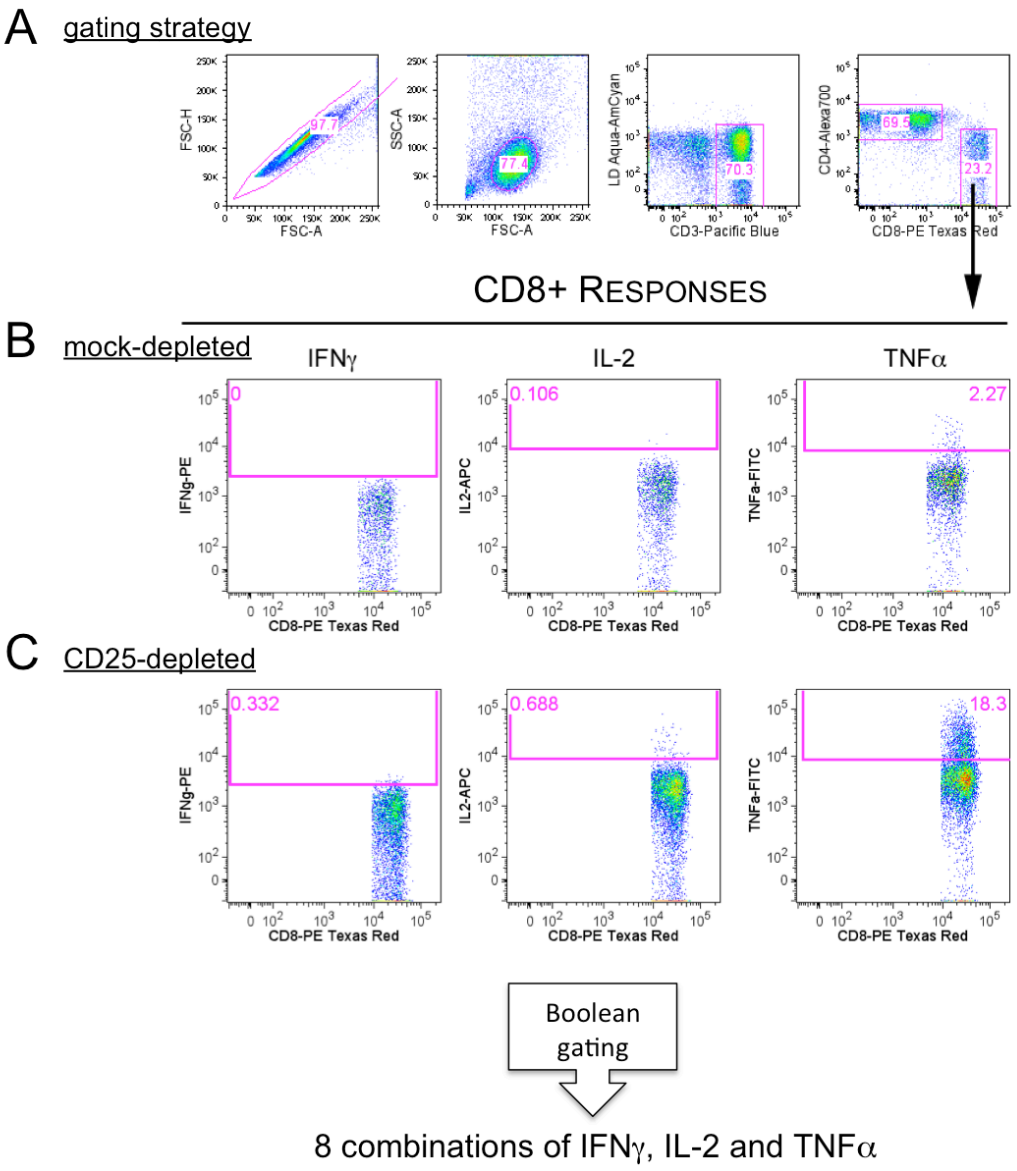


Figure 13. Representative staining of T cell cytokine responses. (A) Samples were first gated on singlets using FSC-H and FSC-A properties, followed by lymphocyte identification using FSC-A and SSC-A. Live T cells were then identified by gating on CD3+ cells with dim staining of LIVE/DEAD Aqua, and further discriminated based on CD4 and CD8 expression. Intracellular cytokine staining was used to detect production of IFNγ, IL-2 and TNFα after stimulation with CD3/CD28 beads or HIV peptides in (B) mock-depleted or (C) CD25-depleted cultures. A boolean gating strategy was applied to identify cells producing all combinations of cytokines. Representative CD8+ T cell responses to CD3/CD28 stimulation in cells from ML1607 (HESN) are used in this example. Parallel approaches were used to evaluate CD4+ and CD8+ T cell cytokine responses after HIV peptide stimulation.

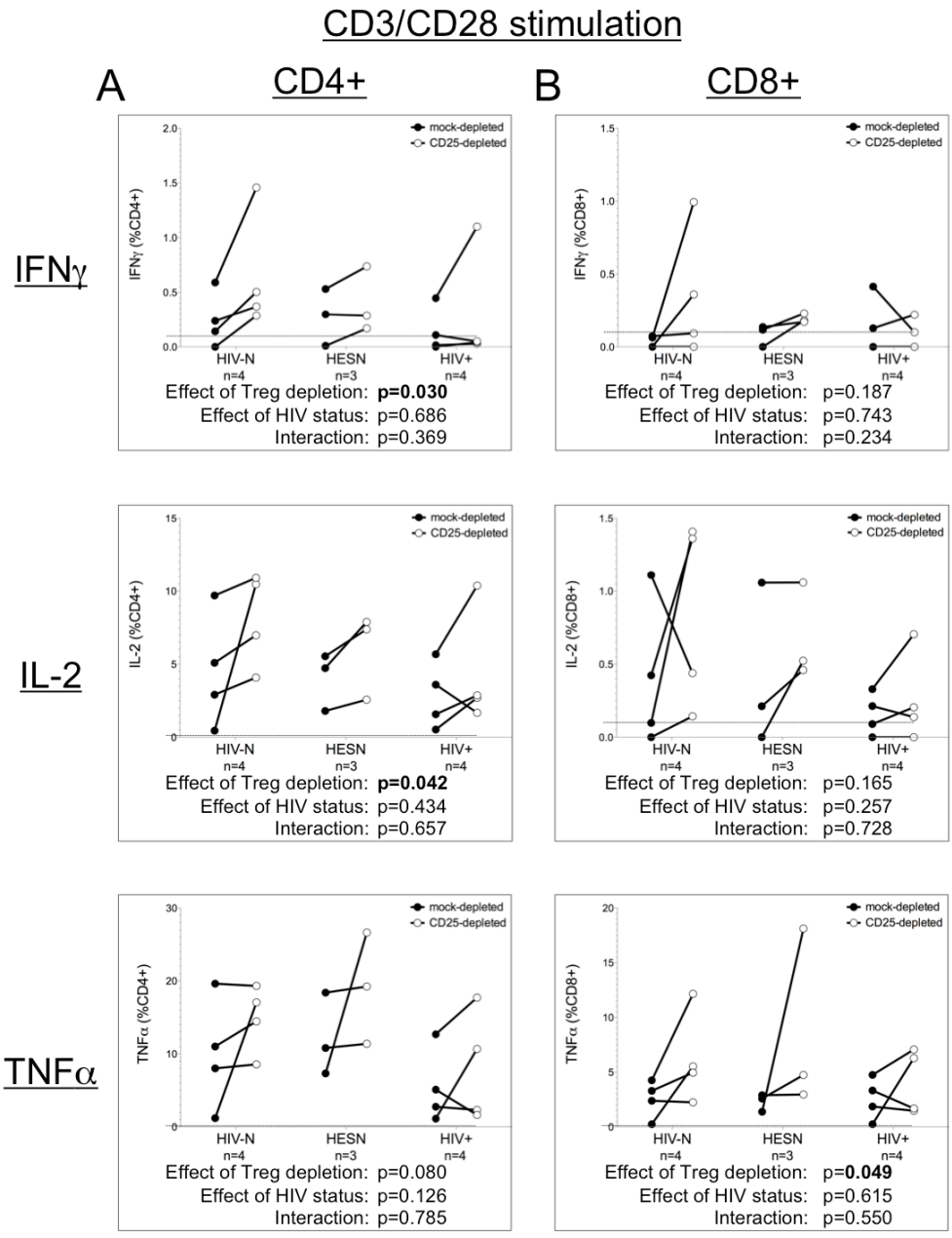


Figure 14. Effect of Treg depletion on T cell cytokine production in response to stimulation with CD3/CD28 beads. (A) Treg depletion resulted in greater CD4+ T cell IFNγ, IL-2 and TNFα production in response to stimulation with CD3/CD28 beads, though exceptions were observed. (B) Treg depletion resulted in greater CD8+ TNFα production in response to stimulation with CD3/CD28 beads. Although Treg depletion boosted CD8+ IFNγ and IL-2 production in several individuals, the increase was not statistically significant. Data includes responses from 4 HIV-N, 3 HESN and 4 HIV+. Results of two-way repeated measures ANOVA are indicated at the bottom of each panel.

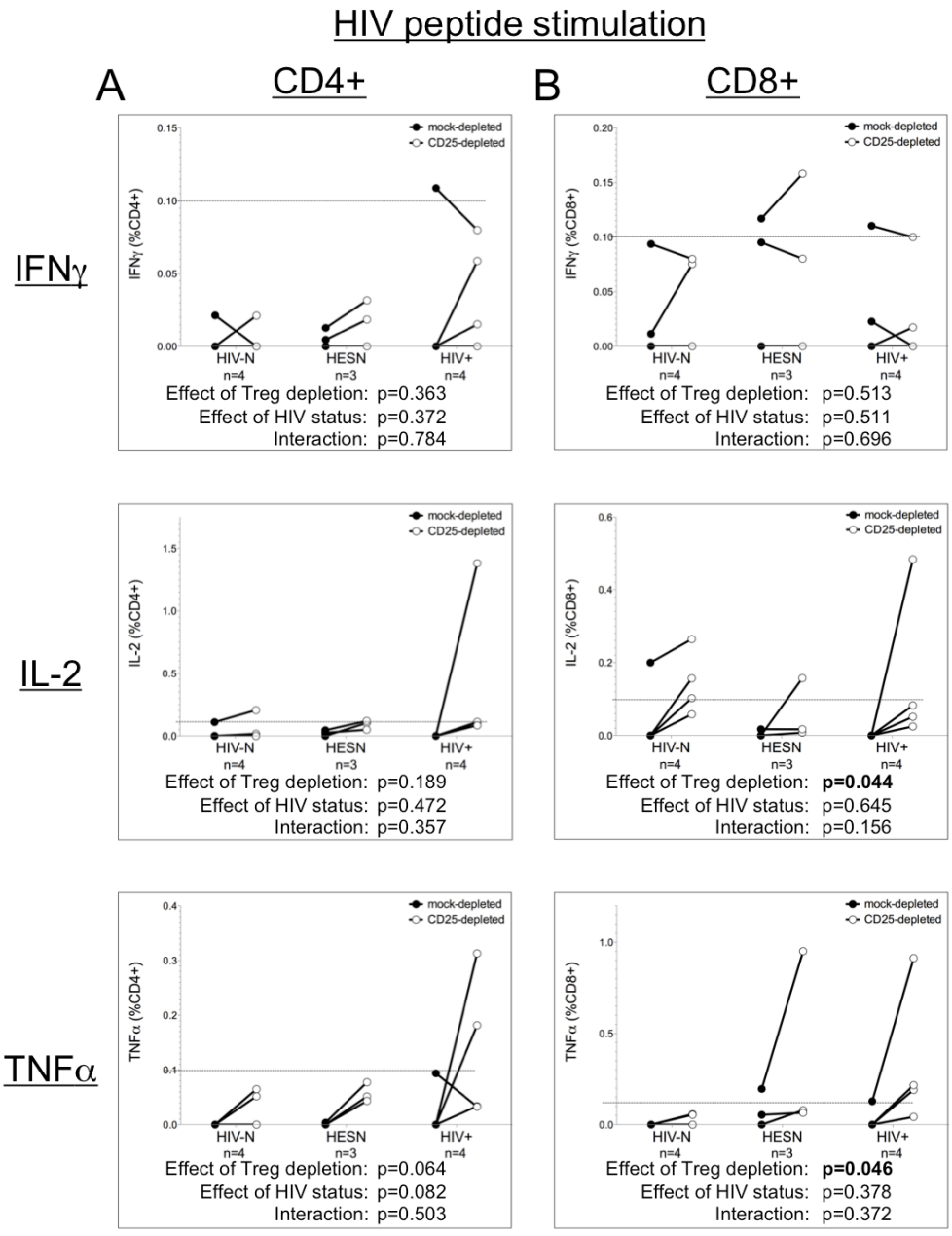


Figure 15. Effect of Treg depletion on T cell cytokine production in response to stimulation with HIV gag peptides. (A) Treg depletion boosted pre-existing and revealed new HIV gag-specific CD4+ IL-2 and TNFα responses in some individuals, but did not boost CD4+ IFNγ responses. (B) Treg depletion resulted in greater HIV gag-specific CD8+ IL-2 and TNFα production in several individuals, but only boosted CD8+ IFNγ production in one case. Data includes responses from 4 HIV-N, 3 HESN and 4 HIV+. Results of two-way repeated measures ANOVA are indicated at the bottom of each panel.

In the CD8+ T cell compartment, 4 of 11 individuals (1 HIV-N, 1 HESN and 2 HIV+) had positive HIV gag-specific cytokine responses prior to Treg depletion. Treg depletion revealed HIV gag-specific cytokine responses in 5 individuals (2 HIV-N, 1 HESN, 2 HIV+) with previously undetectable responses (Figure 16B, top panel). When the total cytokine response was broken down into individual cytokines, the trends that emerged were comparable to those for CD4+ cytokine responses (Figure 16B, lower panels). Treg depletion had no effect on HIV gag-specific CD8+ IFNγ responses, which were present in 1 HESN and 1 HIV+ patient in mock-depleted cultures. Treg depletion resulted in boosting of the CD8+ IFNγ response in the HESN, but dampening of the response in the HIV+ patient. CD8+ IL-2 responses were detectable in 1 HIV-N in mock-depleted cultures. Treg depletion revealed CD8+ IL-2 responses in an additional 4 individuals, including 2 HIV-N, 1 HESN and 1 HIV+ ($p=0.149$). CD8+ TNFα responses were detectable in 1 HESN and 1 HIV+ patient in mock-depleted cultures, and were revealed in an additional 2 HIV+ patients following Treg depletion. These data show that HIV-specific CD4+ and CD8+ T cell cytokine responses can be revealed by Treg depletion, though these increases in detection of cytokine responses did not reach statistical significance.

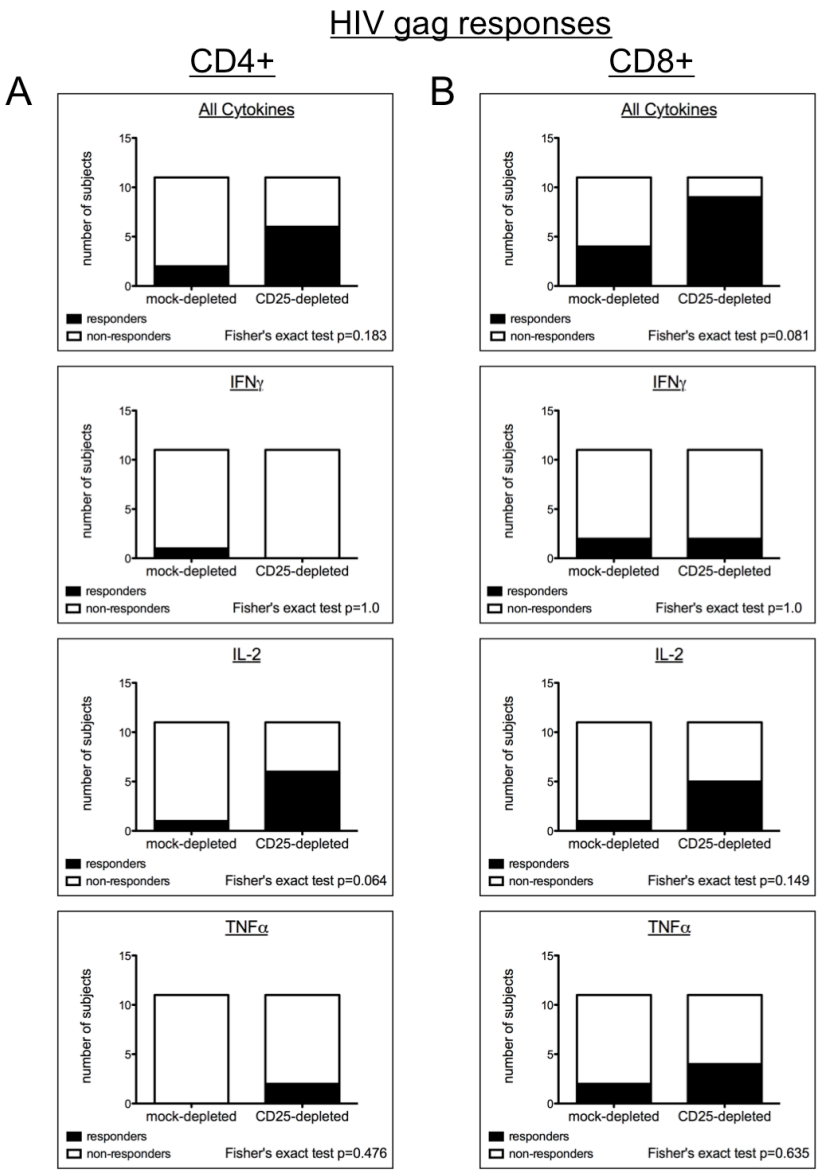


Figure 16. Effect of Treg depletion on detection of positive cytokine responses to HIV gag stimulation. (A) Positive CD4+ T cell cytokine responses were observed in 2/11 individuals in mock-depleted cultures. Following Treg depletion, positive CD4+ T cell cytokine responses were detectable in 6/11 individuals, including 1/11 IFNγ, 1/11 IL-2 and no TNFα responders. Responses in CD25-depleted cultures included 0/11 IFNγ, 6/11 IL-2 and 2/11 TNFα responders. (B) Positive CD8+ T cell cytokine responses were observed in 4/11 individuals in mock-depleted cultures. Following Treg depletion, positive CD8+ T cell cytokine responses were detectable in 9/11 individuals, including 2/11 IFNγ, 1/11 IL-2 and 2/11 TNFα responders. Responses in CD25-depleted cultures included 2/11 IFNγ, 5/11 IL-2 and 4/11 TNFα responders. Participants included 4 HIV-N, 3 HESN and 4 HIV+.

4.4.5 Effect of Treg depletion on polyfunctionality of T cell cytokine responses

To address whether Treg depletion affects polyfunctionality (i.e. the production of more than one cytokine by a single cell), we analyzed the contribution of each cytokine combination to the total HIV gag-specific cytokine response (Figure 17). The HIV gag-specific CD4+ T cell cytokine response was predominantly composed of cells producing a single cytokine (i.e. IFNγ, TNFα or IL-2) in mock-depleted cultures. However, following Treg depletion, the proportion of CD4+ cells producing IL-2 and TNFα increased and a small population of cells co-producing all three cytokines emerged (Figure 17A), though this was not statistically significant (p=0.40). In the CD8+ T cell compartment, Treg depletion resulted in expansion of the IL-2+ TNFα- and IL-2- TNFα+ CD8+ T cell populations (Figure 17A). This change in the distribution of cytokine-producing cells represented a notable trend, though not statistically significant (p=0.07).

We also analyzed cytokine polyfunctionality in individual groups of participants (Figure 17B). In the HIV-N group, Treg depletion resulted in expansion of CD4+ IL-2+ TNFα+, CD4+ TNFα+, CD8+ IL-2+ and CD8+ TNFα+ populations. These changes in cytokine production by HIV-N were not statistically significant in the CD4+ T cell compartment (p=0.70), but a trend was observed in the CD8+ T cell compartment (p=0.08). The changes in cytokine distribution in HESN mirrored those in HIV-N participants, such that Treg depletion resulted in increases in CD4+ IL-2+ TNFα+, CD4+ TNFα+, CD8+ IL-2+ and CD8+ TNFα+ populations. These changes in cytokine production by HESN represented a trend in the CD4+ T cell compartment (p=0.09), but were not statistically significant in

the CD8+ T cell compartment (p=0.50). The effects of Treg depletion on cytokine polyfunctionality differed in HIV+ patients relative to HIV-N and HESN. In the CD4+ T cell compartment, Treg depletion resulted in increased IL-2+ cells, similar to uninfected groups. In addition, Treg depletion resulted in the emergence of a small population of cells producing all three cytokines. These changes to CD4+ T cell cytokine distribution in HIV+ patients were not statistically significant (p=0.37). In the CD8+ T cell compartment, Treg depletion led to an expansion of the IL-2+ TNFα+ population while all other cytokine-producing populations experienced a relative reduction in size, but this change was not statistically significant (p=0.62). This increase in dual-cytokine production by CD8+ T cells is contrary to HIV-uninfected groups, which experience a relative increase in cells producing a single cytokine (IL-2 or TNFα).

4.4.6 Correlation between ex vivo Treg phenotype and effect of Treg depletion

We next addressed whether the magnitude of the change in T cell response resulting from Treg depletion is a factor of the frequency or phenotype of Tregs. The magnitude of the change in T cell response (Δ response) resulting from Treg depletion was calculated as: [%responding T cells in CD25-depleted culture] – [%responding T cells in mock-depleted culture] for each effector function. The calculated difference was then correlated with the *ex vivo* Treg frequency and Treg expression of CTLA-4.

All participants had positive responses to CD3/CD28 stimulation, so this condition was used to examine the relationship between *ex vivo* Tregs and the effects of Treg

depletion on cytokine production (HIV-N n=4, HESN n=3, HIV+ n=4) and proliferation (HIV-N n=4, HESN n=6, HIV+ n=5). As shown in Table 13, Treg frequency correlated with the magnitude of the change in CD8+ TNFα response to CD3/CD28 stimulation (p=0.027, r=0.661). A similar trend was observed for CD4+ TNFα, though this did not reach statistical significance (p=0.086, r=0.541). No associations were found between the magnitude of the change in T cell IFNγ or IL-2 responses and Treg frequency. CTLA-4 expression on Tregs correlated with the magnitude of the change in CD4+ IL-2 response to CD3/CD28 stimulation (p=0.043, r=0.618).

4.5 Summary

Treg depletion increased T cell proliferation and cytokine responses to stimulation with CD3/CD28 beads in several individuals, independent of HIV status. This was consistent with the hypothesis that HIV-N, HESN and HIV+ would demonstrate comparable increases in T cell responses to CD3/CD28 stimulation following Treg depletion.

Treg depletion increased the magnitude of HIV-specific T cell responses, and revealed new responses, in several individuals from HIV-N, HESN and HIV+ groups. The presence of detectable responses and the magnitude of the change following Treg depletion varied widely. Among the three cytokines measured, a preferential increase in IL-2 production was apparent, which was reflected in the cytokine polyfunctionality analysis.

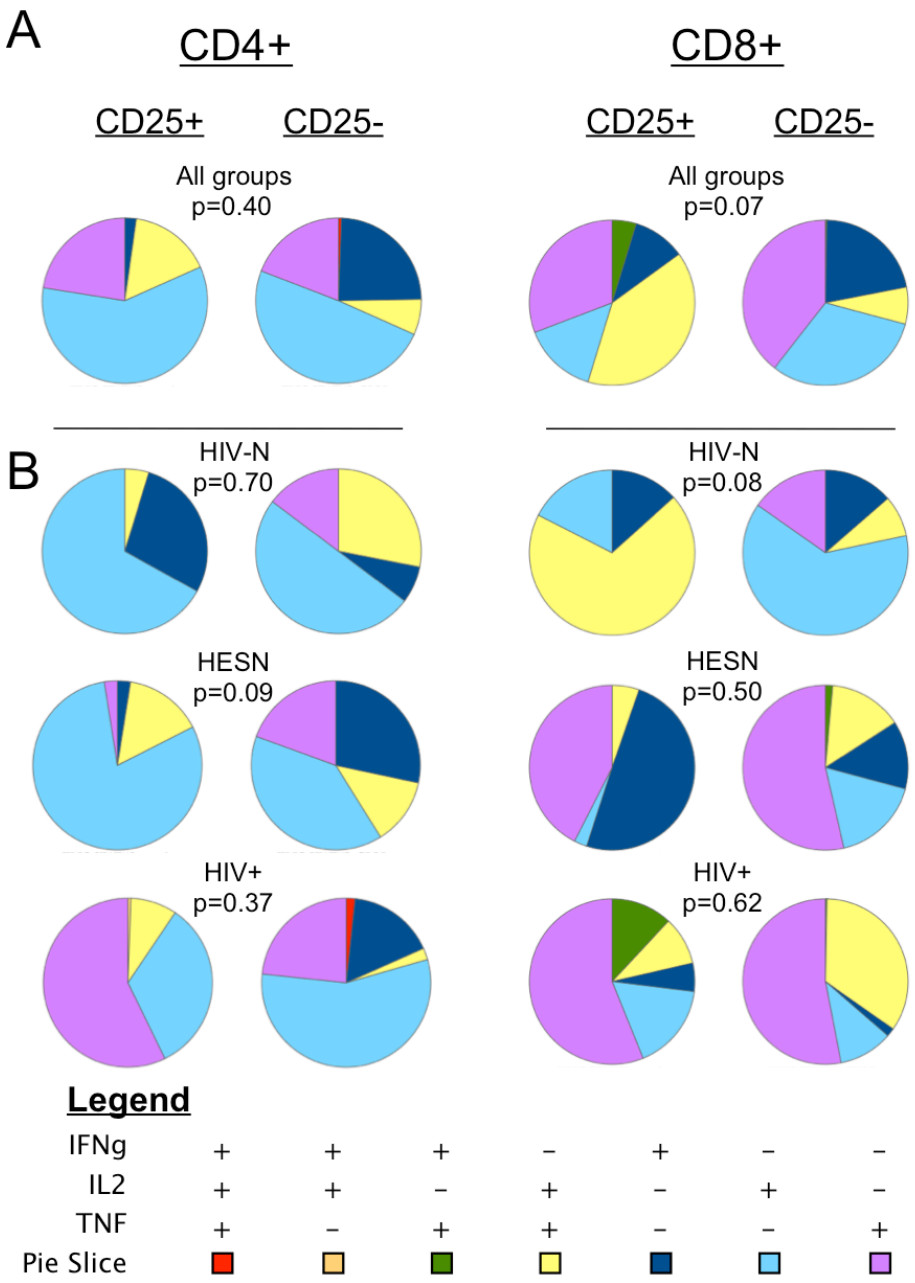


Figure 17. Effect of Treg depletion on cytokine polyfunctionality of HIV-specific T cells. (A) Relative contributions of various cytokine combinations to the total HIV-specific CD4+ and CD8+ T cell responses in the presence or absence of CD25- depletion, independent of HIV status (n=11). (B) Relative contributions of various cytokine combinations to HIV-specific CD4 and CD8 T cell responses for HIV-N (n=4), HESN (n=3) and HIV+ (n=4) groups. Colours of pie slices indicate cytokine combinations as indicated in the legend. Cytokine distributions before and after Treg depletion were compared by permutation testing (10,000 tests). Permutation p-values for each pair of pies are shown.

Table 13. Correlation between Treg phenotype and effect of Treg depletion on responses to CD3/CD28 stimulation[a]

		Treg frequency		CTLA-4 expression on Tregs	
		p-value[b]	r[c]	p-value	r
CD4 responses	IFNγ	0.441		0.441	
	IL-2	0.958		**0.043**	0.618
	TNFα	**0.086**	0.541	0.647	
	Proliferation	0.754		0.824	
CD8 responses	IFNγ	0.343		0.491	
	IL-2	0.307		0.350	
	TNFα	**0.027**	0.661	0.570	
	Proliferation	0.818		0.794	

[a] Effect of Treg depletion calculated as:
[%responding T cells in CD25-depleted culture] – [%responding T cells in mock-depleted culture]
[b] p-values calculated by Spearman rank correlation. Significant ($p<0.05$) or trending ($p<0.10$) p-values are shown in bold text.
[c] r values are Spearman rho correlation coefficients. Spearman r values are only shown in the case of a significant ($p<0.05$) or trending ($p<0.1$) p-value.

The final hypothesis for this study was that amplification of T cell responses following Treg depletion would be strongest in Individuals with high Treg frequencies. Consistent with this, the increase in CD3/CD28-induced TNFα responses after Treg depletion correlated with Treg frequency. However, no other readout demonstrated a comparable correlation. The frequency of CTLA-4+ Tregs correlated with the magnitude of the change in CD4+ IL-2 responses to CD3/CD28, highlighting the importance of CTLA-4 in immunoregulation.

Chapter 5. Immune Quiescence in the Female Genital Tract Mucosa

5.1 Rationale

The results discussed in Chapter 3 of this thesis, along with other emerging data from the Pumwani CSW cohort, suggest that resistance to infection is associated with reduced T cell activation in HESN. However, those data were focused on peripheral blood. We asked whether the reduced activation observed in peripheral blood was mirrored at the female genital tract (FGT) mucosa, which is the primary site of exposure to HIV for these women. To this end, levels of cytokines and chemokines in cervicovaginal lavage (CVL) samples and markers of T cell activation and Tregs in cervical mononuclear cells (CMC) and PBMC were evaluated in HESN and HIV-N participants of the Pumwani cohort.

5.2 Hypotheses

1. Proinflammatory cytokines and chemokines will be reduced in CVL from HESN relative to HIV-N.
2. HESN will have lower T cell activation and higher Tregs in CMC compared to HIV-N.
3. Frequencies of activated T cells and Tregs will be elevated in CMC compared to peripheral blood.

5.3 Objectives

1. Compare concentrations of cytokines and chemokines in CVL between HESN and HIV-N.
2. Compare T cell activation and Tregs in CMC between HESN and HIV-N.

3. Compare markers of T cell activation and Tregs between CMC and PBMC samples of HESN and HIV-N.

5.4 Results

5.4.1 Study populations

CVL samples from a total of 25 HIV-N and 18 HESN from the Pumwani cohort were collected. HESN were older ($p<0.0001$), had longer self-reported duration of prostitution ($p<0.0001$) and duration of follow-up ($p<0.0001$). Study groups did not differ in clients per week, days since last sex, days since last menses, douching practices or proportion of patients with bacterial vaginosis (BV). No patients were diagnosed with Gonorrhea or Chlamydia infections. A higher proportion of HESN were in menopause compared to HIV-N ($p=0.002$) (Table 14).

CMC samples were included in the flow cytometry analysis if they had discernable CD4+ and CD8+ T cells populations. CMC samples from a total of 11 HIV-N and 11 HESN met the inclusion criteria. As expected, HESN were older ($p=0.002$), had longer self-reported duration of prostitution ($p<0.0001$) and duration of follow-up ($p<0.0001$). Groups did not differ in clients per week, days since last sex, days since last menses, douching practices or proportion of participants in menopause or with BV. No patients were diagnosed with Gonorrhea or Chlamydia infections (Table 15).

Table 14. Characteristics of participants included in CVL cytokine and chemokine study			
	HIV-N (n=25)	HESN (n=18)	p-value[e]
Age, years	**33.0 (30.5-37.5)**	**43.5 (37.0-52.0)**	**<0.0001**
Duration of prostitution, years[a]	**5.0 (3.0-10.0)**	**18.5 (12.8-28.0)**	**<0.0001**
Duration of follow-up, years[a]	**1.5 (1.3-2.3)**	**15.5 (8.0-17.6)**	**<0.0001**
Clients/week[a]	13.0 (3.0-25.0)	5.5 (1.8-15.3)	0.062
Time since last sex, days[a]	2.0 (1.0-5.5)	1.5 (1.0-7.3)	0.866
Days since last menses[a, b]	11.0 (4.5-17.0)	11.5 (8.0-14.25)	0.976
Menopause, number (%)[b, c]	**1 (4.0)**	**8 (44)**	**0.002**
Practice douching[c]	25 (100)	15 (83.3)	0.066
Douched on sample date[c]	17 (68.0)	13 (72.2)	1.0
Gonorrhoea infection[c]	0 (0)	0 (0)	1.0
Chlamydia infection[c]	0 (0)	0 (0)	1.0
Bacterial vaginosis[c, d]	12 (48)	5 (29.4)	0.339

[a] Data represent median (interquartile range) values.
[b] Excludes menopausal patients. Data on last menses was unavailable for three subjects.
[c] Data represent number of patients (% of patients).
[d] Patients with positive BV diagnosis had intermediate or BV+ Nugent scores. BV data was unavailable for one HESN patient.
[e] p-values were calculated using Mann Whitney *U* tests for continuous variables and Fisher's exact test for dichotomous variables. Variables that showed statistically significant ($p<0.05$) or trending ($p<0.10$) differences are highlighted in bold text.

Table 15. Characteristics of participants included in CMC T cell phenotyping study			
	HIV-N (n=11)	HESN (n=11)	p-value[e]
Age, years[a]	**33.0 (31.0-38.0)**	**44.0 (41.0-53.0)**	**0.002**
Duration of prostitution, years[a]	**6.0 (3.0-8.0)**	**22.0 (15.0-28.0)**	**<0.0001**
Duration of follow-up, years[a]	**1.5 (1.5-2.0)**	**16.0 (8.0-17.5)**	**<0.0001**
Clients/week[a]	13.0 (3.0-25.0)	6.0 (2.0-14.0)	0.372
Time since last sex, days[a]	1.0 (2.0-5.0)	1.0 (2.0-6.0)	0.835
Days since last menses[a, b]	11.0 (4.5-14.0)	12.5 (6.5-14.75)	0.667
Menopause[b, c]	2 (18.2)	6 (54.5)	0.183
Practice douching[c]	11 (100)	9 (81.9)	0.476
Douched on sample date[c]	7 (63.6)	8 (72.7)	1.0
Gonorrhoea infection[c]	0 (0)	0 (0)	1.0
Chlamydia infection[c]	0 (0)	0 (0)	1.0
Bacterial vaginosis[c, d]	5 (45.5)	4 (50)	1.0

[a] Data represent median (interquartile range) values.
[b] Excludes menopausal patients. Data on last menses was unavailable for three subjects.
[c] Data represent number of patients (% of patients).
[d] Patients with positive BV diagnosis had intermediate or BV+ Nugent scores. BV data was unavailable for one HESN patient.
[e] p-values were calculated using Mann Whitney *U* tests for continuous variables and Fisher's exact test for dichotomous variables. Variables that showed statistically significant ($p<0.05$) or trending ($p<0.10$) differences are highlighted in bold text.

5.4.2 Concentrations of cytokines and chemokines in CVL

The concentrations of 23 cytokines and chemokines were evaluated in CVL samples from HIV-N (n=25) and HESN (n=18) participants. Analytes measured included fractalkine (CX3CL1), interferon (IFN)-α2, IFNγ, interleukin (IL)-1α, IL-1β, IL-1 receptor agonist (ra), IL-2, soluble IL-2 receptor (sIL-2R)-α, IL-6, IL-7, IL-8, IL-10, IL-15, IL-17, IFNγ-induced protein (IP)-10 (CXCL10), monocyte chemotactic protein (MCP)-1 (CCL2), MCP-3 (CCL7), MDC (CCL22), macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4), soluble CD40 ligand (sCD40L), tumor necrosis factor (TNF)-α and transforming growth factor beta (TGF)-β. Concentrations of IFNγ, IL-2, sIL-2Rα, IL-6, IL-10, IL-15, IL-17, TNFα and TGFβ were undetectable in >80% of samples.

Concentrations of the 14 analytes that met the criteria for analysis (detectable in >20% of samples) are shown in Table 16. The concentration of the proinflammatory cytokine IL-1α was significantly lower in CVL from HESN compared to HIV-N (p=0.009). A similar trend was observed for the chemokine IL-8/CXCL8, but the association did not reach statistical significance (p=0.083) (Figure 18A). The proportions of participants with detectable levels of IL-1α and IL-8 in CVL were also evaluated (Figure 18B). Fewer HESN subjects had detectable levels of IL-1α (>3.45 pg/ml) than HIV-N (p=0.020), whereas the proportion of subjects with detectable levels of IL-8 (>3.25 pg/ml) was comparable between groups.

Table 16. Cytokine and chemokine levels in CVL samples from HESN and HIV-N

Analyte	Concentration[a]		p-value[b]
	HIV-N (n=25)	HESN (n=18)	
Chemokines			
Fractalkine/CXCL1	3.6 (0.0-38.9)	5.9 (0.0-18.2)	0.900
IL-8/CXCL8	**131.6 (7.6-691.2)**	**24.6 (0.0-181.0)**	**0.083**
IP-10/CXCL10	12.4 (0.0-57.5)	12.4 (0.0-74.6)	0.990
MCP-1/CCL2	0.0 (0.0-24.8)	0.0 (0.0-39.4)	0.979
MCP-3/CCL7	0.0 (0.0-4.7)	0.0 (0.0-4.0)	0.904
MDC/CCL22	11.2 (0.0-24.9)	5.4 (2.5-24.9)	1.00
MIP-1α/CCL3	0.0 (0.0-7.8)	0.0 (0.0-0.0)	0.539
MIP-1β/CCL4	0.0 (0.0-9.3)	0.0 (0.0-9.3)	0.725
Proinflammatory and anti-viral cytokine response			
IFNα2	0.0 (0.0-6.4)	2.1 (0.0-12.3)	0.538
IL-1α	**28.3 (7.3-132.0)**	**0.0 (0.0-19.4)**	**0.009**
IL-1β	0.0 (0.0-1.3)	0.0 (0.0-7.1)	0.337
sCD40L	0.0 (0.0-25.9)	0.0 (0.0-5.4)	0.349
Anti-inflammatory cytokine response			
IL-1ra	2882 (1034-4136)	4327 (2031-4648)	0.181
Homeostatic Cytokines			
IL-7	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.653

[a] Concentration is listed as median pg/ml. Interquartile range is shown in brackets.
[b] p-values were calculated using Mann-Whitney *U* tests. Variables that showed statistically significant ($p<0.05$) or trending ($p<0.10$) differences are highlighted in bold text.

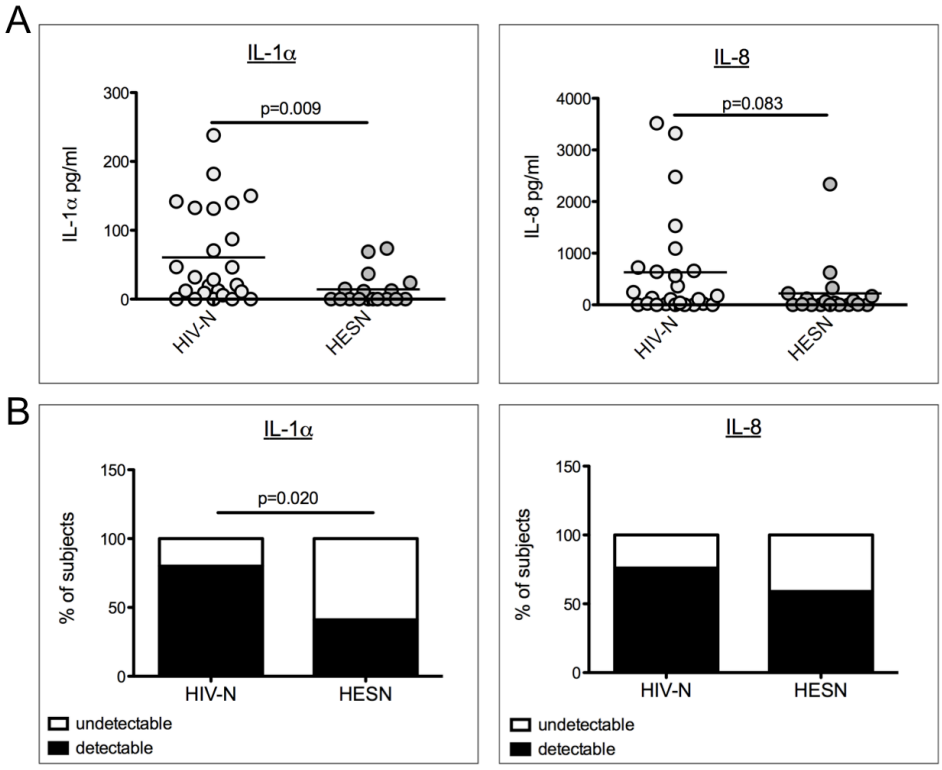


Figure 18. Comparison of IL-1α and IL-8 expression in CVL samples between HIV-N and HESN. Lines indicate mean values. (A) HESN (n=25) had lower concentrations of IL-1α and IL-8 in CVL samples than HIV-N (n=18). P-values were calculated using the Mann-Whitney *U* test. (B) The proportion of subjects with detectable levels of IL-1α (>3.45 pg/ml) was lower for HESN than HIV-N. The proportion of subjects with detectable levels of IL-8 (>3.25 pg/ml) did not differ between groups. P-values were calculated using Fisher's exact test.

5.4.3 T cell activation in CMC

To determine levels of mucosal CD4+ and CD8+ T cell activation, expression of CD69, HLA DR and CD38 were measured on CMC from HIV-N (n=5) and HESN (n=6). Representative staining of activation markers on T cells in CMC is shown in Figure 19. No significant differences were found in the expression of CD69, CD38 or HLA DR on CD4+ (Figure 20A) or CD8+ (Figure 21A) T cells from CMC. In addition to measuring expression of individual activation markers on CD4+ and CD8+ T cells, coexpression of activation markers was examined using a boolean gating analysis strategy, allowing evaluation of T cell populations expressing every combination of CD69, CD38 and HLA DR. No significant differences were observed between HESN and HIV-N in the frequencies of CD4+ (Figure 20B) or CD8+ (Figure 21B) T cells expressing any specific combination of activation markers. Although HESN had lower levels of total activated CD4+ (Figure 20B) and CD8+ (Figure 21B) T cells (i.e. the sum of all activated subsets) than HIV-N, the differences were not statistically significant.

These results are in contrast to the observed differences in T cell activation between HESN and HIV-N in PBMC, as discussed in Chapter 3. However, the small sample size (HIV-N n=5, HESN n=6) may preclude detection of differences between groups. Indeed, analysis of T cell activation in matched PBMC from the same individuals did not reveal any differences between groups (Table 17). Based on the observed differences between groups in PBMC activation, this study was only 15.5% and 43% powered to detect differences in CD4+ and CD8+ T cell activation, respectively.

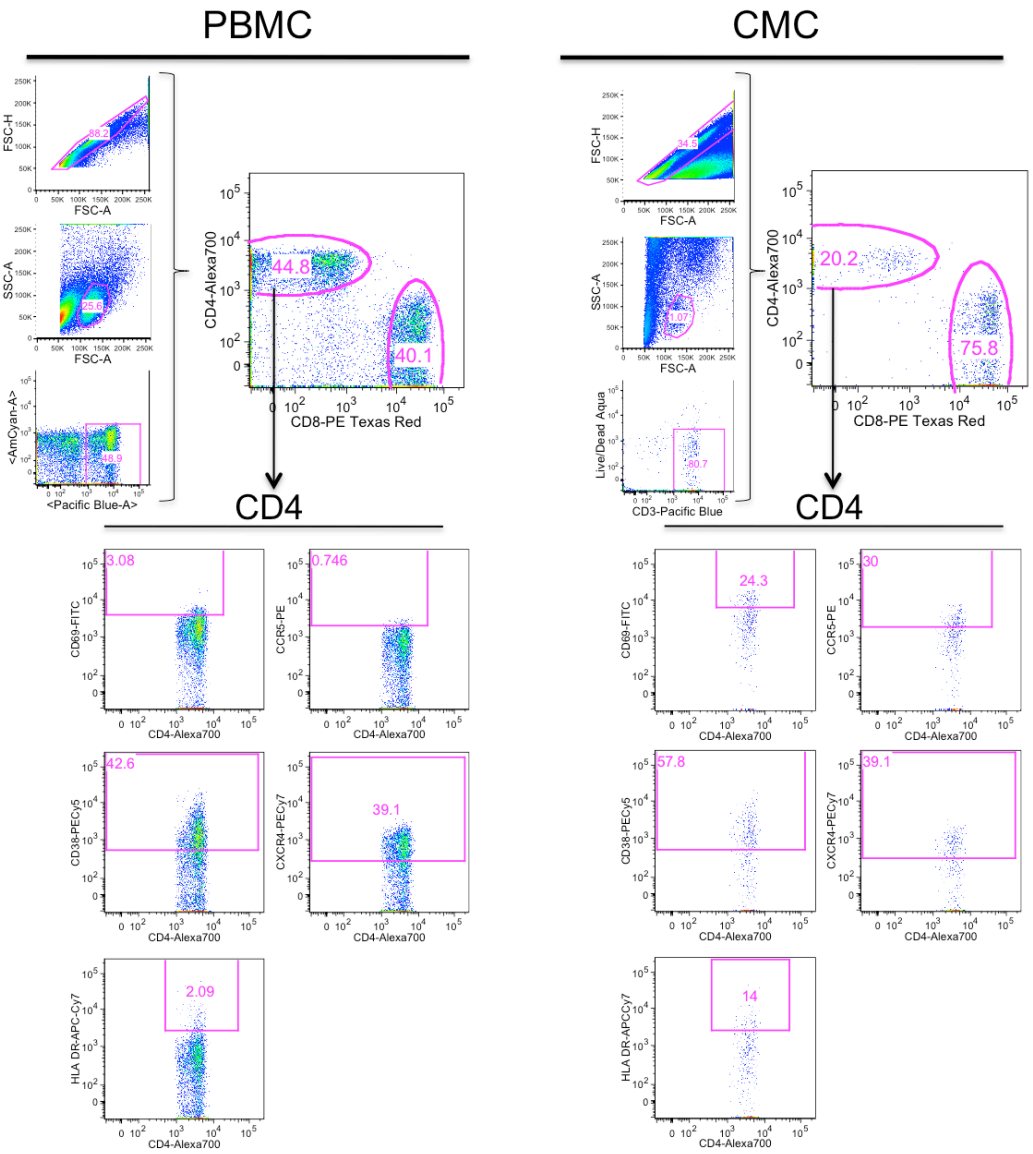


Figure 19. Representative staining of T cell activation and HIV coreceptor expression in CMC and PBMC. Samples were first gated on singlets using FSC-H and FSC-A properties, followed by lymphocyte identification using FSC-A and SSC-A. Live T cells were then identified by low expression of LIVE/DEAD Aqua and expression of CD3, and further discriminated based on CD4 and CD8 expression. Expression of CD69, CD38, HLA DR, CCR5 and CXCR4 was then evaluated for CD4+ and CD8+ T cells. A Boolean gating strategy was applied to evaluate co-expression of multiple markers. Gating on CD4+ T cells is shown. A parallel approach was used to evaluate CD8+ T cell activation. Data from ML3064 (HIV-N) is used in this example.

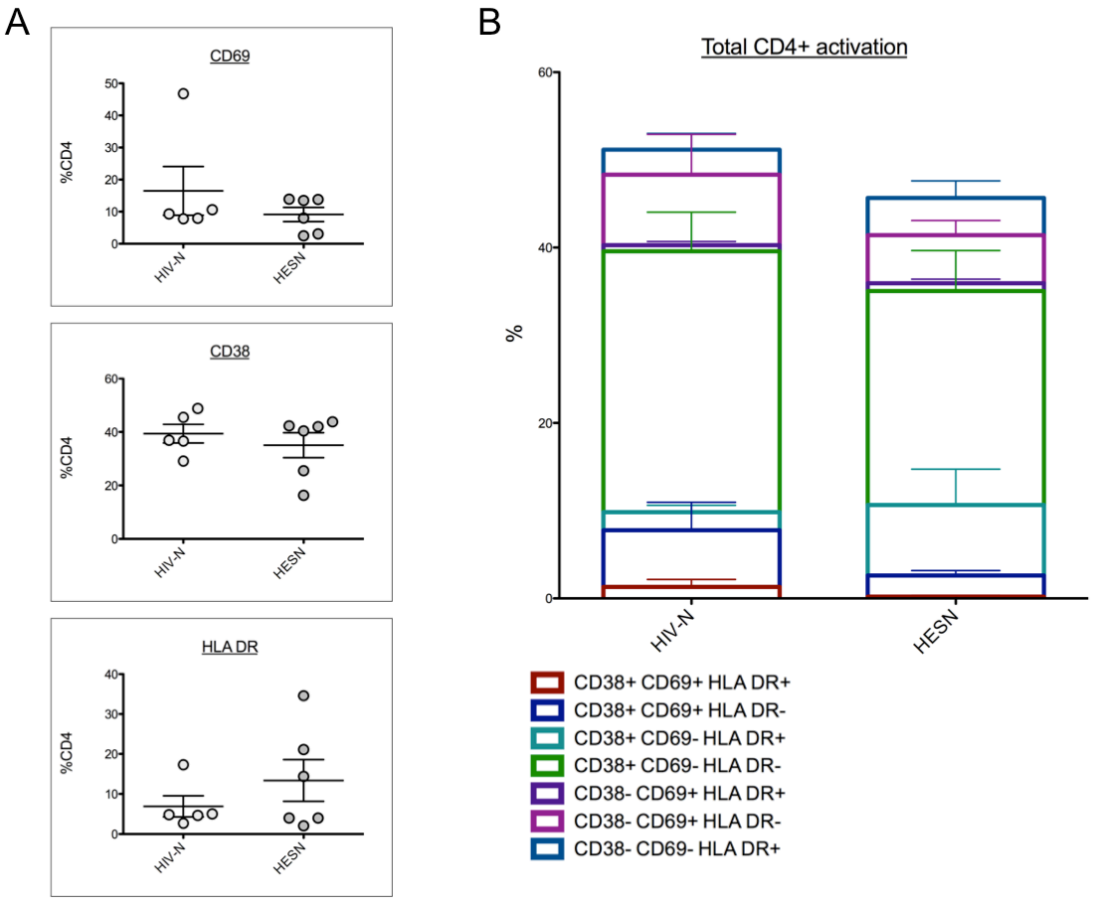


Figure 20. Comparison of CD4+ T cell activation in CMC between HIV-N and HESN. (A) Frequencies of CD4+ T cells expressing CD69, CD38 or HLA DR in CMC were not significantly different between HIV-N (n=5) and HESN (n=6). (B) The contribution of various subsets of activated T cells (based on co-expression of CD69, CD38 and HLA DR) to the total pool of activated CD4+ T cells was examined. HIV-N and HESN did not differ in the proportions of specific activated subsets or in the level of total CD4+ T cell activation in CMC.

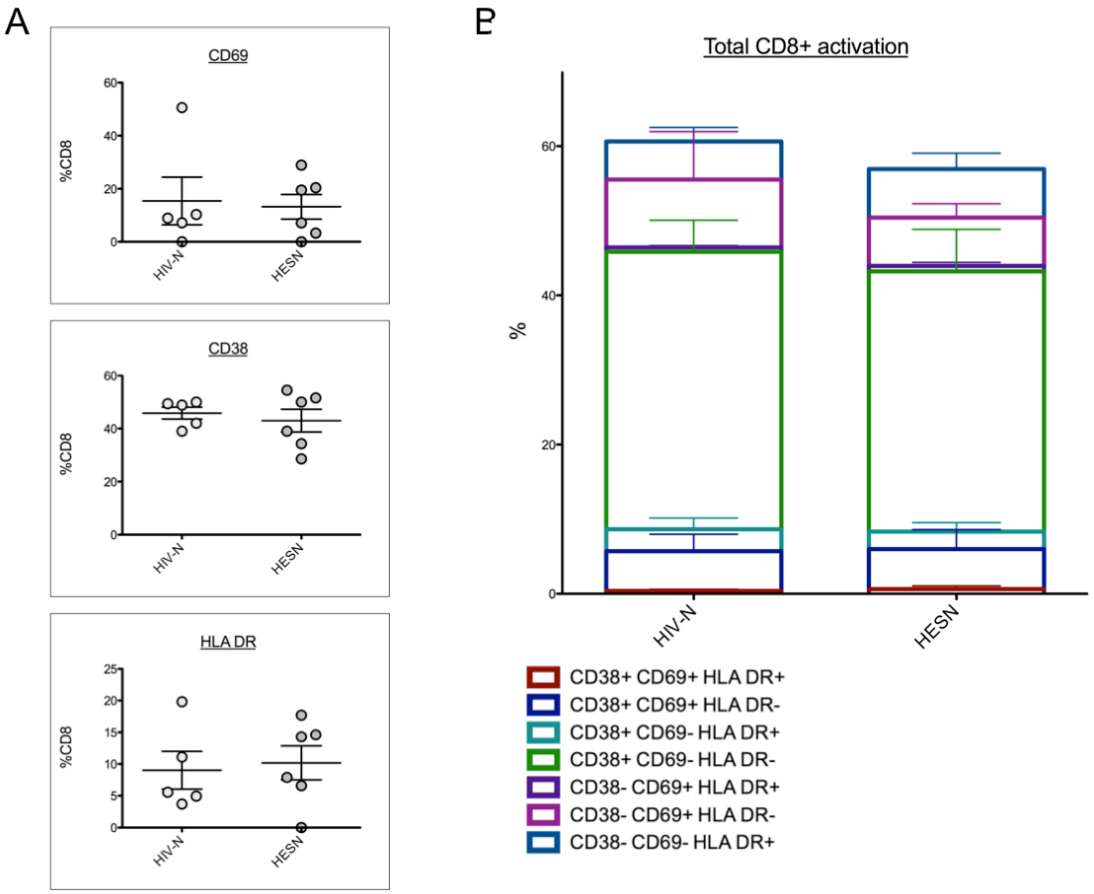


Figure 21. Comparison of CD8+ T cell activation in CMC between HIV-N and HESN. (A) Frequencies of CD8+ T cells expressing CD69, CD38 or HLA DR in CMC were not significantly different between HIV-N (n=5) and HESN (n=6). (B) The contribution of various subsets of activated T cells (based on co-expression of CD69, CD38 and HLA DR) to the total pool of activated CD8+ T cells was examined. HIV-N and HESN did not differ in the proportions of specific activated subsets or in the level of total CD8+ T cell activation in CMC.

Table 17. Expression of activation markers on T cells in CMC and PBMC from HIV-N and HESN

Subset	Phenotype	CMC			PBMC		
		Expression[a]		p-value[b]	Expression[a]		p-value[b]
		HIV-N (n=5)	HESN (n=6)		HIV-N (n=4)	HESN (n=6)	
CD4+ T Cells	CD38+ CD69+ HLADR+	0.53 (0.15-2.85)	0.00 (0.00-0.43)	0.104	0.10 (0.01-0.26)	0.01 (0.00-0.08)	0.271
	CD38+ CD69+ HLADR-	3.57 (2.00-12.36)	2.62 (0.81-3.75)	0.429	3.26 (0.79-5.41)	0.22 (0.11-2.63)	0.171
	CD38+ CD69- HLADR+	2.1 (0.45-3.65)	2.53 (0.93-19.5)	0.537	1.08 (0.93-2.37)	0.96 (0.46-1.21)	0.352
	CD38+ CD69- HLADR-	30.60 (19.90-39.20)	20.20 (16.18-36.03)	0.537	39.90 (32.93-48.75)	27.05 (15.69-60.10)	0.476
	CD38- CD69+ HLADR+	0.00 (0.00-1.72)	0.42 (0.00-1.88)	0.774	0.07 (0.03-0.20)	0.05 (0.00-0.08)	0.352
	CD38- CD69+ HLADR-	3.48 (1.96-16.41)	5.85 (1.12-8.92)	1.00	1.5 (0.79-4.07)	0.79 (0.13-3.04)	0.476
	CD38- CD69- HLADR+	1.85 (0.00-6.20)	2.05 (0.62-10.15)	0.645	0.86 (0.71-3.57)	1.39 (0.77-3.08)	0.700
	Total CD38+	36.90 (32.85-47.15)	41.2 (23.20-42.68)	0.662	42.15 (40.05-56.63)	32.00 (17.32-60.75)	0.476
	Total CD69+	9.30 (7.86-28.70)	10.75 (2.93-13.83)	0.931	4.94 (1.63-9.96)	1.03 (0.30-5.80)	0.257
	Total HLADR+	4.83 (3.68-11.17)	9.21 (3.52-24.48)	0.931	1.98 (1.85-6.34)	2.30 (1.57-4.23)	1.00
	Total CD4+ activation	48.94 (44.99-58.46)	46.12 (38.75-51.45)	0.537	47.67 (43.10-56.82)	37.52 (21.42-61.64)	0.762
CD8+ T Cells	CD38+ CD69+ HLA DR+	0.26 (0.00-0.81)	0.00 (0.00-1.49)	0.765	0.11 (0.04-0.33)	0.04 (0.00-0.12)	0.257
	CD38+ CD69+ HLA DR-	4.44 (1.55-9.57)	3.32 (0.00-11.27)	1.00	0.63 (0.53-4.83)	0.36 (0.18-1.12)	0.257
	CD38+ CD69- HLA DR+	2.22 (0.65-5.59)	1.11 (0.00-5.40)	0.575	3.72 (1.93-5.01)	2.49 (1.80-3.24)	0.352
	CD38+ CD69- HLA DR-	34.80 (29.5-46.10)	30.10 (23.48-50.75)	0.537	35.45 (34.20-40.15)	36.35 (31.85-43.85)	1.00
	CD38- CD69+ HLA DR+	0.40 (0.13-1.17)	0.00 (0.00-1.96)	0.632	0.14 (0.08-0.32)	0.03 (0.00-0.11)	0.114
	CD38- CD69+ HLA DR-	3.53 (1.67-19.28)	7.51 (1.65-10.3)	0.714	0.79 (0.71-5.23)	0.62 (0.32-1.26)	0.171
	CD38- CD69- HLA DR+	5.56 (0.95-9.04)	6.15 (1.97-10.90)	0.714	4.05 (1.62-6.40)	2.81 (1.39-4.30)	0.476
	Total CD38+	48.90 (40.50-49.70)	44.5 (32.88-52.33)	0.927	42.50 (39.90-44.50)	40.40 (35.43-45.50)	0.761
	Total CD69+	8.89 (3.58-30.45)	13.32 (2.48-22.53)	1.00	1.62 (1.49-10.69)	1.16 (0.51-2.52)	0.171
	Total HLA DR+	5.56 (4.32-15.45)	11.10 (4.94-15.38)	0.662	8.27 (3.81-11.68)	5.26 (3.27-6.41)	0.352
	Total CD8+ activation	61.08 (50.09-70.95)	57.25 (53.39-60.35)	0.537	47.23 (46.53-52.50)	44.00 (39.94-48.53)	0.257

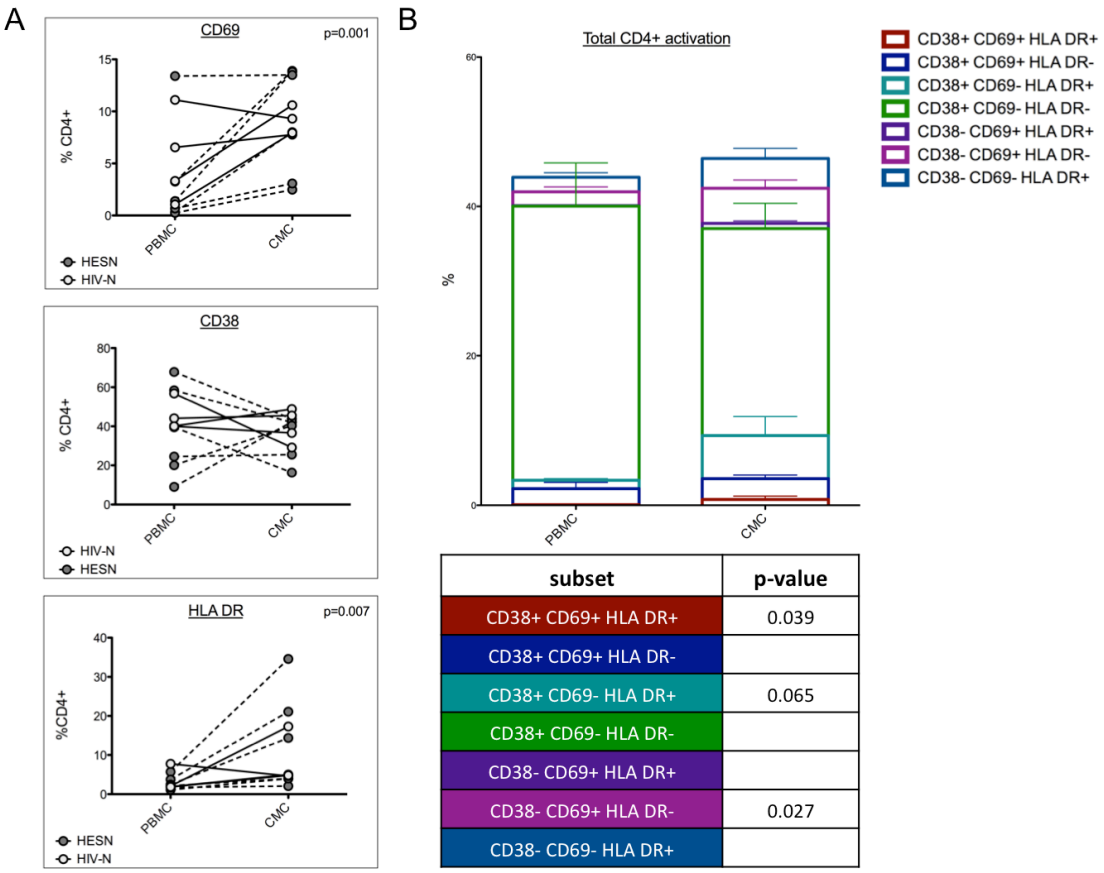
[a] Expression of phenotypic markers is listed as median of percent positive (+). Interquartile range is shown in parentheses.

[b] p-values were calculated using non-parametric Mann-Whitney *U* tests.

5.4.4 T cell activation – PBMC vs. CMC

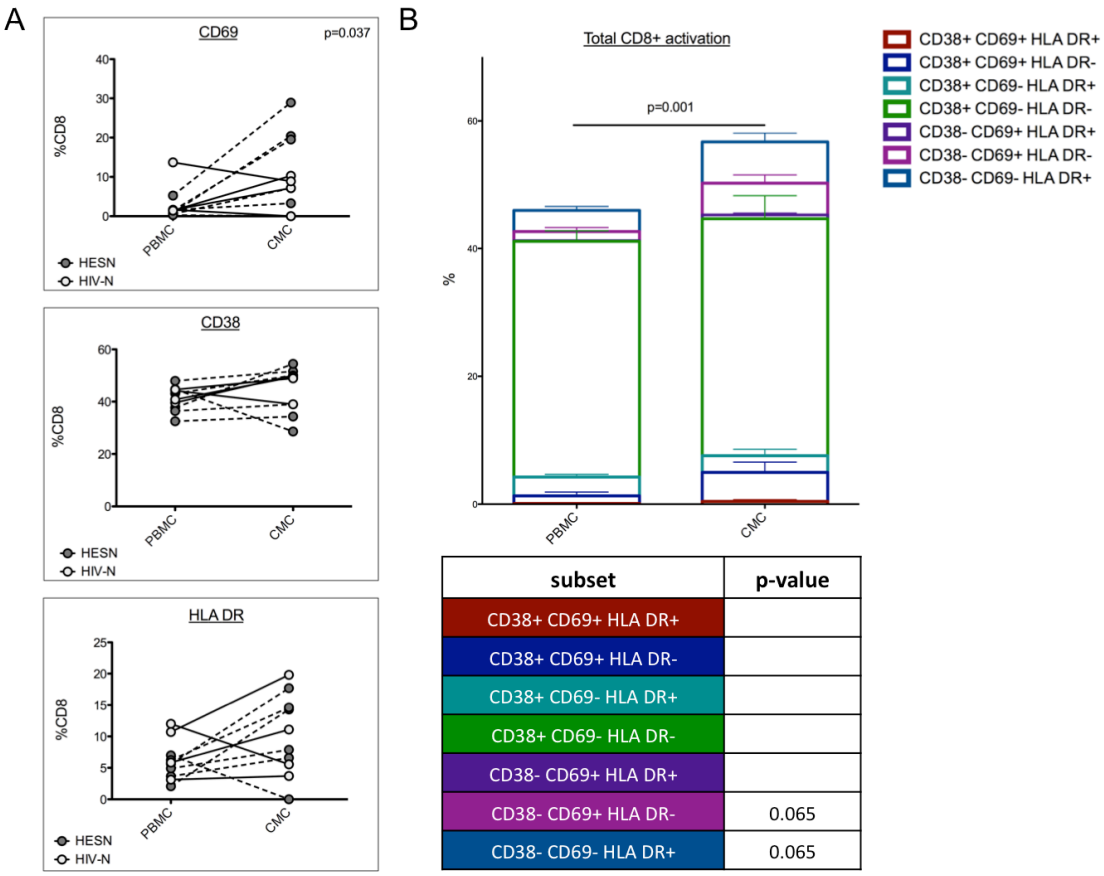
T cell activation was compared between CMC and matched PBMC from a total of 10 participants (HIV-N n=4, HESN n=6). CD4+ T cells expressing CD69 and HLA DR were elevated in CMC (p=0.001 and p=0.007, respectively), but CD38+ cells were comparable between the two cell types (Figure 22A). The contribution of subsets of activated CD4+ T cells (based on coexpression of CD69, CD38 and HLA DR) to the total pool of activated CD4+ T cells was also compared between CMC and PBMC (Figure 22B). Frequencies of CD38+ CD69+ HLA DR+, CD38+ CD69- HLA DR+ and CD38- CD69+ HLA DR- cells were elevated in CMC relative to PBMC (p=0.039, p=0.065 and p=0.027, respectively). However, the total pool of activated CD4+ T cells did not differ between the two compartments. The elevated levels of CD38+ CD69- HLA DR- cells in PBMC relative to CMC (medians 34.35% and 26.25%, respectively) may have offset differences in the other subsets. This high level of CD38+ cells in PBMC may be due to the high expression of CD38 on T_{NAIVE} CD4+ T cells, which were elevated in PBMC compared to CMC.

In the CD8+ T cell subset, CD69+ T cells were elevated in CMC relative to PBMC (p=0.037), whereas CD38+ and HLA DR+ cells did not differ between compartments (Figure 23A). The contribution of subsets of CD8+ T cells to the total pool of activated CD8+ T cells was compared between CMC and PBMC (Figure 23B). Trends toward elevated CD38- CD69+ HLA DR- and CD38- CD69- HLA DR+ cells in CMC relative to PBMC were observed (both p=0.065). In addition, the total pool of activated CD8+ T cells was elevated in CMC relative to PBMC (0=0.001).



subset	p-value
CD38+ CD69+ HLA DR+	0.039
CD38+ CD69+ HLA DR-	
CD38+ CD69- HLA DR+	0.065
CD38+ CD69- HLA DR-	
CD38- CD69+ HLA DR+	
CD38- CD69+ HLA DR-	0.027
CD38- CD69- HLA DR+	

Figure 22. Comparison of CD4+ T cell activation between PBMC and CMC. Matched PBMC and CMC were compared in a total of 11 participants (5 HIV-N and 6 HESN). (A) CD4+ CD69+ and CD4+ HLA DR+ cells were significantly elevated in CMC relative to PBMC. The frequency of CD4+ CD38+ T cells did not differ between cell compartments. Solid lines indicate HIV-N samples. Dotted lines indicate HESN samples. (B) The contribution of various subsets CD4+ T cells (based on coexpression of CD69, CD38 and HLA DR) to the total pool of activated CD4+ T cells was examined in PBMC and CMC. Frequencies of CD38+ CD69+ HLA DR+, CD38+ CD69- HLA DR+ and CD38- CD69+ HLA DR- cells were elevated in CMC relative to PBMC. The total pool of activated CD4+ T cells did not differ between PBMC and CMC. P-values were calculated using the Wilcoxon signed rank test.



subset	p-value
CD38+ CD69+ HLA DR+	
CD38+ CD69+ HLA DR-	
CD38+ CD69- HLA DR+	
CD38+ CD69- HLA DR-	
CD38- CD69+ HLA DR+	
CD38- CD69+ HLA DR-	0.065
CD38- CD69- HLA DR+	0.065

Figure 23. Comparison of CD8+ T cell activation between PBMC and CMC. PBMC and CMC were compared in a total of 11 participants (5 HIV-N and 6 HESN). (A) CD8+ CD69+ cells were significantly elevated in CMC relative to PBMC. The frequencies of CD8+ CD38+ and CD8+ HLA DR T cells did not differ between cell compartments. Solid lines indicate HIV-N samples. Dotted lines indicate HESN samples. (B) The contribution of various subsets of CD8+ T cells (based on coexpression of CD69, CD38 and HLA DR) to the total pool of activated CD8+ T cells was examined in PBMC and CMC. Frequencies of CD38- CD69+ HLA DR- and CD38- CD69- HLA DR cells were elevated in CMC relative to PBMC. The total pool of activated CD8+ T cells was significantly larger in CMC relative to PBMC. P-values were calculated using the Wilcoxon signed rank test.

5.4.5 Treg phenotypes and memory subsets in CMC

Mucosal and tissue-resident Tregs lacking CD25 expression have previously been described [200, 363]. Therefore, Tregs were identified as FOXP3+ CD4+ T cells in CMC from HIV-N (n=7) and HESN (n=10), then were further discriminated based on expression of CD25, CD103 ($\alpha E\beta 7$ integrin), CTLA-4 and memory subset markers CD45RA and CCR7. Staining of Tregs in CMC is shown in Figure 24. Although HESN tended to have elevated FOXP3+ CD4+ cells relative to HIV-N (medians 6.7% and 3.1%, respectively), the difference was not statistically significant (p=0.288) (Figure 25A). Based on the observed difference between groups, this analysis was only 32% powered to detect a statistically significant difference. Assuming standard deviations below 5, an α error of 0.05 and a β error of 0.2, a sample size of 31 individuals in each group would be required to observe significant differences in CD4+ FOXP3+ T cells between HESN and HIV-N CMC samples.

CD4+ FOXP3+ T cells were then distinguished based on CD25 expression (Figure 25B), which was comparable between groups. Treg phenotypes were further examined by assessing CD103 and CTLA-4 expression and memory subset distribution on various populations of CD4+ T cells, including bulk CD4+, CD4+ FOXP3+, CD4+ FOXP3+ CD25+ and CD4+ FOXP3+ CD25- T cells (Figure 25C). HESN tended to have more CD103+ CD4+ FOXP3+ CD25+ T cells than HIV-N (p=0.095). Memory subset distribution of CD4+ FOXP3+ CD25- T cells was also found to differ between HIV-N and HESN, such that HESN had fewer T_{EM} (p=0.044) and more T_{CM} CD4+ FOXP3+ CD25- cells (p=0.056) than HIV-N.

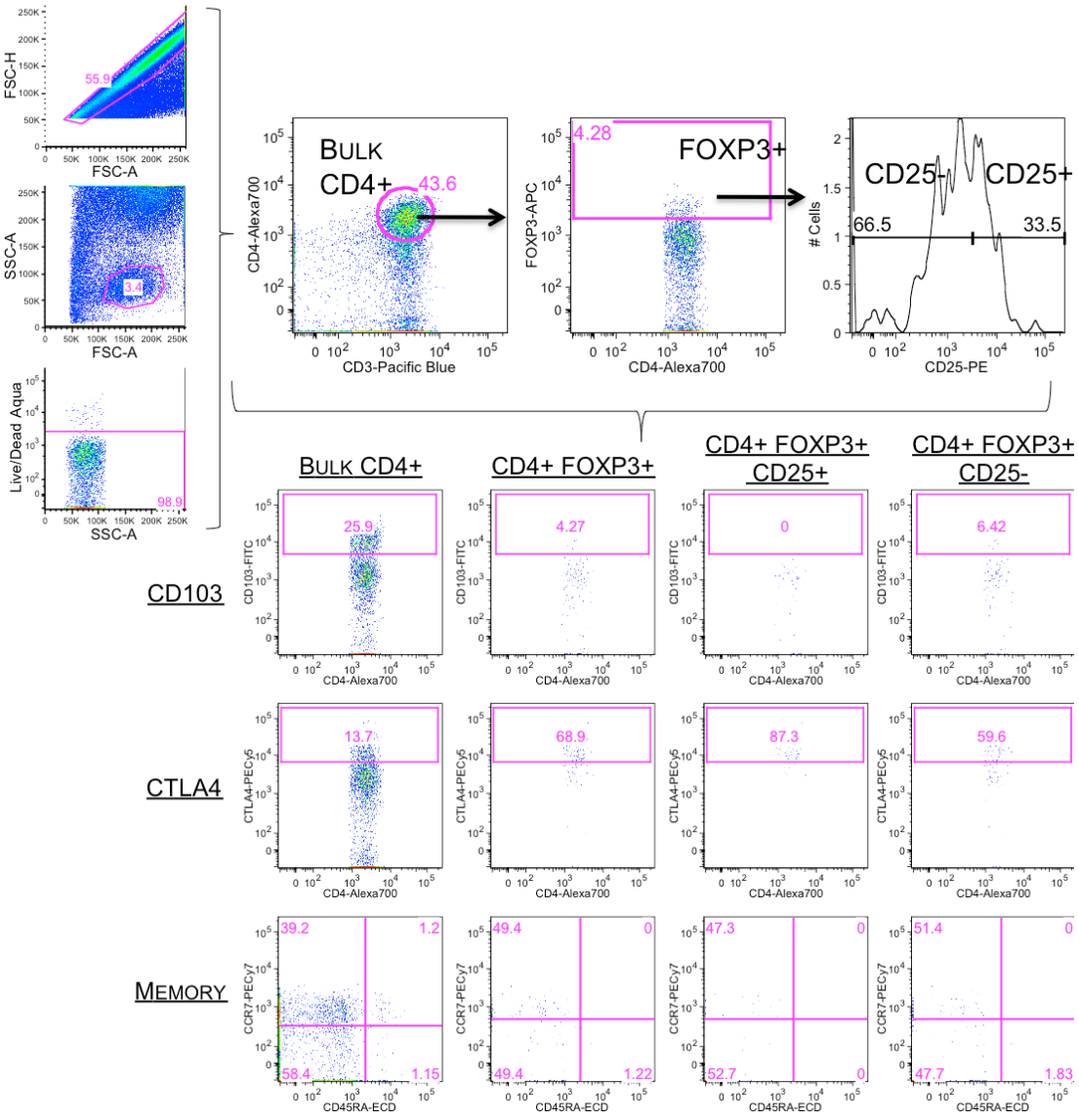


Figure 24. Representative staining of Tregs in CMC samples. Samples were first gated on singlets using FSC-H and FSC-A properties, followed by lymphocyte identification using FSC-A and SSC-A. Live cells were then identified by low expression of LIVE/DEAD Aqua. CD4+ T cells were identified by coexpression of CD3 and CD4, followed by further discrimination based on FOXP3 and CD25. Bulk CD4+, CD4+ FOXP3+, CD4+ FOXP3+ CD25+ and CD4+ FOXP3+ CD25- T cells were analyzed for expression of CD103, CTLA-4 and memory subset phenotype. Data from ML3091 (HIV-N) is used in this example.

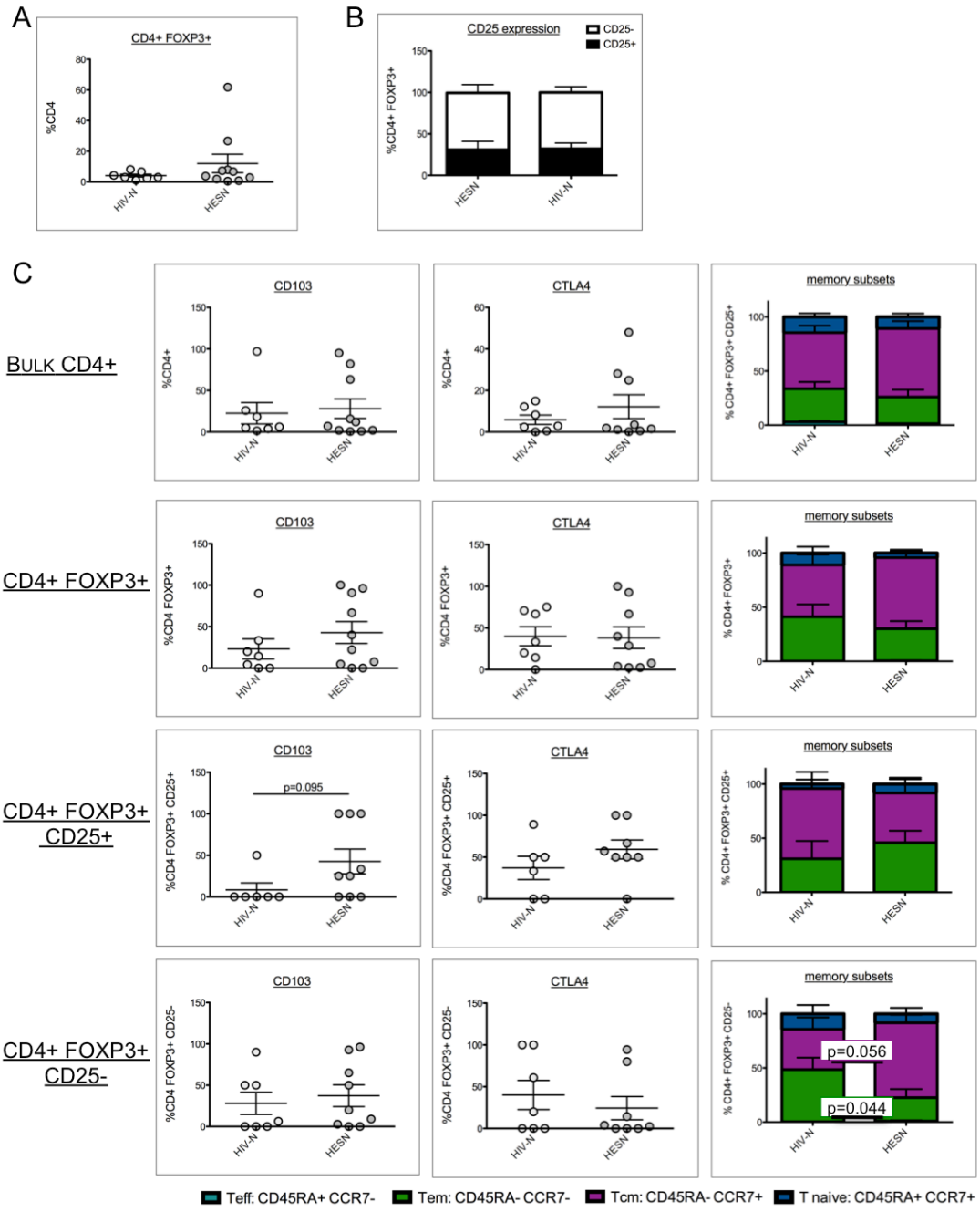


Figure 25. Comparison of Treg phenotypes in CMC between HIV-N and HESN. (A) Frequencies of CD4+ T cells expressing FOXP3 in CMC were not significantly different between HIV-N (n=7) and HESN (n=10). (B) CD25 expression on CD4+ FOXP3+ cells was comparable between HIV-N and HESN. (C) HESN had more CD103+ CD4+ FOXP3+ CD25+ T cells than HIV-N. Memory subset distribution of CD4+ FOXP3+ CD25- T cells also differed between HIV-N and HESN, such that HESN had fewer TEM and more TCM CD4+ FOXP3+ CD25- cells than HIV-N. P-values were calculated using the Mann-Whitney *U* test.

5.4.6 Treg phenotypes and memory subsets – PBMC vs. CMC

Treg phenotypes and CD4+ memory cell subsets were compared between PBMC and CMC in a total of 17 HIV-uninfected participants (HIV-N n=7, HESN n=10). A greater frequency of CD4+ T cells expressed FOXP3 in CMC than PBMC (p=0.0007) (Figure 26A). CD25 expression on CD4+ FOXP3+ cells was lower in CMC compared to PBMC (p=0.002) (Figure 26B). Bulk CD4+, CD4+ FOXP3+, CD4+ FOXP3+ CD25+ and CD4+ FOXP3+ CD25- T cells from PBMC and CMC were analyzed for expression of CD103 and CTLA-4. CMC had elevated frequencies of CD103+ cells in the bulk CD4+ and CD4+ FOXP3+ subsets (p=0.0003 and p=0.005, respectively), which is to be expected because CD103 is associated with mucosal trafficking [364]. A greater frequency of bulk CD4+ T cells expressing CTLA-4 was also observed in CMC compared to PBMC (p=0.021), which may reflect the elevated FOXP3+ population, which tends to express CTLA-4. A similar trend was observed in the CD4+ FOXP3+ CD25+ T cell subset, but this trend did not reach statistical significance (p=0.10).

The memory subset distribution of bulk CD4+, CD4+ FOXP3+, CD4+ FOXP3+ CD25+ and CD4+ FOXP3+ CD25- cells also varied between PBMC and CMC (Figure 27). Bulk CD4+ T_{NAIVE} cells (CD45RA+ CCR7+) were lower in CMC than in PBMC (p=0.023). Similarly, although T_{EFF} cells accounted for a low proportion of all CD4+ subsets in both PBMC and CMC, they were consistently lower in CMC than PBMC in the bulk CD4+ (p=0.014), CD4+ FOXP3+ (p=0.007), CD4+ FOXP3+ CD25+ (p=0.0005) and CD4+ FOXP3+ CD25- (p=0.017) cell populations.

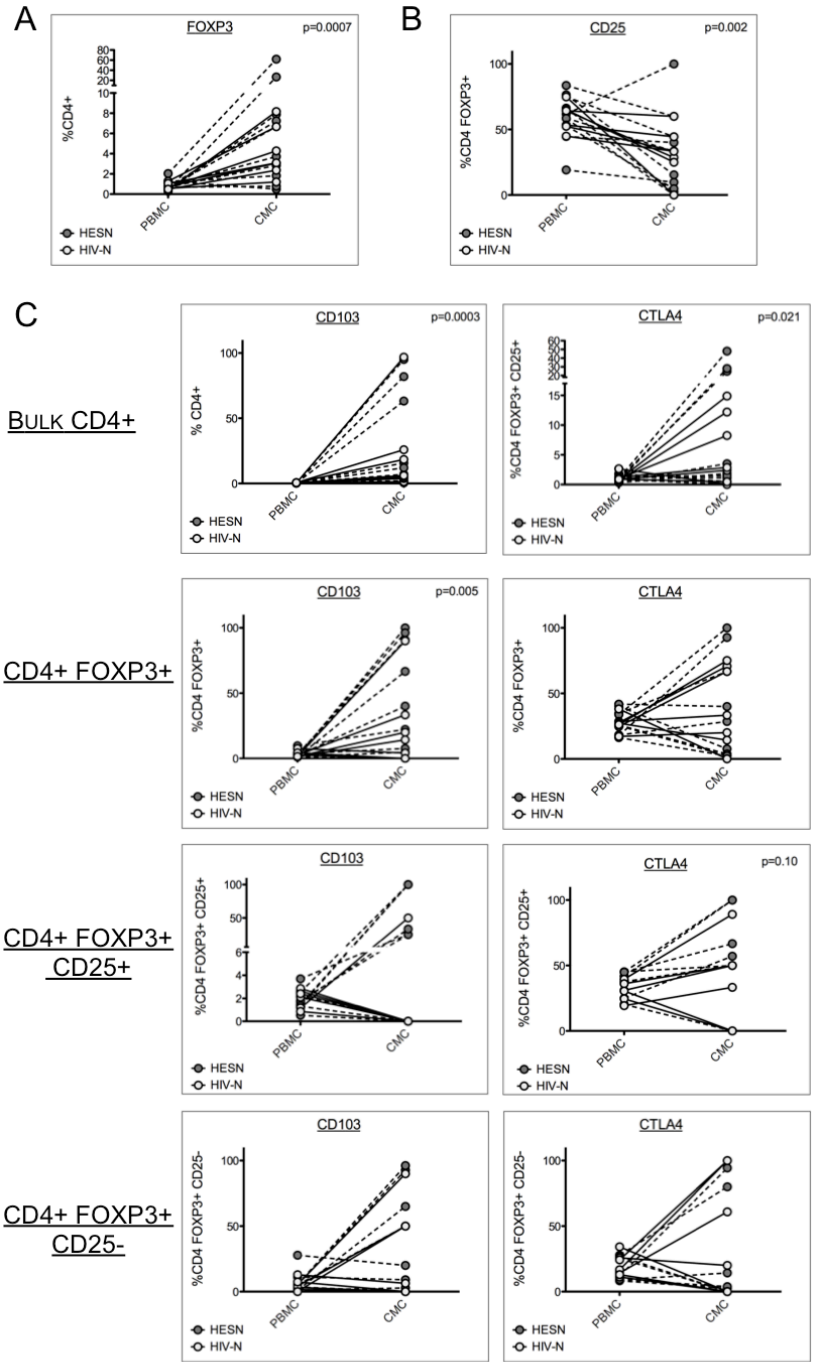
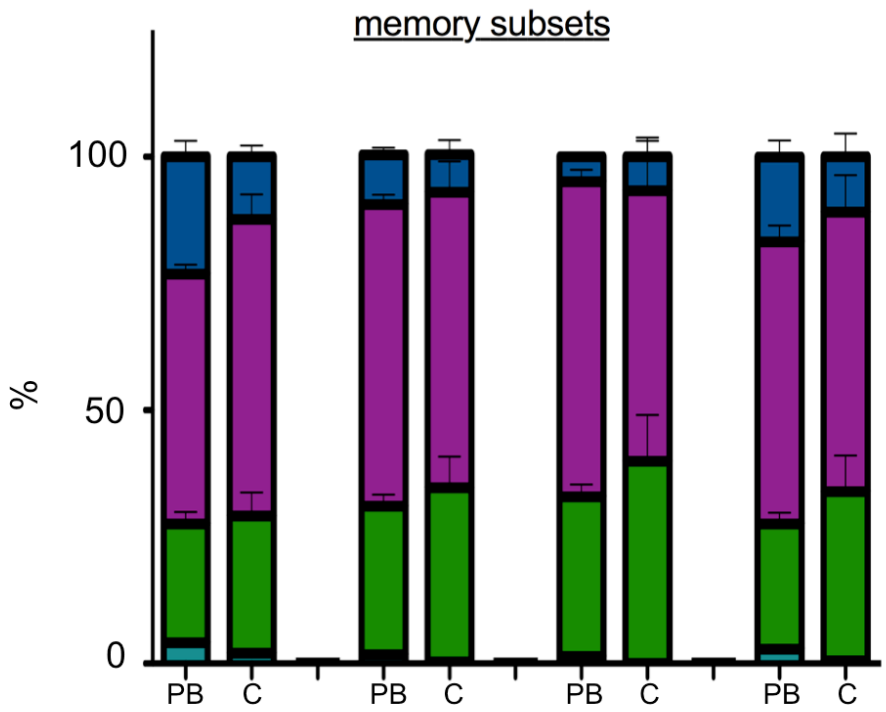


Figure 26. Comparison of Treg phenotypes between PBMC and CMC. PBMC and CMC were compared in a total of 17 participants (7 HIV-N and 10 HESN). Solid lines indicate HIV-N samples. Dotted lines indicate HESN samples. (A) FOXP3+ CD4+ T cells were higher in CMC than PBMC. (B) Fewer CD4+ FOXP3+ cells expressed CD25 in CMC compared to PBMC. (C) CMC had higher frequencies of bulk CD4+ and CD4+ FOXP3+ T cells expressing CD103 than PBMC. A higher frequency of bulk CD4+ T cells expressing CTLA-4 was observed in CMC compared to PBMC. P-values were calculated using the Wilcoxon signed rank test.



Subset	CD45RA	CCR7	CD4+ T cells	CD4+ FOXP3+	CD4+ FOXP3+ CD25+	CD4+ FOXP3+ CD25-
TNAIVE	+	+	0.023			
TCM	-	+				
TEM	-	-				
TEFF	+	-	0.014	0.007	0.0005	0.017

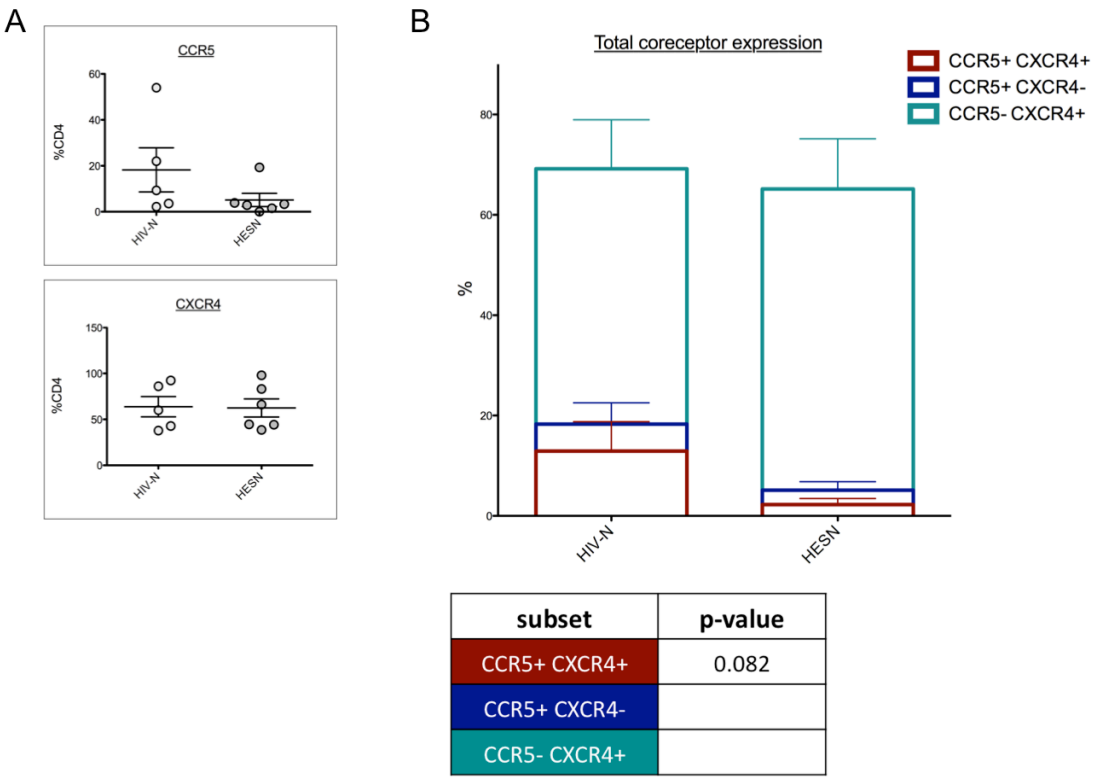
Figure 27. Comparison of CD4+ T cell memory subsets between PBMC and CMC. PBMC and CMC were compared in a total of 17 participants (7 HIV-N and 10 HESN). Bulk CD4+, CD4+ FOXP3+, CD4+ FOXP3+ CD25+ and CD4+ FOXP3+ CD25- T cells were analyzed for memory subset distribution based on expression of CD45RA and CCR7. Bulk CD4+ TNAIVE cells were lower in CMC (C) than in PBMC (PB). TEFF cells accounted for a low proportion of all CD4+ subsets in both PBMC and CMC, but were consistently higher in PBMC than CMC for all subsets. P-values were calculated using the Wilcoxon signed rank test.

5.4.7 Expression of HIV coreceptors on CD4+ T cells in CMC

The expression of the HIV coreceptors CCR5 and CXCR4 was measured on CD4+ T cells in CMC from HIV-N (n=5) and HESN (n=6). HIV-N and HESN did not differ significantly in the frequency of CD4+ T cells expressing CCR5 or CXCR4 (Figure 28A), although HESN tended to have lower frequencies of CD4+ CCR5+ T cells than HIV-N (medians 3.1% and 9.3%, respectively). In addition to measuring expression of total CCR5 and CXCR4 on CD4+ T cells, coexpression of HIV coreceptors was examined using a boolean gating analysis strategy, and the contribution of each subset to the total pool of coreceptor-expressing CD4+ T cells was examined. CCR5+ CXCR4- and CCR5- CXCR4+ populations did not differ between groups, but HESN had fewer CCR5+ CXCR4+ CD4+ T cells than HESN, though the trend was not statistically significant (p=0.082). The total pool of coreceptor-expressing CD4+ T cells (sum of all coreceptor-expressing populations) did not differ between HIV-N and HESN (Figure 28B).

5.4.8 HIV coreceptor expression on CD4+ T cells – PBMC vs. CMC

A trend toward elevated CD4+ CCR5+ T cells in CMC relative to PBMC was observed (p=0.10), but the frequencies of CD4+ CXCR4+ T cells did not differ between groups (Figure 29A). PBMC and CMC did not differ significantly in the proportions of specific coreceptor-expressing CD4+ T cell subsets (based on co-expression of CCR5 and CXCR4) or in the level of total coreceptor expression on CD4+ T cells (Figure 29B).



subset	p-value
CCR5+ CXCR4+	0.082
CCR5+ CXCR4-	
CCR5- CXCR4+	

Figure 28. Comparison of HIV coreceptor expression on CD4+ T cells in CMC between HIV-N and HESN. (A) Frequencies of CD4+ T cells expressing CCR5 or CXCR4 in CMC were not significantly different between HIV-N (n=5) and HESN (n=6). (B) The contribution of various subsets of CD4+ T cells (based on coexpression of CCR5 and CXCR4) to the total pool of coreceptor-expressing CD4+ T cells was examined. HESN had fewer CCR5+ CXCR4+ CD4+ T cells than HESN, though the difference was not statistically significant. The total pool of coreceptor-expressing CD4+ T cells did not differ between HIV-N and HESN. P-values were calculated using the Wilcoxon signed rank test.

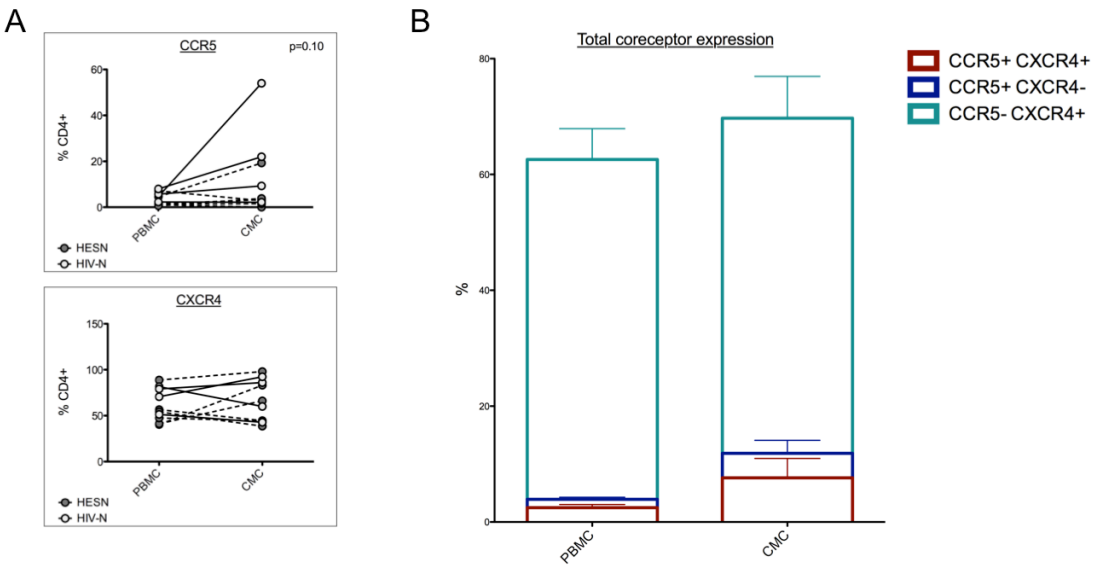


Figure 29. Comparison of HIV coreceptor expression on CD4+ T cells between PBMC and CMC. PBMC and CMC were compared in a total of 11 participants (5 HIV-N and 6 HESN). (A) CD4+ CCR5+ T cells were elevated in CMC relative to PBMC. The frequencies of CD4+ CXCR4+ T cells did not differ between compartments. Solid lines indicate HIV-N samples. Dotted lines indicate HESN samples. (B) The contribution of various subsets of CD4+ T cells (based on coexpression of CCR5 and CXCR4) to the total pool of coreceptor-expressing CD4+ T cells was examined in PBMC and CMC. PBMC and CMC did not differ in the proportions of specific coreceptor-expressing CD4+ T cell subsets or in the level of total coreceptor expression. P-values were calculated using the Wilcoxon signed rank test.

5.4.9 Effects of participant characteristics on phenotype

The study groups included in the CVL cytokine and chemokine study differed in age, self-reported duration of prostitution, and proportion of menopausal patients. Study groups did not differ in self-reported clients per week, days since last sex, days since last menses, douching practices or BV status, but these factors could also affect the inflammatory milieu evaluated in CVL samples. As such, these patient characteristics were evaluated for their relationship with IL-1α and IL-8 in CVL of HIV-N and HESN. Age was found to be inversely associated with IL-8 in the HIV-N ($p=0.010$, $r=-0.503$), but not HESN ($p=0.136$, $r=-0.314$) group. This suggests that the observation of lower levels of IL-8 in CVL from HESN may be due the older age of this group. No other associations were found between patient characteristics and concentrations of IL-1α or IL-8 in CVL.

The study groups included in the CMC T cell phenotyping study differed in age and self-reported duration of prostitution. Although study groups did not differ in clients per week, days since last sex, days since last menses, douching practices or proportions of participants in menopause or with BV, these characteristics may be expected to influence T cell activation and Treg phenotype in CMC. However, few phenotypic characteristics of CMC T cells were found to differ between HIV-N and HESN. Specifically, HESN had more CD103+ CD4+ FOXP3+ CD25+ and T_{CM} CD4+ FOXP3+ CD25- cells, and fewer T_{EM} CD4+ FOXP3+ CD25- and CD4+ CCR5+ CXCR4+ cells than HIV-N subjects. As such, the relationships between these parameters and patient characteristics were evaluated. Due to the low sample numbers, HIV-N and HESN were

evaluated as a single group for the purpose of this analysis. No associations were found between any of the phenotypic parameters evaluated and age, duration of prostitution, clients per week, days since last sex, days since last menses, douching practices or BV status. However, participants who were menopausal had significantly higher CD4+ FOXP3+ CD25+ CD103+ T cells than participants were not in menopause (p=0.015). This may explain the observed difference in the frequency of these cells between HIV-N and HESN in CMC.

5.5 Summary

This study aimed to determine whether immune quiescence could be observed at the level of the female genital tract in HESN women from the Pumwani cohort. To this end, levels of soluble and cellular markers of activation were evaluated in mucosal samples.

A panel of cytokines and chemokines was assayed in CVL samples to evaluate the inflammatory microenvironment in the FGT of HESN and HIV-N women. Consistent with the hypothesis that proinflammatory cytokines and chemokines would be reduced in HESN relative to HIV-N, IL-1α and IL-8 were found at lower concentrations in HESN women. In addition, fewer HESN had detectable levels of IL-1α than HIV-N women. These findings were confirmed in a recent independent study of inflammatory biomarkers in CVL from HESN from the Pumwani cohort [365].

The next hypothesis tested was that HESN would have elevated Tregs and reduced T cell activation in CMC compared to HIV-N. Contrary to this hypothesis, HESN had comparable levels of T cell activation as HIV-N women. Likewise, levels of Tregs did not differ significantly between groups. However, the HIV coreceptor CCR5, which is also a T cell activation marker, was lower in CD4+ T cells from HESN compared to HIV-N. Due to challenges associated with working with mucosal samples, these analyses were restricted to small sample sizes, such that we were not sufficiently powered to detect phenotypic differences between groups.

Matched PBMC were also collected from the study participants and T cell activation and Tregs were compared between PBMC and CMC compartments. Consistent with the hypothesis that frequencies of activated T cells and Tregs would be elevated in CMC compared to PBMC samples, several subsets of activated CD4+ and CD8+ T cells were higher in CMC. In particular, CD69+, HLA DR+ and CCR5+ T cell subsets were elevated in the CMC compartment. In addition, the total pool of activated CD8+ T cells was significantly higher in CMC relative to PBMC. These findings point to trafficking of activated T cells to the mucosa, where pathogens are encountered.

Chapter 6. Immune Quiescence and Susceptibility to HIV Infection *in vitro*

6.1 Rationale

HIV preferentially establishes productive infection in activated T cells due its dependency on host substrates for efficient viral replication [102, 331-334]. The low levels of activation and inflammation observed in HESN may therefore protect against infection by limiting the pool of activated target cells. Consistent with this, a study of discordant couples from the Central African Republic demonstrated that unstimulated PBMC from HESN had reduced susceptibility to *in vitro* HIV infection, which was associated with low levels of CD4+ T cell activation. However, mitogenic stimulation of the PBMC prior to infection abrogated the difference between HESN and controls, suggesting differences in cellular susceptibility to infection that exist due to low levels of target cell activation may be masked by assays that measure infectability following activation [342].

A previous study conducted in the Pumwani CSW cohort found that PBMC from HESN are equally susceptible to *in vitro* HIV infection as PBMC from control groups [266]. However, in those experiments, PBMC were stimulated with PHA prior to infection, so any differences in T cell activation between HESN and controls would be abated before exposure to the virus. In the present study, the infectability of unstimulated PBMC and the role of target cell activation on susceptibility to infection were assessed in HESN and HIV-N CSW from the Pumwani cohort.

6.2 Hypotheses

1. Unstimulated PBMC from HESN will demonstrate reduced susceptibility to *in vitro* infection relative to HIV-N, but stimulation of PBMC with PHA prior to infection will abrogate differences between groups.
2. Elevated levels of T cell activation *ex vivo* will be associated with increased susceptibility to *in vitro* infection of unstimulated PBMC.
3. Activated CD4+ T cells will be preferentially infected by HIV.

6.3 Objectives

1. Perform *in vitro* infections of unstimulated and PHA-stimulated PBMC from HESN and HIV-N with the HIV_{ML1956} primary HIV isolate and measure the infection frequency of replicate cultures, the amount of virus produced by infected cells and the frequency of infected (HIV p24+) CD4+ T cells.
2. Correlate *ex vivo* T cell activation and Treg phenotypes with the amount of HIV p24 produced in infection cultures.
3. Compare T cell activation and Treg phenotypes among uninfected (HIV p24-) and infected (HIV p24+) CD4+ T cells, and infected CD4+ T cells inoculated at varying multiplicities of infection.

6.4 Results

6.4.1 Study participants

Samples from 11 HESN and 10 HIV-N women from the Pumwani cohort were included in this study. As expected, HESN were older (p=0.001), had longer self-reported duration of prostitution (p=0.002) and duration of follow-up within the cohort (p=0.0001). Study groups did not differ in self-reported clients per week (Table 18).

6.4.2 HIV p24 production

Unstimulated PBMC from 11 HESN and 10 HIV-N were inoculated with HIV_{ML1956} using five multiplicities of infection (MOI) ranging from 0.1 to 0.0004 $TCID_{50}$ per cell. Supernatants were harvested on days 9 and 16 post-inoculation, and levels of HIV replication were determined by HIV p24 ELISA. PHA-stimulated PBMC were infected with virus in parallel, and supernatants were harvested for virus quantification on days 6 and 13 post-inoculation. Due to technical complications of maintaining virus cultures, not all cultures were carried through all time points.

The infection frequency, i.e. the percentage of replicate wells that demonstrated detectable p24 levels over background, was compared between HESN and HIV-N. At day 9 post-inoculation, unstimulated PBMC from HESN demonstrated lower infection frequencies than HIV-N (Figure 30A). The difference was statistically significant at a MOI of 0.0063 (p=0.029). By day 16 post-inoculation, unstimulated PBMC from HESN and HIV-N had comparable infection frequencies (Figure 30A).

The level of HIV p24 production in productively infected wells was also compared between groups. No significant differences in HIV p24 production were observed between unstimulated cells of HESN and HIV-N (Figure 31A) at any time point. Likewise, PHA-stimulated PBMC from HESN and HIV-N tended to produce similar levels of virus once infected, although on day 6 post-inoculation, infected wells from HESN had lower concentrations of p24 than HIV-N at MOI 0.1 (Figure 31B).

6.4.3 Effect of ex vivo T cell phenotype on cellular susceptibility to infection

PBMC were phenotyped *ex vivo* to address the roles of T cell activation and Tregs in cellular susceptibility to infection. On day 9 post-inoculation, 7 individuals did not demonstrate any productive infection of unstimulated PBMC, even at the highest MOI. As such, the T cell phenotypes of *ex vivo* PBMC were compared between individuals with no detectable infection and those demonstrating productive infection on day 9 (Table 19). As shown in Figure 32A, individuals with undetectable levels of virus production by day 9 had lower levels of CD4+ CD69+ T cells ($p=0.028$). In addition, a trend was observed in which lack of infection was associated with a modest elevation in Treg frequency ($p=0.093$) (Figure 32B).

Table 18. Characteristics of participants included in the *in vitro* HIV infection study[a]			
	HIV-N (n=10)	HESN (n=11)	p-value[b]
Age, years	**32.5 (31.0-39.0)**	**44.0 (41.0-49.0)**	**0.001**
Duration of prostitution, years	**7.5 (3.0-10.25)**	**15.0 (11.0-28.0)**	**0.002**
Duration of follow-up, years	**1.5 (1.38-1.63)**	**13.0 (8.0-16.0)**	**0.0001**
Clients/week	8.5 (2.0-25.0)	2.0 (1.0-12.0)	0.164

[a] Data represent median (interquartile range) values.

[b] p-values were calculated using Mann Whitney *U* tests. Variables that showed statistically significant ($p<0.05$) or trending ($p<0.10$) differences are highlighted in bold text.

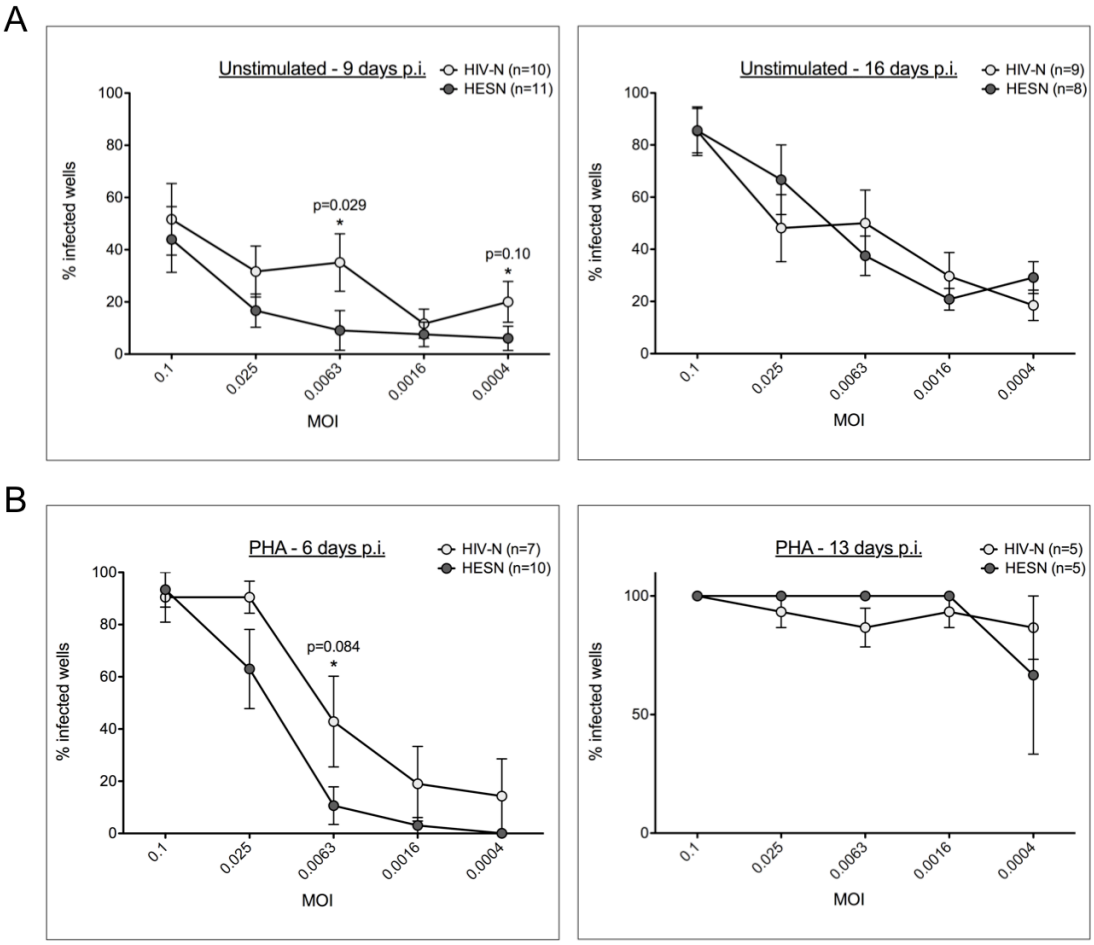


Figure 30. Infection frequencies of unstimulated and PHA-stimulated PBMC inoculated with HIV_{ML1956}. Unstimulated and PHA-stimulated PBMC from HIV-N and HESN were inoculated with HIV_{ML1956} at MOIs ranging from 0.1 to 0.0063. The infection frequency is the percent of replicate wells with detectable levels of HIV p24 production in harvested supernatants. (A) On day 9 post-inoculation, unstimulated PBMC from HESN (n=11) demonstrated significantly lower infection frequencies than HIV-N (n=10) at a MOI of 0.0063. A similar trend was observed at a MOI of 0.0004. On day 16 post-inoculation, unstimulated PBMC from HESN (n=8) and HIV-N (n=9) had comparable infection frequencies at all MOIs. (B) On day 6 post-inoculation, PHA-stimulated PBMC from HESN (n=7) demonstrated lower infection frequencies than HIV-N (n=10) at a MOI of 0.0063. On day 13 post-inoculation, infection frequencies were comparable between HESN (n=5) and HIV-N (n=5). P-values indicate results of Mann-Whitney *U* tests.

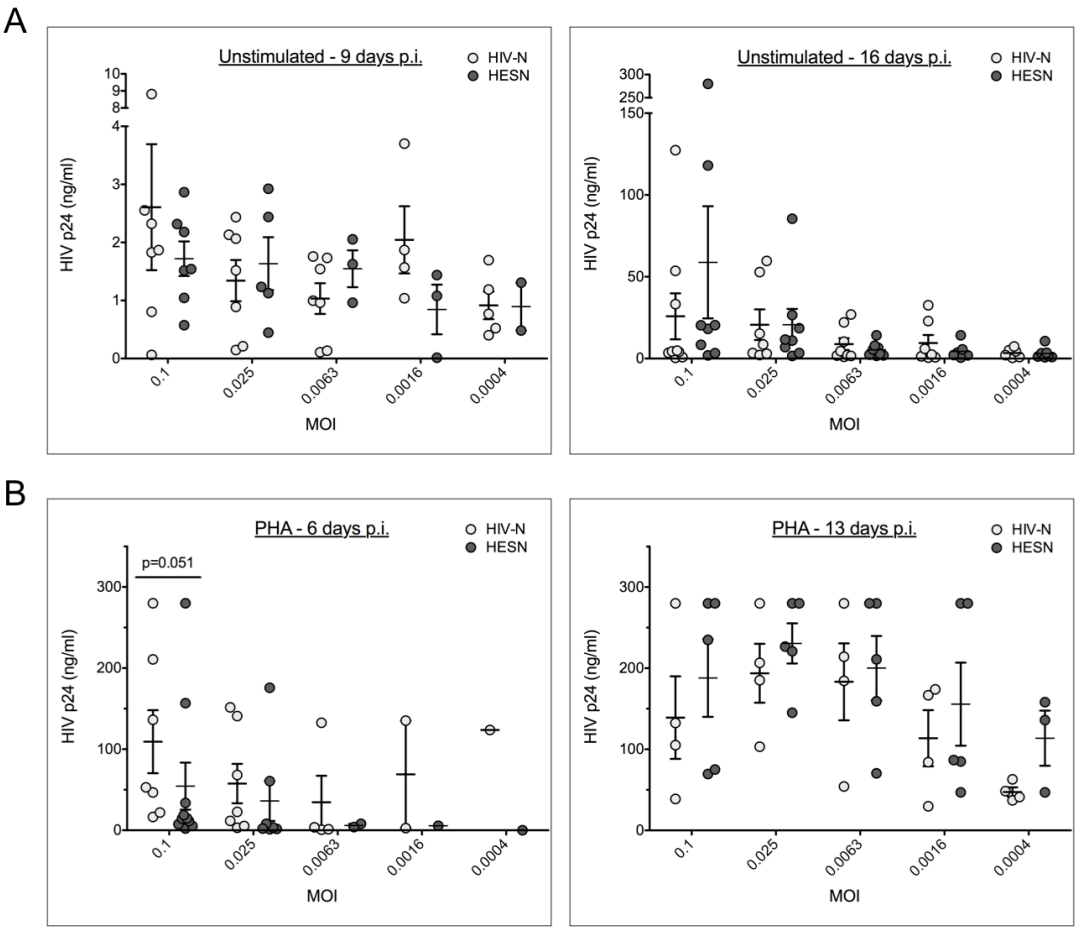


Figure 31. HIV p24 production by unstimulated and PHA-stimulated PBMC inoculated with HIV_{ML1956}. Unstimulated and PHA-stimulated PBMC from HIV-N and HESN were inoculated with HIV_{ML1956} at MOIs ranging from 0.1 to 0.0063. Mean concentration of HIV p24 in replicate wells demonstrating productive infection is shown. (A) Infected unstimulated cells from HESN and HIV-N produce similar amounts of p24 on days 9 and 16 post-inoculation. (B) On day 6 post-inoculation, infected PHA-stimulated cells from HESN had lower levels of p24 compared to HIV-N at a MOI of 0.1. HIV p24 production was similar between HESN and HIV-N at all other MOIs on day 6 post-inoculation and at all MOIs on day 13 post-inoculation. P-values indicate results of Mann-Whitney *U* tests.

Table 19. Effect of *ex vivo* T cell phenotype on susceptibility of unstimulated PBMC to infection with HIV_{ML1956} virus at a MOI of 0.1

T cell phenotype	Expression[a]		p-value[b]
	No Infection (n=7)	Infection (n=14)	
CD4+ CD69+	**0.41 (0.24-1.00)**	**1.45 (0.48-2.31)**	**0.028**
CD4+ CD38+	38.8 (23.8-49.6)	42.3 (34.9-46.2)	0.628
CD4+ HLA DR+	1.56 (0.80-2.32)	1.65 (1.24-2.48)	0.479
CD4+ CD38+/HLA DR+	0.75 (0.28-1.20)	0.73 (0.61-1.24)	0.737
CD4+ CCR5+	0.70 (0.46-1.56)	0.99 (0.63-1.35)	0.628
CD4+ CXCR4+	73.6 (35.2-86.1)	54.0 (40.8-84.0)	0.970
CD4+ CCR5+/CXCR4+	0.46 (0.23-0.49)	0.47 (0.30-0.80)	0.391
CD8+ CD69+	1.06 (0.53-1.88)	1.91 (0.67-4.59)	0.192
CD8+ CD38+	47.2 (41.1-48.2)	44.9 (43.3-46.8)	0.852
CD8+ HLA DR+	4.90 (2.92-8.60)	4.52 (3.56-5.28)	0.794
CD8+ CD38+/HLA DR+	2.81 (1.58-4.50)	2.44 (1.92-2.74)	0.682
Treg frequency	**1.07 (1.00-1.43)**	**0.94 (0.78-1.12)**	**0.093**
Treg/CTLA4+	23.2 (11.1-26.4)	22.0 (18.9-27.7)	0.682

[a] Expression of phenotypic markers is listed as median of percent positive (+) cells. Interquartile range is shown in brackets

[b] p-values calculated using non-parametric Mann-Whitney U test. Parameters that demonstrated statistically significant ($p<0.05$) or trending ($p<0.10$) p-values are highlighted in bold text.

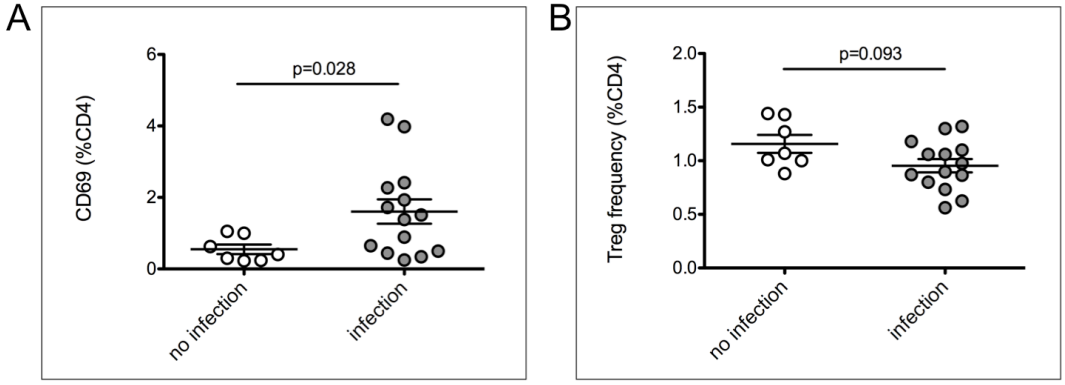


Figure 32. T cell phenotypes of unstimulated PBMC with differential susceptibility to *in vitro* infection with HIV_{ML1956}. PBMC were immunophenotyped *ex vivo* to assess T cell activation and Tregs prior to inoculation with HIV_{ML1956} *in vitro*. PBMC with no detectable infection (n=7) were compared to those demonstrating productive infection (n=14) at 9 days post-inoculation. (A) PBMC with undetectable levels of virus production by day 9 post-inoculation had lower frequencies of CD4+ CD69+ T cells. (B) PBMC with undetectable levels of virus production by day 9 post-inoculation had higher frequencies of Tregs, but the difference was not statistically significant. P-values were calculated using the Mann-Whitney *U* test.

The effects of T cell activation and Tregs on levels of virus production were also examined in individuals with detectable virus production in unstimulated cultures at days 9 and 16 post-inoculation (Table 20). At day 9, HIV p24 was positively correlated with CD8+ CD69+ T cells (p=0.037, r=0.717) at a MOI of 0.0063. At day 16, HIV p24 was positively associated with CD4+ CXCR4+ T cells (p=0.004, r=0.697) at a MOI of 0.025. However, these associations were not consistent across dilutions or time points.

6.4.4 Detection of infection by flow cytometry

When infection cultures were terminated, cells were harvested and stained for HIV p24 and markers of T cell activation and Tregs (Figure 33A). The frequencies of infected (HIV p24+) CD4+ T cells detected by flow cytometry correlated with levels of HIV p24 detected in infection supernatants by ELISA (p=0.0002, r=0.7764), validating the use of flow cytometry as a tool for evaluating cellular infection (Figure 33B). The frequency of p24+ CD4+ T cells was comparable between HESN and HIV-N when cells were PHA-stimulated (HIV-N n=6, HESN n=5), or unstimulated and infected with a MOI of 0.1 (HIV-N n=9, HESN n=8) (Figure 33C). In a subset of participants (HIV-N n=3, HESN n=6), frequencies of p24+ CD4+ T cells were also compared between groups at a MOI of 0.0063. At the lower MOI, HESN had fewer p24+ CD4+ T cells than HIV-N (p=0.095). Since cells from replicate wells had to be pooled for flow cytometry, the lower frequency of p24+ CD4+ T cells may reflect the lower infection frequency of replicate wells in HESN at MOI 0.0063 (Figure 30A, discussed above).

Table 20. Effect of T cell phenotype on virus production in unstimulated cells productively infected with HIV_{ML1956} virus at days 9 and 16 post-inoculation.

		Day 9									
		MOI 0.1 (n=14)		MOI 0.025 (n=12)		MOI 0.0063 (n=9)		MOI 0.0016 (n=7)		MOI 0.0004 (n=7)	
Subset	Parameter	p-value[a]	r[b]	p-value	r	p-value	r	p-value	r	p-value	r
CD4	CD69	0.737		0.417		0.336		0.203		0.193	
	CD38	0.122		0.485		0.148		0.171		0.939	
	HLA DR	0.771		0.633		0.948		0.589		0.294	
	HLA DR+/ CD38+	0.658		0.931		0.336		0.645		0.482	
	CCR5	0.653		0.077		0.410		0.383		0.216	
	CXCR4	0.846		0.463		0.912		0.333		0.645	
	Tregs	0.151		0.302		0.336		0.180		0.879	
	CTLA4+ Tregs	0.422		0.354		0.194		0.253		0.253	
CD8	**CD69**	0.144		0.191		**0.037**	**0.717**	0.337		0.702	
	CD38	0.383		0.812		0.359		0.534		0.702	
	HLA DR	0.947		0.948		0.776		0.482		0.193	
	HLA DR+/ CD38+	0.681		0.829		0.776		0.879		0.216	
		Day 16									
Subset	Parameter	MOI 0.1 (n=17)		MOI 0.025 (n=15)		MOI 0.0063 (n=16)		MOI 0.0016 (n=14)		MOI 0.0004 (n=13)	
CD4	CD69	0.328		0.173		0.778		0.503		0.181	
	CD38	0.844		0.860		0.957		0.982		0.154	
	HLA DR	0.358		0.742		0.374		0.358		0.280	
	HLA DR+/ CD38+	0.495		0.771		0.324		0.417		0.339	
	CCR5	0.874		0.483		0.144		0.670		0.859	
	CXCR4	0.220		**0.004**	**0.697**	0.424		0.299		0.266	
	Tregs	0.837		0.390		0.721		0.227		0.845	
	CTLA4+ Treg	0.586		0.880		0.854		0.887		0.591	
CD8	CD69	0.593		0.341		0.485		0.782		0.831	
	CD38	0.146		0.174		0.228		0.273		0.487	
	HLA DR	0.195		0.960		0.158		0.594		0.986	
	HLA DR+/ CD38+	0.687		0.771		0.217		0.637		0.845	

[a] p-values calculated by Spearman rank correlation. Parameters demonstrating statistically significant ($p<0.05$) correlations with virus production are highlighted in bold text.

[b] r values are Spearman rho correlation coefficients shown in the case of a significant ($p<0.05$) p-value.

6.4.5 Phenotypes of infected CD4+ T cells

On day 16 post-inoculation, markers of T cell activation and Tregs were compared between infected (HIV p24+) and uninfected (HIV p24-) CD4+ T cells from unstimulated infection cultures inoculated at a MOI of 0.1 (n=12). A higher proportion of infected cells were CD69+ HLA DR+ (p=0.005), CD69+ HLA DR- (p=0.0005) or CD69- HLA DR+ (p=0.0005) compared to uninfected cells (Figure 34A). In a subset of participants (n=6), T cell activation was compared between infected CD4+ T cells inoculated at MOIs of 0.1 and 0.0063. Infected CD4+ T cells inoculated at a MOI of 0.0063 demonstrated higher frequencies of CD69+ HLA DR+ (p=0.031) and CD69- HLA DR+ (p=0.063) cells compared to infected CD4+ T cells inoculated at a MOI of 0.1 (Figure 34B). Collectively, these data are consistent with the preferential infection of activated cells by HIV. Furthermore, these data suggest that at a low MOI, HIV may be more selective of activated target cells, whereas at a high MOI, resting cells are more readily infected due to target cell availability.

In addition to demonstrating elevated levels of T cell activation, a greater percentage of infected cells demonstrated a Treg phenotype ($CD25^{hi}$ $CD127^{lo}$) compared to uninfected cells (p=0.0005) (Figure 34C). In addition, infected cells inoculated at a MOI of 0.0063 had higher Treg frequencies than infected cells inoculated at a MOI of 0.1 (p=0.031) (Figure 34D). These results suggest that Tregs are readily infected by HIV *in vitro*. This finding is not surprising, as Tregs are known to express HIV co-receptors. However, it should be noted that in this study, Tregs were defined by expression of high levels of

CD25 and low levels of CD127/IL-7Rα, and not by FOXP3, due to complications of co-staining FOXP3 and HIV p24. Since activated cells upregulate CD25 and downregulate CD127, the Treg populations evaluated here may not be pure, and likely contain a high number of activated cells.

6.5 Summary

This study was aimed at determining if unstimulated cells from HESN and HIV-N have differential susceptibility to *in vitro* infection with a primary HIV isolate, and elucidating the effects of T cell activation and Tregs on cellular susceptibility to infection. Nine days post-inoculation, unstimulated PBMC from HESN demonstrated lower infection frequencies than HIV-N a MOI of 0.0063. These data are supported by reduced frequencies of HIV p24+ CD4+ T cells in HESN compared to HIV-N at a MOI of 0.0063, and are consistent with the hypothesis that unstimulated PBMC from HESN would demonstrate reduced susceptibility to *in vitro* infection relative to HIV-N. The amount of HIV p24 produced by productively-infected cells was comparable between groups, suggesting that once infected, cells from HESN can support HIV replication as well as cells from HIV-N. However, contrary to the hypothesis that stimulation of PBMC with PHA prior to infection would abrogate differences between groups, PHA-stimulated PBMC from HESN demonstrated lower infection frequencies than HIV-N, though differences between groups were not statistically significant. These data suggest that PHA stimulation does not consistently rescue productive infection of PBMC at early time points.

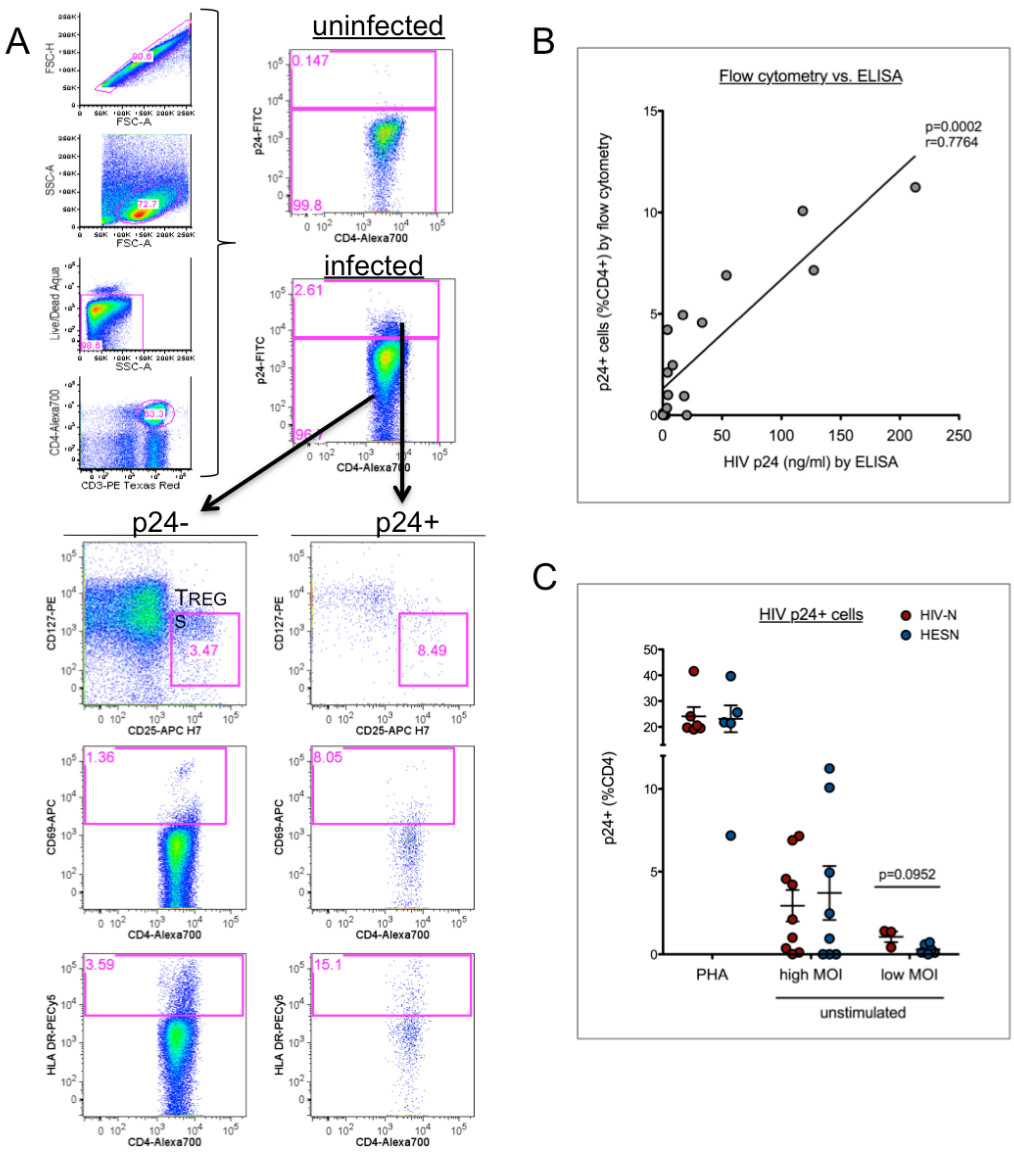


Figure 33. Measurement of HIV-infected cells by flow cytometry. (A) Samples were first gated on singlets and lymphocytes using FSC and SSC properties. Live cells were identified by dim staining of LIVE/DEAD Aqua, then CD4+ T cells were identified and evaluated for infection by intracellular staining for HIV p24. Infected and uninfected cells were phenotyped for markers of Tregs ($CD25^{hi}$, $CD127^{lo}$) and T cell activation (CD69, HLA DR). Representative data from ML1860 (HESN) is used in this example. (B) Frequencies of infected (p24+) CD4+ T cells correlate with levels of p24 detected in infection supernatants by ELISA. Spearman's rho correlation coefficient and p-value from non-parametric correlation are shown. (C) The frequency of p24+ CD4+ T cells was comparable between HESN and HIV-N when cells were PHA-stimulated (HIV-N n=6, HESN n=5) or unstimulated and infected with a MOI of 0.1 (HIV-N n=9, HESN n=8). At a MOI of 0.0063, HESN (n=6) had fewer p24+ CD4+ T cells than HIV-N (n=3). P-value calculated using the Mann-Whitney *U* test.

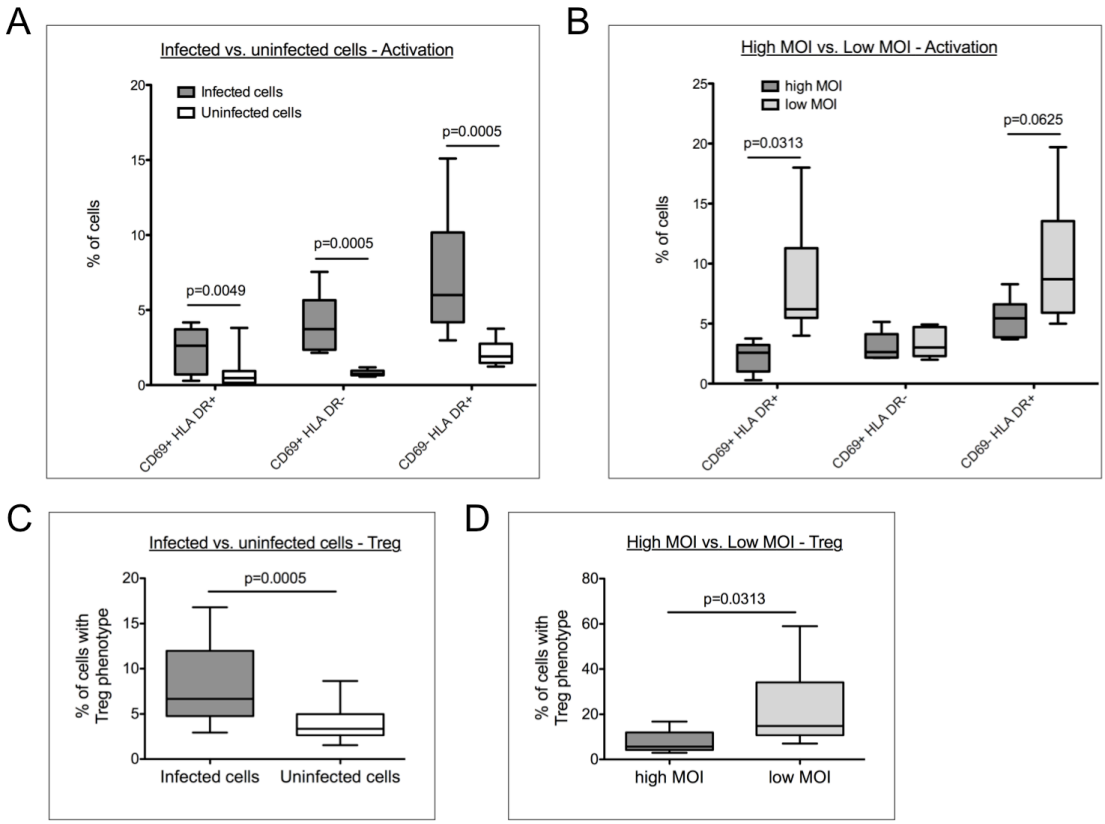


Figure 34. Phenotypes of HIV-infected unstimulated CD4+ T cells. (A) T cell activation was compared between Infected and uninfected CD4+ T cells inoculated at a MOI of 0.1 (n=12). Higher frequencies of Infected cells were activated (CD69+ HLA DR+, CD69+ HLA DR- or CD69- HLA DR+) compared to uninfected cells. (B) T cell activation was compared between infected CD4+ T cells inoculated at a MOI of 0.1 and 0.0063 (n=6). Higher frequencies of infected cells inoculated at MOI 0.0063 were activated (CD69+ HLA DR+ or CD69- HLA DR+) compared to infected cells incoulated at MOI 0.1. (C) Tregs were compared between Infected and uninfected CD4+ T cells inoculated at a MOI of 0.1 (n=12). Higher frequencies of infected cells had a Treg phenotype ($CD25^{hi}$, $CD127^{lo}$) compared to uninfected cells. (D) Tregs were compared between infected CD4+ T cells inoculated at a MOI of 0.1 and 0.0063 (n=6). Higher frequencies of infected cells inoculated at MOI 0.0063 had a Treg phenotype ($CD25^{hi}$, $CD127^{lo}$) compared to infected cells inoculated at MOI 0.1. P-values were calculated using the Wilcoxon signed-rank test for matched pairs.

In line with the hypothesis that T cell activation would be associated with increased susceptibility to *in vitro* infection of unstimulated PBMC, individuals with detectable levels of HIV replication by day 9 post-inoculation had higher levels of CD4+ CD69+ T cells and lower levels of Tregs *ex vivo* than those with no detectable infection by day 9. However, no other markers of T cell activation were associated with susceptibility to infection, and no T cell activation or Treg markers were consistently correlated with levels of virus production.

Finally, the phenotypes of infected CD4+ T cells were assessed to test the hypothesis that activated CD4+ T cells would be preferentially infected by HIV. Consistent with this, the p24+ CD4+ T cell population contained higher levels of activated T cells than the p24- CD4+ T cell population. Tregs were also enriched within the p24+ CD4+ T cell population, although the Treg population may also include activated T cells due to the phenotyping strategy. The phenotypes of infected CD4+ T cells were also compared between high and low MOIs. The p24+ CD4+ T cell population from cultures infected with the low MOI had higher levels of activated T cells and Tregs than the p24+ CD4+ T cells from the high MOI culture. These data suggest that at low doses of virus, activated cells are preferentially targeted, but at higher virus doses, infection is also established in resting cells. Collectively, these data support the hypothesis that activated T cells are preferentially targeted, but highlight the role of target cell availability as a determinant of infection.

Chapter 7. Correlates of Protection in HIV-controllers

7.1 Rationale

The data presented in Chapters 3-6 explored immune quiescence as a mechanism of resistance to HIV infection in HESN women. Immune quiescence may also play a role in delaying disease progression, as T cell activation drives viral replication. Indeed, levels of immune activation are drastically reduced in HIV-controllers in comparison to non-controllers [138, 196, 208, 212, 366]. The role of Tregs in maintaining reduced T cell activation in HIV-controllers is unclear, although several studies point to a relative maintenance of this subset in controllers compared to non-controllers [197, 210, 212].

Within the Manitoba HIV-controller population, there is potential for identification of unique correlates of protection and variation from known associations of HIV control (e.g. HLA type) due to the diverse ethnic backgrounds of the cohort participants. Thus, we sought to comprehensively evaluate multiple potential correlates of immune control within this unique population of HIV-controllers. To this end, we measured blood plasma levels of 22 cytokines and chemokines and expression of markers of T cell activation and Tregs in peripheral blood mononuclear cells (PBMC) from HIV-infected controllers and non-controllers. In a subset of patients, HIV-specific cytokine and proliferative T cell responses were also evaluated.

7.2 Hypotheses

1. HIV-controllers will express lower levels of pro-inflammatory cytokines and chemokines than non-controllers.
2. T cells from HIV-controllers will demonstrate lower levels of activation and elevated Tregs compared to non-controllers.
3. HIV-controllers will maintain robust HIV-specific T cell responses that are qualitatively distinct from non-controllers.

7.3 Objectives

1. Measure concentrations of plasma cytokines and chemokines in HIV-controllers, non-controllers and uninfected controls.
2. Characterize T cell activation and Tregs in HIV-controllers, non-controllers and uninfected controls.
3. Compare the ability of T cells from HIV-controllers and non-controllers to produce cytokines and proliferate in response to HIV peptide stimulation.

7.4 Results

7.4.1 Study participants

A total of 31 HIV-infected individuals enrolled in this study, including 5 elite controllers (HIV+ EC), 14 viremic controllers (HIV+ VC) and 12 non-controllers (HIV+ NC). The majority of analyses combined HIV+ EC and HIV+ VC into a single group (HIV-controllers,

HIV+ C) due to low participant numbers in the HIV+ EC group. HIV-uninfected healthy subjects (HIV-N, n=20) were also included as controls.

Characteristics of HIV+ study participants are shown in Table 21. No differences were observed between groups in terms of age, gender or years since HIV diagnosis. As expected, CD4 count and plasma viral load differed significantly between groups ($p=0.0004$ and $p<0.0001$, respectively), such that HIV+ NC had the lowest CD4 counts and highest viral loads. The ethnic representation of the cohort was comparable to the greater HIV-infected population in Manitoba. The cohort included individuals of Canadian Aboriginal (First Nations and Métis), Caucasian and African descent (48.4%, 35.5% and 16.1%, respectively). This is in contrast to studies conducted in other HIV-controller cohorts, which primarily enrolled Caucasian and African American individuals, but included minimal or no Aboriginal representation [219, 345, 367].

7.4.2 Cytokine and chemokine expression

Plasma concentrations of 22 cytokines and chemokines were compared between HIV-N (n=13), HIV+ C (n=18) and HIV+ NC (n=11). Nine of 22 analytes were found to vary between groups (Table 22). These included various chemokines (IP-10, IL-8/CXCL8, MCP-1, MIP-1α), proinflammatory cytokines (IFNγ, IL-17), anti-inflammatory cytokines (TGFβ) and homeostatic cytokines (IL-2, IL-7). Post-test comparisons revealed that the differences in IL-8, IFNγ, IL-17, IL-2 and IL-7 concentrations were attributable to differences between the HIV-N and HIV+ NC groups. However, the concentrations of IP-

10 and MCP-1 were significantly lower in HIV+ C (p=0.003 and p=0.017, respectively) compared to HIV+ NC (Figure 35A). A similar trend was observed with TGFβ, but this association did not reach statistical significance (p=0.085). MIP-1α was found to be significantly higher in HIV+ C relative to HIV+ NC (p=0.013).

The relationships between these four analytes and clinical parameters were also evaluated (Figure 35B). CD4 count correlated inversely with IP-10 (r=-0.36, p-0.017), but positively with MIP-1α concentration (r=0.46, p=0.001). Although CD4 count is important for evaluating disease progression, factors that may play a role in control of viral replication, as indicated by plasma viral load, were of specific interest to this study. Plasma viral load correlated positively with levels of IP-10 and TGFβ (r=0.59, p=0.003 and r=0.46, p=0.005, respectively) and inversely with MIP-1α (r=-0.50, p=0.003). Together, these results suggest that elevated levels of MIP-1α and lower levels of IP-10 and TGFβ associate with control of viral replication.

7.4.3 ex vivo T cell phenotypes

Levels of T cell activation were evaluated by measuring expression of the surface markers CD38 and HLA-DR on bulk CD4+ and CD8+ T cells and CD4+ and CD8+ memory T cell subsets from HIV-N (n=12), HIV+ C (n=15) and HIV+ NC (n=11) (Figure 36). Frequencies of bulk CD4+ and CD8+ T cells expressing each marker and median fluorescence intensities (MFI) are summarized in Table 23.

Table 21. Characteristics of HIV+ study participants of the MECC study.

	HIV+ EC (n=5)	HIV+ VC (n=14)	HIV+ NC (n=12)	p-value[a]
Age[b]	33 (26-42.5)	37 (30.5-49.8)	48 (32.8-52.8)	0.148
Female participants[c]	2 (40)	7 (50)	5 (41.7)	0.885
Ethnicity – Aboriginal[c]	3 (60)	7 (50)	5 (41.7)	
Ethnicity – Caucasian[c]	0 (0)	5 (35.7)	6 (50)	0.288
Ethnicity – African[c]	2 (40)	2 (14.3)	1 (8.3)	
CD4 count (cells/mm^3)[b]	567 (509-627)	662 (522.5-899.3)	350 (303.5-494)	**0.0004**
Plasma HIV RNA copies/ml[b]	<50	322.5 (106.3-783.8)	26650 (10675-51400)	**<0.0001**
Years since HIV diagnosis[b]	6 (4.5-10)	7.5 (5-11)	9 (5.3-11)	0.579

[a] P-values of continuous variables calculated by Kruskal-Wallis ANOVA. P-values of dichotomous variables calculated by Chi-square test.
[b] Data represent median (interquartile range) values.
[c] Data represent number of patients (percent of patients).

Table 22. Cytokine and chemokine levels in plasma samples from HIV-uninfected, HIV-controller and non-controller subjects.

	Concentration[a]			ANOVA[b]	Mann-Whitney post-tests[c]		
	HIV-N (n=13)	HIV-C (n=17)	HIV-NC (n=12)	p-value	HIV-N vs. HIV+ C	HIV-N vs. HIV+ NC	HIV+ C vs. HIV+ NC
Chemokines							
Fractalkine/CXCL1	281.0 (220.2-461.4)	328.3 (285.9-425.1)	285.9 (220.3-326.0)	0.172			
IL-8/CXCL8	**32.4 (9.2-48.1)**	**69.5 (29.4-323.0)**	**239.2 (24.0-429.9)**	**0.051**	**0.030**	**0.047**	0.719
IP-10/CXCL10	**657.2 (511.4-946.7)**	**733.5 (568.3-1121.0)**	**3025.0 (1936.0-4331.0)**	**<0.0001**	0.368	**0.0002**	**0.0003**
MCP-1/CCL2	**328.7 (250.8-444.6)**	**334.8 (221.8-435.4)**	**488.2 (351.1-774.7)**	**0.033**	0.738	**0.041**	**0.017**
MCP-3/CCL7	27.2 (16.9-122.9)	36.9 (16.6-96.8)	45.8 (19.1-108.1)	0.916			
MDC/CCL22	1510.0 (1314.0-2010.0)	1482.0 (1162.0-1861.0)	1300.0 (1065.0-1922.0)	0.509			
MIP-1α/CCL3	**30.9 (21.7-73.7)**	**18.7 (13.9-33.3)**	**0.0 (0.0-19.2)**	**0.002**	0.112	**0.002**	**0.013**
MIP-1β/CCL4	49.1 (28.0-109.6)	63.0 (49.8-93.4)	49.1 (31.9-77.4)	0.543			
Proinflammatory and anti-viral cytokine response							
IFNα2	39.1 (28.0-56.8)	44.8 (33.7-64.7)	30.6 (23.6-52.7)	0.218			
IFNγ	**25.2 (10.9-141.8)**	**9.4 (6.0-22.7)**	**10.3 (4.8-20.8)**	**0.038**	**0.033**	**0.026**	0.865
IL-1α	15.3 (7.6-85.6)	13.5 (9.3-32.2)	24.8 (15.4-37.1)	0.762			
IL-1β	5.4 (4.4-12.5)	4.8 (3.6-7.8)	4.8 (0.0-12.6)	0.669			
sIL-2Rα	12.4 (0.0-27.3)	25.6 (0.0-63.5)	37.1 (7.1-54.0)	0.258			
IL-6	12.5 (0.0-46.8)	0.0 (0.0-11.6)	3.7 (0.0-7.9)	0.401			
IL-17	**19.0 (8.7-85.7)**	**10.1 (7.2-13.7)**	**6.5 (4.9-11.4)**	**0.016**	**0.035**	**0.011**	0.330
TNFα	11.5 (9.2-21.7)	11.8 (10.1-16.3)	15.7 (13.0-18.6)	0.209			
Anti-inflammatory cytokine response							
IL-1ra	29.1 (19.7-56.3)	33.9 (25.1-45.6)	29.1 (16.6-40.6)	0.528			
IL-10	9.07 (5.1-14.8)	7.8 (4.9-9.6)	9.2 (6.8-11.5)	0.494			
TGFβ	**30361 (22289-39196)**	**47154 (34458-53734)**	**55927 (45609-68843)**	**0.003**	**0.014**	**0.002**	0.085
Homeostatic Cytokines							
IL-2	**5.7 (0.0-11.7)**	**0.0 (0.0-5.8)**	**0.0 (0.0-0.0)**	**0.038**	0.225	**0.015**	0.112
IL-7	**11.2 (7.7-19.2)**	**9.0 (5.8-12.9)**	**6.0 (0.0-10.4)**	**0.040**	0.167	**0.013**	0.287
IL-15	3.1 (0.0-12.1)	0.0 (0.0-3.9)	0.0 (0.0-2.3)	0.154			

[a] Concentration is listed as median pg/ml. Interquartile range is shown in brackets.

[b] p-values were calculated using Kruskall-Wallis non-parametric ANOVA. Parameters that demonstrated statistically significant ($p<0.05$) or trending ($p<0.1$) differences are highlighted in bold text.

[c] Pairwise comparisons of individual groups were done using non-parametric Mann-Whitney *U* tests if ANOVA results were statistically significant ($p<0.05$) or trending ($p<0.1$)

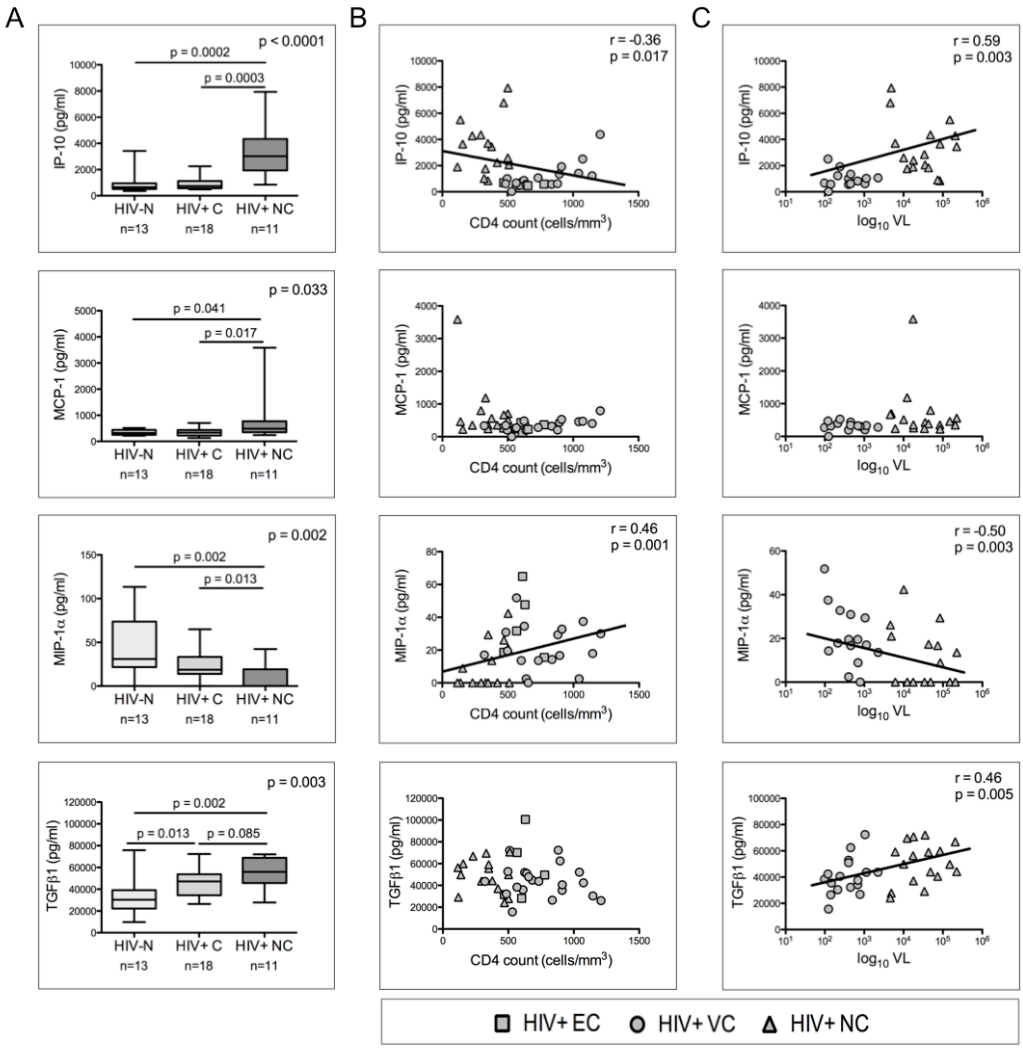


Figure 35. Plasma cytokines and chemokines in HIV-controllers and non-controllers. (A) Concentrations of select cytokines and chemokines. P-values in upper right corner of each graph were calculated using Kruskal-Wallis non-parametric ANOVA. Mann-Whitney *U* tests were used to calculate p-values for pair-wise comparisons. HIV+ C had lower levels of IP-10, MCP-1 and TGFβ and elevated levels of MIP-1α. (B) Correlations between CD4 count (cells/mm^3) and analyte concentrations. CD4 count correlated positively with MIP-1α and inversely with IP-10 concentration. (C) Correlations between detectable $\log_{10}$ plasma viral load (RNA copies/ml) and analyte concentrations. Detectable plasma viral load correlated positively with IP-10 and TGFβ and inversely with MIP-1α concentration. Numbers in upper right corner of correlation graphs indicate Spearman's rho correlation coefficient and p-value from non-parametric correlations.

CD4+ T cells expressing CD38 and HLA DR varied significantly between groups. Most notably, HIV+ NC had elevated frequencies of HLA DR+ and CD38+/HLA DR+ CD4+ T cells relative to HIV+ C (p=0.035 and p=0.007, respectively). Similar differences were observed in the CD8+ T cell subset, where differences between groups were reflected not only in the frequencies of cells expressing these markers (CD38+, HLA DR+ and CD38+/HLA DR+), but also in the median fluorescence intensities (MFI) of the positive cells. The frequencies of CD8+ T cells expressing CD38 (p=0.002) and co-expressing CD38 and HLA DR (p=0.020) were elevated in HIV+ NC relative to HIV+ C. Consistent with this upregulation of CD8+ T cell activation, the MFI of CD38 was higher in the CD8+ T cell subset of non-controllers compared to controllers.

T cell activation markers were also measured on memory CD4+ and CD8+ T cell subsets, as delineated by expression of CD45RA and CCR7. Frequencies of activated memory subset CD4+ and CD8+ T cells are summarized in Tables 24 and 25, respectively. Evaluation of T cell activation markers on memory subsets revealed differences between groups that were undetectable in bulk T cells. For example, while no significant differences were observed in the frequency of bulk CD4+ CD38+ T cells between HIV+C and HIV+ NC, CD38+ CD4+ T_{CM} and T_{EM} cells were lower in HIV+ C than HIV+ NC (p=0.033 and p-0.025, respectively). Similarly, the frequency of bulk CD8+ HLA DR+ T cells did not differ significantly between HIV+ C and HIV+ NC, but HLA DR+ CD8+ T_{CM} were lower in HIV+ C than HIV+ NC (p=0.045).

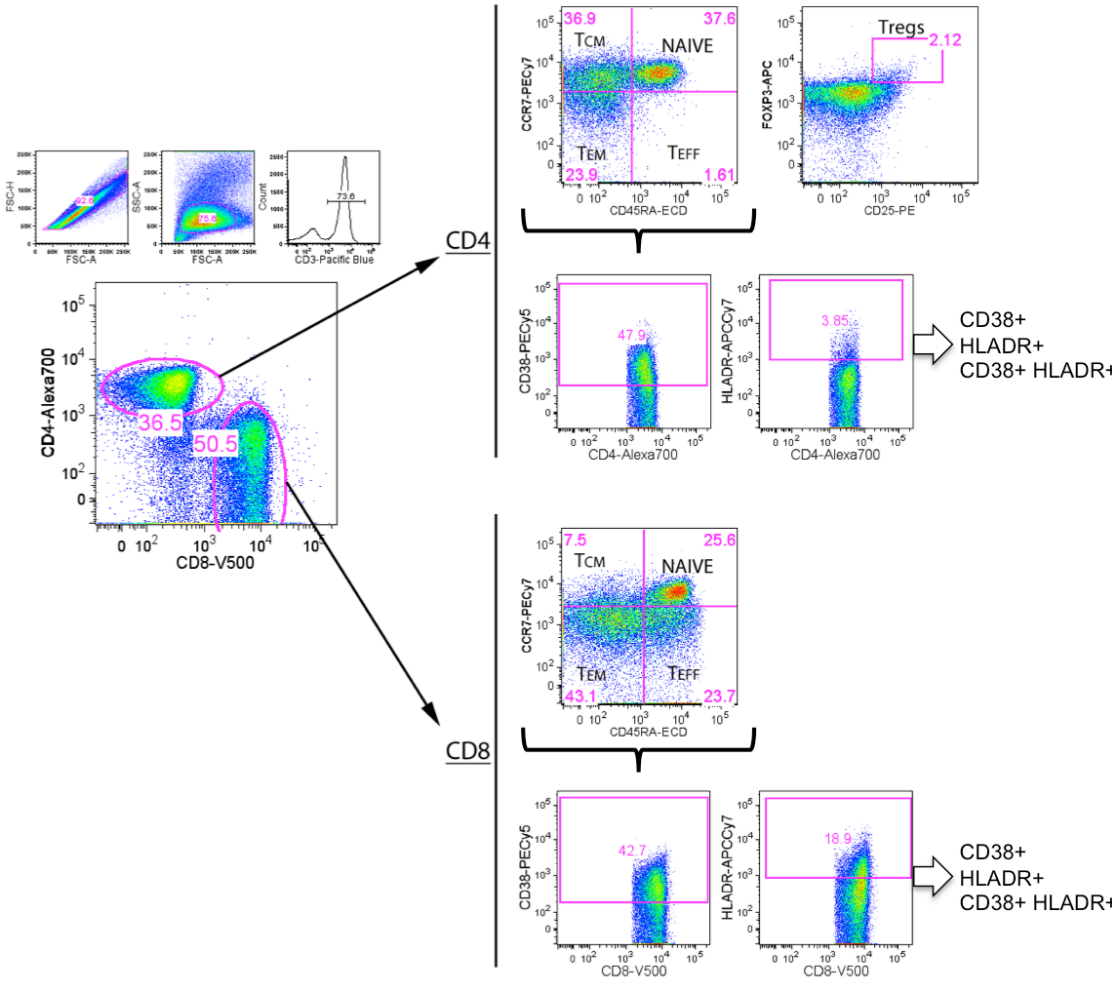


Figure 36. Representative staining of T cell memory and activation markers on PBMC from participants of the MECC study. Samples were first gated on singlets using FSC-H and FSC-A properties, followed by lymphocyte identification using FSC-A and SSC-A. CD3+ T cells were then identified and further discriminated based on CD4 and CD8 expression. Memory CD4+ and CD8+ subsets were identified by relative expression of CD45RA and CCR7. Expression of CD38 and HLA DR was then evaluated for bulk CD4+ and CD8+ T cells and T cell memory subsets. Representative data from AZ1980 (HIV+ VC) is used in this example.

Co-expression of CD38 and HLA DR was used to investigate the relationship between levels of T cell activation and CD4 count or detectable plasma viral load (Figure 37). CD4+ and CD8+ T cell activation correlated inversely with CD4 count ($r=-0.51$, $p=0.0008$ and $r=-0.47$, $p=0.003$, respectively) and positively with plasma viral load ($r=0.76$, $p<0.0001$ and $r=0.67$, $p=0.0002$, respectively). These results lend support to previous observations that viral control is associated with low levels of baseline T cell activation [138, 196, 208, 212, 366, 368].

Levels of Tregs were assessed in a subset of subjects (HIV-N n=5, HIV+ C n=14, HIV+ NC n=11) (Figure 36). No significant differences in frequency of Tregs within the CD4+ T cell compartment were observed (Figure 38A), and Treg frequency did not correlate with CD4 count or detectable plasma viral load (Figure 38B). To examine the effect of CD4 decline on the Treg population, CD4 counts and Treg frequencies (expressed as a percentage of CD4+ T cells) were used to calculate absolute Treg counts in HIV-infected patients (HIV+ C n=11 and HIV+ NC n=11). Absolute Treg counts were elevated in HIV+ C compared to HIV+ NC ($p=0.044$) (Figure 38A). These observations are in line with other studies of Tregs in HIV disease [196, 197, 211]. Absolute Treg counts correlated positively with CD4 count ($r=0.44$, $p=0.014$) and inversely with detectable plasma viral load ($r=-0.42$, $p=0.047$) (Figure 38B). These data suggest that the decline of peripheral Tregs is comparable to that of other CD4+ T cell subsets. Though Treg frequencies within the CD4+ T cell compartment are relatively maintained at various stages of disease, the decline in absolute numbers of Tregs may affect regulation of T cell activation.

Table 23. Expression of activation and memory markers on T cells in HIV-uninfected, HIV-controller and non-controller subjects.

Subset	Parameter	Expression[a]			ANOVA[b]	Mann-Whitney post-tests[c]		
		HIV-N	HIV-C	HIV-NC	p-value	HIV-N vs. HIV+ C	HIV-N vs. HIV+ NC	HIV+ C vs. HIV+ NC
Total CD4+ T cells	**CD38+ (%)**	**42.5 (37.2-48.6)**	**47.1 (40.0-53.7)**	**50.5 (46.0-58.2)**	**0.052**	0.330	**0.019**	0.103
	CD38 MFI	461.5 (431.8-534.0)	502.0 (455.5-593.0)	516.0 (469.3-587.5)	0.380			
	HLA DR+ (%)	**2.5 (1.5-2.9)**	**4.1 (2.9-5.8)**	**6.1 (4.3-8.9)**	**0.002**	**0.023**	**0.001**	**0.035**
	HLA DR MFI	1809 (1704-2646)	2482 (2027-2798)	2531 (2150-2649)	0.174			
	CD38+ HLA DR+ (%)	**0.8 (0.6-1.5)**	**1.8 (1.2-2.5)**	**3.1 (2.5-5.0)**	**<0.0001**	**0.002**	**0.0001**	**0.007**
	CD45RA+ CCR7+ (%)	36.1 (28.6-46.6)	30.6 (21.6-42.4)	30.0 (19.5-44.1)	0.424			
	CD45RA- CCR7+ (%)	41.8 (33.7-46.2)	45.3 (35.7-50.7)	47.6 (35.8-67.8)	0.261			
	CD45RA- CCR7- (%)	19.9 (14.7-25.0)	19.9 (17.0-26.7)	16.1 (10.4-20.7)	0.219			
	CD45RA+ CCR7- (%)	2.2 (1.7-3.7)	1.3 (0.6-2.1)	2.0 (0.5-3.3)	0.167			
Total CD8+ T cells	**CD38+ (%)**	**34.5 (33.2-39.0)**	**42.8 (38.2-47.1)**	**54.4 (49.6-61.5)**	**<0.0001**	**0.002**	**<0.0001**	**0.002**
	CD38 MFI	**428.5 (410.3-501.8)**	**542.0 (461.5-589.0)**	**637.5 (536.8-736.0)**	**0.0008**	**0.005**	**0.001**	0.060
	HLA DR+ (%)	**3.6 (1.8-5.0)**	**13.9 (5.1-18.9)**	**15.4 (7.7-22.3)**	**0.0002**	**0.001**	**0.0001**	0.514
	HLA DR MFI	**2024 (1874-2686)**	**2632 (1991-2983)**	**2851 (2644-3049)**	**0.068**	0.080	**0.041**	0.380
	CD38+ HLA DR+ (%)	**1.7 (1.0-2.4)**	**6.7 (3.3-9.9)**	**13.1 (6.3-15.4)**	**<0.0001**	**0.0002**	**<0.0001**	**0.020**
	CD45RA+ CCR7+ (%)	**33.1 (23.8-45.3)**	**25.4 (16.2-38.8)**	**21.0 (9.0-25.2)**	**0.015**	**0.080**	**0.006**	0.126
	CD45RA- CCR7+ (%)	9.2 (6.8-11.9)	9.1 (5.1-11.8)	10.1 (3.9-21.6)	0.829			
	CD45RA- CCR7- (%)	29.7 (20.8-36.9)	37.3 (27.7-47.6)	37.2 (23.5-53.1)	0.168			
	CD45RA+ CCR7- (%)	24.5 (17.1-36.7)	21.3 (17.2-33.9)	27.0 (17.0-34.8)	0.975			

[a] Expression of phenotypic markers is listed as median of percent positive (+) cells or median fluorescence intensity (MFI). Interquartile range is shown in parentheses.
[b] p-values were calculated using Kruskall-Wallis non-parametric ANOVA. Parameters demonstrating statistically significant ($p<0.05$) or trending ($p<0.1$) p-values are highlighted in bold text.
[c] Pairwise comparisons of individual groups were done using non-parametric Mann-Whitney U tests if ANOVA results were statistically significant ($p<0.05$) or trending ($p<0.1$).

Table 24. Expression of activation markers on CD4+ T cell memory subsets cells in HIV-uninfected, HIV-controller and non-controller subjects.

Subset	Parameter	Expression[a]			ANOVA[b]	Mann-Whitney post-tests[c]		
		HIV-N	HIV-C	HIV-NC	p-value	HIV-N vs. HIV+ C	HIV-N vs. HIV+ NC	HIV+ C vs. HIV+ NC
CD4+ Naïve (CD45RA+ CCR7+)	CD38+	63.2 (57.5-68.3)	64.0 (60.6-67.6)	59.6 (58.5-63.1)	0.368			
	HLA DR+	**0.2 (0.2-0.4)**	**0.4 (0.2-0.6)**	**1.6 (0.5-3.1)**	**0.0007**	0.177	**0.0009**	**0.002**
	CD38+ HLA DR+	**0.2 (0.1-0.2)**	**0.3 (0.1-0.3)**	**1.1 (0.4-2.3)**	**0.0006**	0.184	**0.0009**	**0.002**
CD4+ TCM: Central Memory (CD45RA- CCR7+)	**CD38+**	**34.8 (31.5-40.4)**	**40.0 (35.1-49.6)**	**46.7 (45.3-53.4)**	**0.002**	0.077	**0.0007**	**0.033**
	HLA DR+	**1.8 (1.2-3.8)**	**3.2 (2.2-4.4)**	**6.8 (4.2-8.6)**	**0.002**	0.054	**0.003**	**0.008**
	CD38+ HLA DR+	**0.9 (0.5-1.5)**	**1.5 (1.1-2.2)**	**3.4 (2.3-5.3)**	**0.0005**	**0.035**	**0.0008**	**0.005**
CD4+ TEM: Effector Memory (CD45RA- CCR7-)	**CD38+**	**19.9 (16.4-26.0)**	**28.8 (21.2-35.1)**	**38.1 (33.0-43.0)**	**0.0001**	**0.007**	**<0.0001**	**0.025**
	HLA DR+	**3.8 (2.5-5.4)**	**8.9 (4.8-11.4)**	**13.3 (9.3-16.7)**	**0.0002**	**0.003**	**0.0003**	**0.047**
	CD38+ HLA DR+	**1.1 (0.8-1.6)**	**3.7 (2.2-4.9)**	**7.1 (4.0-8.3)**	**<0.0001**	**0.0003**	**0.0002**	**0.013**
CD4+ TEFF: Terminal effector (CD45RA+ CCR7-)	CD38+	50.6 (39.0-59.6)	50.2 (43.7-56.9)	46.7 (41.3-54.1)	0.871			
	HLA DR+	**1.2 (0.4-2.1)**	**3.9 (2.9-7.2)**	**6.9 (4.6-8.8)**	**0.0001**	**0.0005**	**0.0003**	0.238
	CD38+ HLA DR+	**0.4 (0.2-1.0)**	**2.1 (1.2-3.0)**	**3.5 (1.9-4.3)**	**<0.0001**	**0.0003**	**0.0003**	0.097

[a] Expression of phenotypic markers is listed as median of percent positive (+) cells. Interquartile range is shown in parentheses.

[b] p-values were calculated using Kruskall-Wallis non-parametric ANOVA. Parameters demonstrating statistically significant ($p<0.05$) or trending ($p<0.1$) p-values are highlighted in bold text.

[c] Pairwise comparisons of individual groups were done using non-parametric Mann-Whitney U tests if ANOVA results were statistically significant ($p<0.05$) or trending ($p<0.1$)

Table 25. Expression of activation markers on CD8+ T cell memory subsets cells in HIV-uninfected, HIV-controller and non-controller subjects.

Subset	Parameter	Expression[a]			ANOVA[b]	Mann-Whitney post-tests[c]		
		HIV-N	HIV-C	HIV-NC	p-value	HIV-N vs. HIV+ C	HIV-N vs. HIV+ NC	HIV+ C vs. HIV+ NC
CD8+ Naïve (CD45RA+ CCR7+)	**CD38+**	**42.3 (41.2-43.3)**	**45.3 (43.8-49.2)**	**49.1 (45.8-53.7)**	**0.0009**	**0.017**	**0.0004**	0.075
	HLA DR+	**0.8 (0.4-1.5)**	**2.0 (1.4-3.9)**	**3.7 (2.3-11.1)**	**0.002**	**0.006**	**0.004**	0.093
	CD38+ HLA DR+	**0.3 (0.2-0.7)**	**1.0 (0.6-1.9)**	**2.1 (1.5-6.7)**	**0.0003**	**0.001**	**0.002**	**0.031**
CD8+ TCM: Central Memory (CD45RA- CCR7+)	**CD38+**	**29.4 (26.4-32.2)**	**40.5 (33.7-47.5)**	**53.2 (49.5-66.7)**	**<0.0001**	**0.001**	**<0.0001**	**0.0009**
	HLA DR+	**6.3 (4.6-10.2)**	**16.0 (11.6-22.9)**	**23.8 (18.5-40.9)**	**<0.0001**	**0.0002**	**0.0001**	**0.045**
	CD38+ HLA DR+	**3.2 (2.6-4.8)**	**10.5 (7.5-17.5)**	**19.1 (13.7-30.0)**	**<0.0001**	**<0.0001**	**<0.0001**	**0.010**
CD8+ TEM: Effector Memory (CD45RA- CCR7-)	**CD38+**	**27.1 (20.3-31.0)**	**41.2 (33.2-46.7)**	**55.0 (51.2-63.8)**	**<0.0001**	**0.0002**	**<0.0001**	**0.001**
	HLA DR+	**7.6 (4.0-10.2)**	**27.2 (14.1-32.4)**	**34.0 (20.3-44.9)**	**<0.0001**	**<0.0001**	**0.0001**	0.145
	CD38+ HLA DR+	**3.2 (2.3-5.0)**	**13.2 (8.5-24.1)**	**24.2 (17.4-29.9)**	**<0.0001**	**<0.0001**	**<0.0001**	**0.047**
CD8+ TEFF: Terminal effector (CD45RA+ CCR7-)	**CD38+**	**38.4 (36.8-42.3)**	**45.0 (40.0-49.6)**	**53.3 (48.8-61.2)**	**<0.0001**	**0.004**	**0.0003**	**0.008**
	HLA DR+	**4.2 (2.4-4.6)**	**18.1 (10.0-25.6)**	**18.0 (8.7-31.4)**	**0.0001**	**<0.0001**	**0.0009**	0.940
	CD38+ HLA DR+	**1.8 (1.0-2.3)**	**9.0 (5.6-15.3)**	**12.2 (6.4-16.9)**	**<0.0001**	**<0.0001**	**0.0003**	0.530

[a]Expression of phenotypic markers is listed as median of percent positive (+) cells. Interquartile range is shown in parentheses.
[b]p-values were calculated using Kruskall-Wallis non-parametric ANOVA. Parameters demonstrating statistically significant ($p<0.05$) or trending ($p<0.1$) p-values are highlighted in bold text.
[c]Pairwise comparisons of individual groups were done using non-parametric Mann-Whitney U tests if ANOVA results were statistically significant ($p<0.05$) or trending ($p<0.1$).

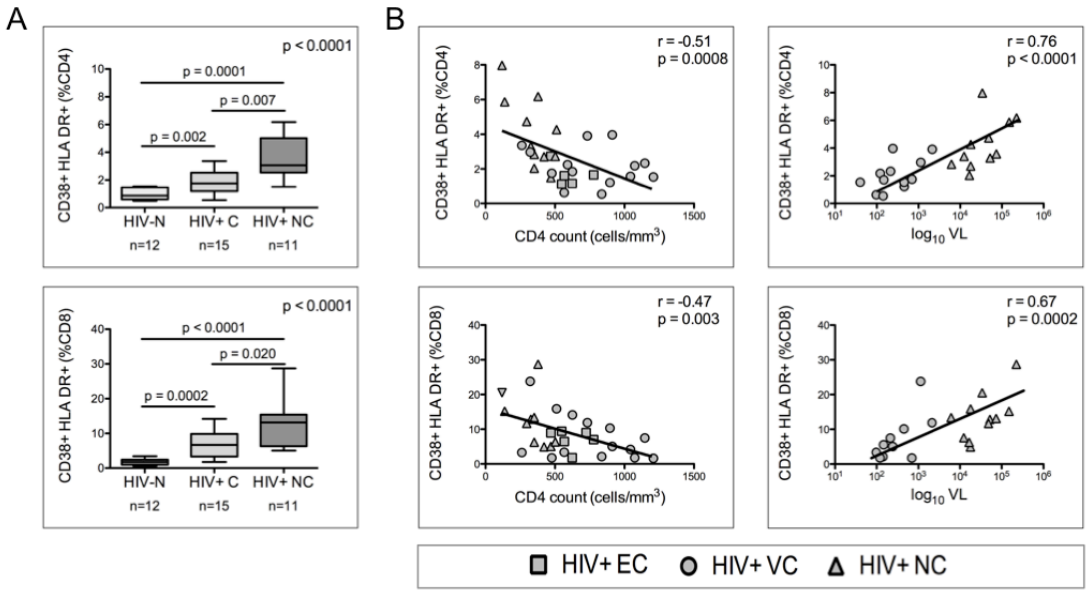


Figure 37. CD4+ and CD8+ T cell activation phenotypes in HIV-controllers, non-controllers and uninfected individuals. (A) Frequencies of activated (CD38+/HLA DR+) T cells. P-value in upper right corner of each graph indicates results of Kruskal-Wallis non-parametric ANOVA. Mann-Whitney *U* tests were used to calculate p-values for pair-wise comparisons. HIV+ NC have more activated CD4+ and CD8+ T cells than HIV+ C and HIV-N. HIV+ C have elevated CD4+ and CD8+ T cell activation relative to HIV-N. (B) Numbers in upper right corner of each graph indicate Spearman's rho correlation coefficient and p-value from non-parametric correlations. Frequencies of activated CD4+ and CD8+ T cells are negatively correlated with CD4 counts and positively correlated with plasma viral loads.

Indeed, absolute Treg counts correlated inversely with frequencies of activated CD8+ T cells (r=-0.62, p=0.0003). A similar relationship was observed between absolute Treg counts and activated CD4+ T cells, but this correlation did not reach statistical significance (r=-0.31, p=0.097) (Figure 38C). These data support the hypothesis that progressive Treg loss may be one factor contributing to aberrant immune activation, a well-established driving factor in HIV disease progression.

7.4.4 T cell responses

Robust CD8+ T cell responses directed against multiple epitopes within HIV gag are thought to be a major mechanism by which HIV-controllers maintain low levels of viral replication [369, 370]. Full characterization of HLA-restricted epitope-specific responses was beyond the scope of this study, but bulk HIV gag-specific CD4+ and CD8+ T cell responses were characterized in a subset of patients. PBMC were stimulated with a pool of HIV gag peptides, followed by measurement of cytokine (IFNγ, IL-2) and proliferation responses. Stimulation with CD3/CD28 beads was used as a positive control.

Intracellular cytokine staining was used to detect the presence of IFNγ and IL-2 production by CD4+ and CD8+ T cells in response to stimulation with CD3/CD28 beads or HIV gag peptides in HIV+ C (n=12) and HIV+ NC (n=6). Representative gating of cytokine responses is shown in Figure 39. All patients responded to CD3/CD28 stimulation, and no differences were observed in the magnitude of T cell cytokine responses (Figure 40).

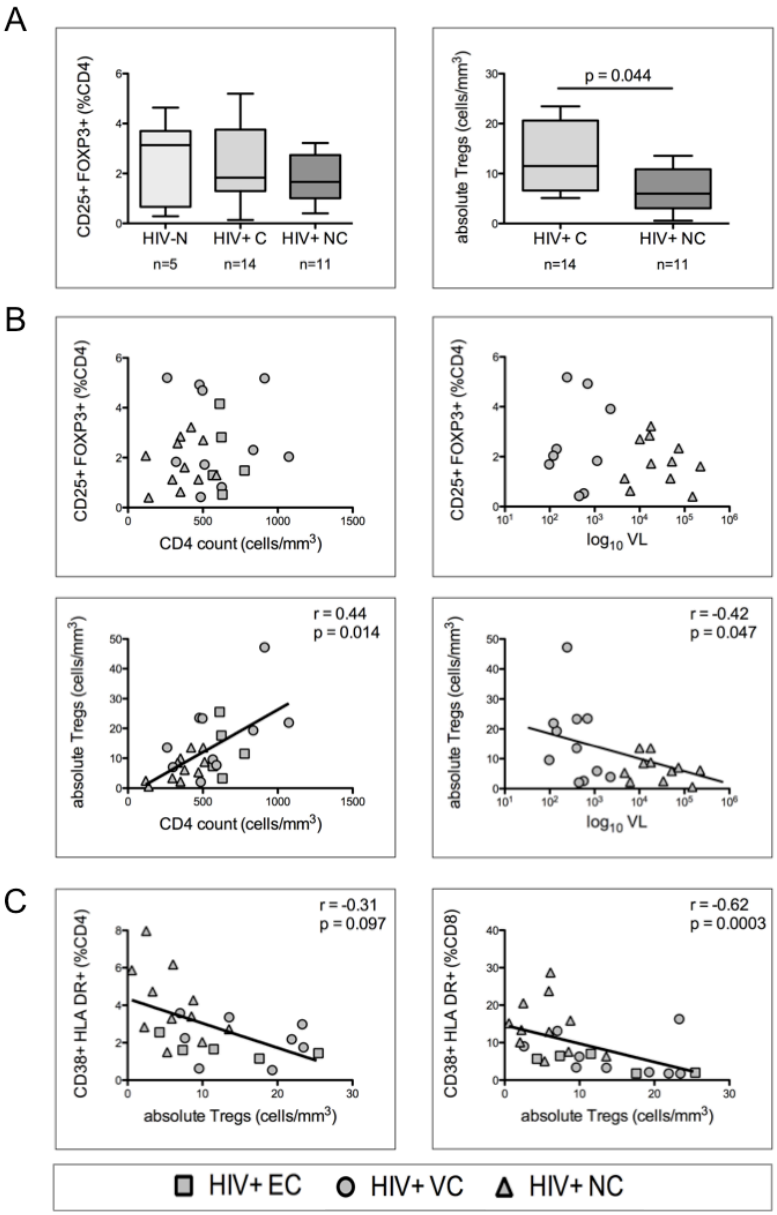


Figure 38. Regulatory T cells in HIV-controllers, non-controllers and uninfected individuals. (A) Treg frequency and absolute Treg count. Treg frequency did not differ between groups, but HIV+ C had higher absolute numbers of Tregs compared to HIV+ NC. (B) Frequencies of Tregs within the CD4+ T cell compartment did not correlate with CD4 count or viral load. Absolute counts of Tregs correlated positively with CD4 count and inversely with plasma viral load. (C) Absolute counts of Tregs correlated inversely with frequencies of activated CD8+ T cells. A similar relationship was observed between absolute Treg count and frequencies of activated CD4+ T cells. Numbers in upper right corner of correation graphs indicate Spearman's rho correlation coefficient and p-value from non-parametric correlations.

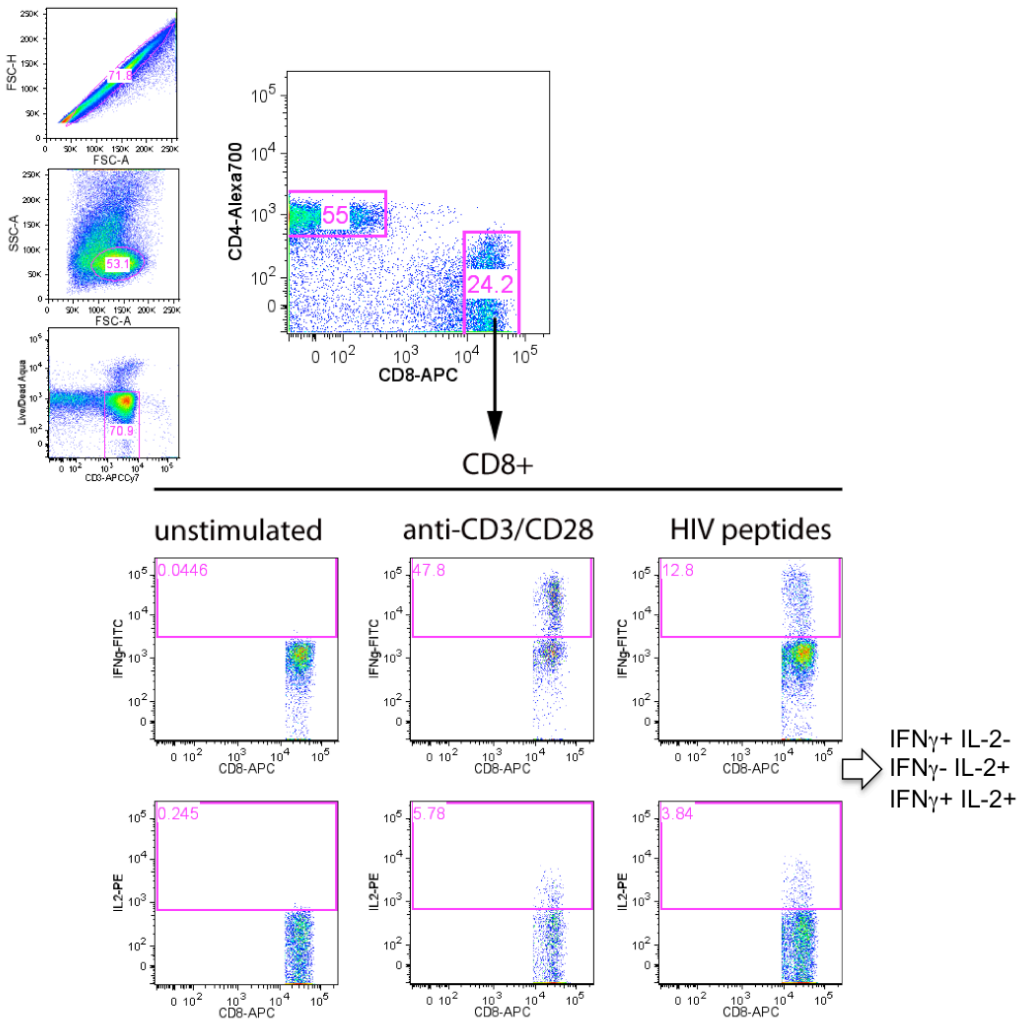


Figure 39. Representative staining of T cell cytokine responses evaluated in the MECC study. Samples were first gated on singlets using FSC-H and FSC-A properties, followed by lymphocyte identification using FSC-A and SSC-A. Live T cells were then identified by gating on CD3+ cells with dim staining of LIVE/DEAD Aqua, and further discriminated based on CD4 and CD8 expression. Intracellular cytokine staining was used to detect production of IFNγ and IL-2 14 hours post-stimulation with CD3/CD28 beads or HIV peptides. A boolean gating strategy was applied to identify cells producing all combinations of cytokines. Gating on CD8+ T cells is shown. A parallel approach was used to evaluate CD4+ T cell cytokine production. Representative data from NG1985 (HIV+ EC) is used in this example.

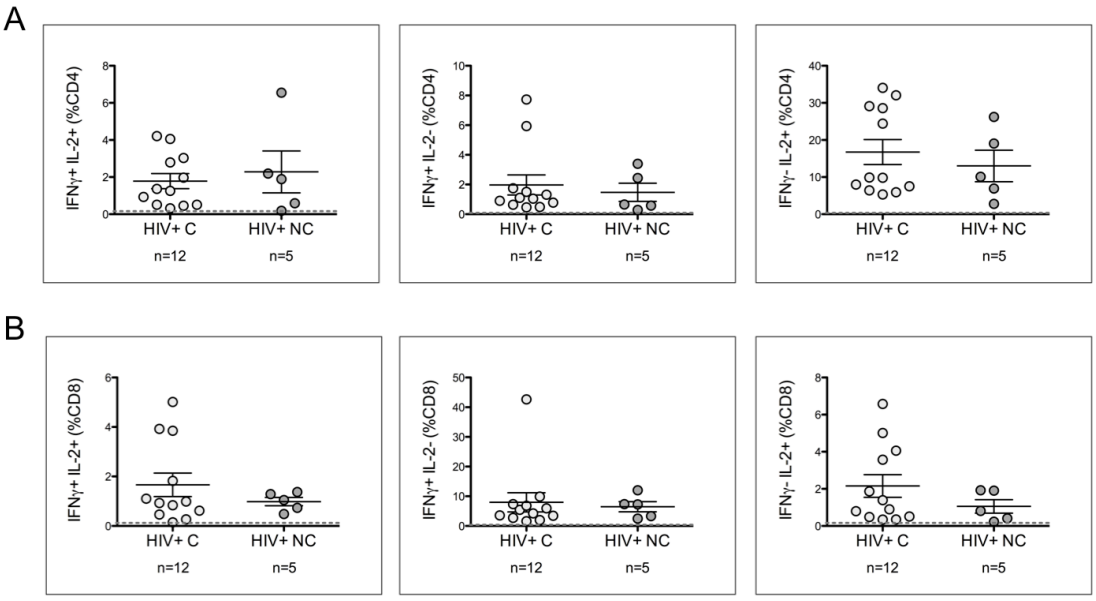


Figure 40. CD4+ and CD8+ T cell cytokine responses to CD3/CD28 stimulation in HIV-controllers and non-controllers. (A, B) Percentages of CD4+ (A) and CD8+ (B) T cells expressing IFNγ and/or IL-2 in response to stimulation with CD3/CD28 beads. Dashed line indicates threshold level of response as determined by responses measured in HIV-negative volunteers. Data points above the dashed line indicate a positive response. All individuals demonstrated positive cytokine responses to stimulation. No differences in the magnitude of response were observed between HIV+ C and HIV+ NC.

A trend was observed in which HIV+ C had stronger HIV-specific CD4+ IFNγ+ IL-2- responses than HIV+ NC (p=0.09) (Figure 41A). The frequency of positive responses (i.e. proportion of individuals responding by a particular cytokine combination) was also compared between groups. No significant differences were observed between HIV+ C and HIV+ NC, although CD4+ IFNγ- IL-2 responses were only detected in the HIV+ C group (Figure 41B). When the HIV+ C group was broken down into Elite Controllers (n=5) and Viremic Controllers (n=7), a linear trend was observed in the frequency of HIV-specific CD4+ IFNγ- IL-2+ responses observed (EC > VC > NC), but this trend did not reach statistical significance (p=0.10) (Figure 41C).

No significant differences between HIV+ C and HIV+ NC were observed with respect to HIV-specific CD8+ IFNγ+ IL-2+ or IFNγ+ IL-2- responses. However, HIV-specific CD8+ IFNγ- IL-2+ responses were stronger in HIV+ C than HIV+ NC (p=0.016) (Figure 42A). No differences in the frequency of positive CD8+ T cell responses were observed between groups (Figure 42B), and no trends were revealed when the HIV+ C group was broken down into elite controllers and viremic controllers (Figure 42C). However, as was seen with CD4+ T cells, non-controllers completely lacked CD8+ IFNγ- IL-2+ responses.

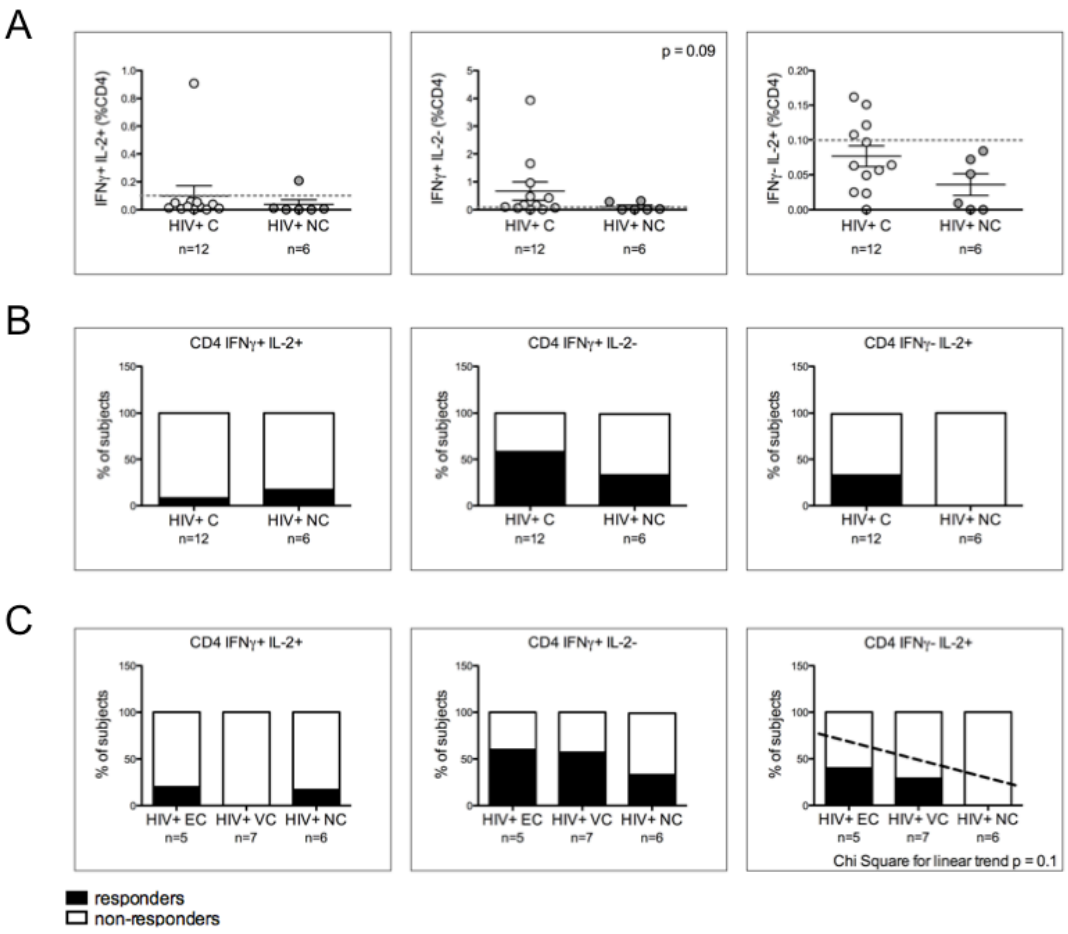


Figure 41. HIV-specific CD4+ T cell cytokine responses in HIV-controllers and non-controllers. (A) Percentages of CD4+ T cells expressing IFNγ and/or IL-2 in response to stimulation with HIV gag peptides. Dashed line indicates threshold level of response. Data points above the dashed line indicate a positive response. A trend was observed in which HIV+ C had a stronger HIV-specific CD4+ IFNγ+ IL-2- response than HIV+ NC. (B) No differences were observed in the frequency of positive HIV-specific CD4+ cytokine responses between HIV+ C and HIV+ NC. (C) When the HIV+ C group was broken down based on level of HIV control (EC <50 RNA copies/ml, n=5; VC 50-2000 RNA copies/ml, n=7), a linear trend was observed in the frequency of HIV-specific CD4+ IFNγ- IL-2+ responses observed (EC > VC > NC).

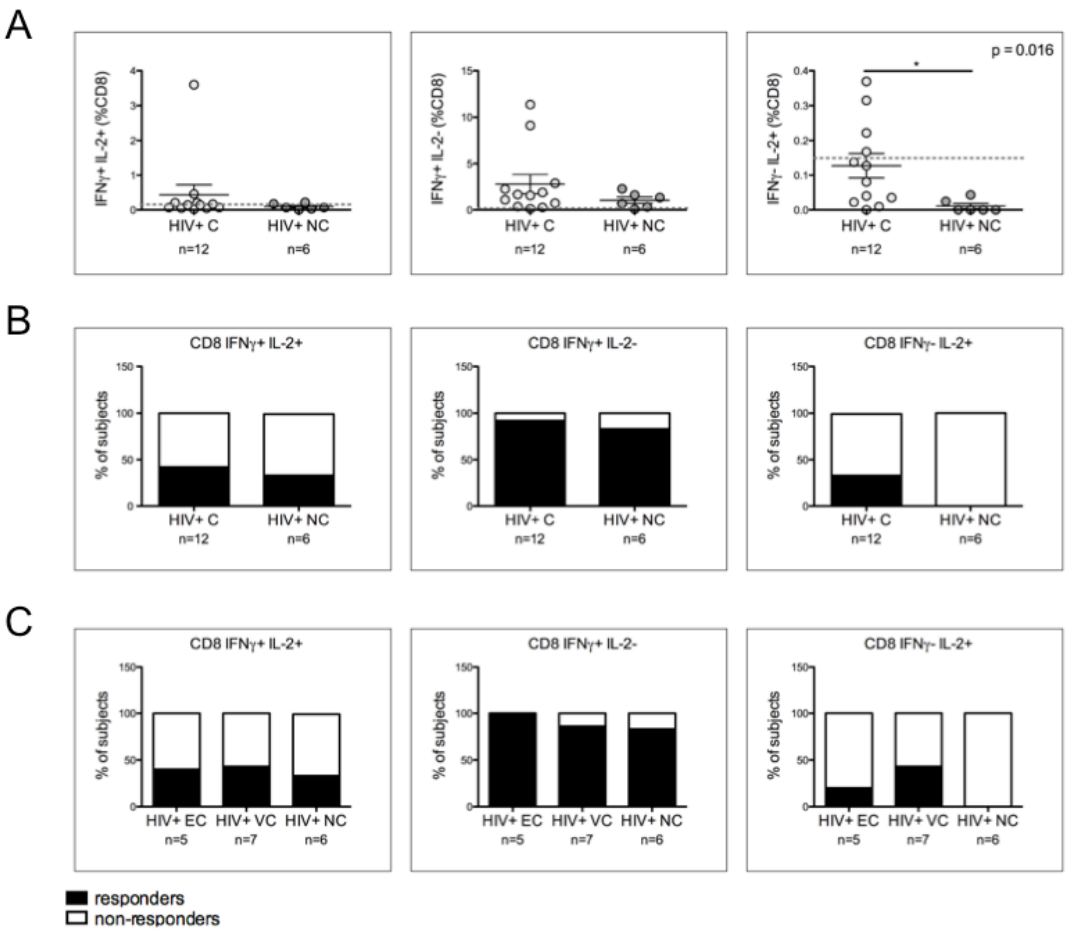


Figure 42. HIV-specific CD8+ T cell cytokine responses in HIV-controllers and non-controllers. (A) Percentages of CD8+ T cells expressing IFNγ and/or IL-2 in response to stimulation with HIV gag peptides. Dashed line indicates threshold level of response. Data points above the dashed line indicate a positive response. HIV+ C had a stronger HIV-specific CD8+ IFNγ- IL-2+ response than HIV+ NC. (B) No differences were observed in the frequency of positive HIV-specific CD8+ cytokine responses between HIV+ C and HIV+ NC. (C) When the HIV+ C group was broken down based on level of HIV control (EC <50 RNA copies/ml, n=5; VC 50-2000 RNA copies/ml, n=7), no differences were observed in the frequency of positive HIV-specific CD8+ cytokine responses between HIV+ EC, HIV+ VC and HIV+ NC.

T cell proliferation was measured six days post-stimulation using CFSE dye dilution in HIV+ C (n=10) and HIV+ NC (n=4). Representative gating of proliferation responses is shown in Figure 43. All patients responded to CD3/CD28 stimulation, and HIV+ C and HIV+ NC did not differ in the magnitude of CD4+ or CD8+ T cell proliferation responses (Figure 44A). HIV-specific CD4+ proliferation responses were significantly stronger (p=0.01) and tended to be seen more frequently (p=0.07) in HIV+ C compared to HIV+ NC (Figure 44B,C). When the HIV+ C group was broken down into elite controllers (n=5) and viremic controllers (n=5), a significant linear relationship was observed in which the presence of positive CD4+ proliferation responses decreased with progressive loss of viral control (p=0.035) (Figure 44D).

Similar associations between HIV control and proliferation responses were observed in the CD8+ T cell compartment as were seen in the CD4+ T cell compartment. HIV-specific CD8+ proliferation responses were stronger in HIV+ C than HIV+ NC (p=0.04) (Figure 44B). No significant differences in frequency of CD8+ proliferation responses were observed between HIV+ C and HIV+ NC (Figure 44C). However, a linear trend was observed in the frequency of HIV-specific CD8+ proliferation when the HIV+ C group was broken down into elite and viremic controllers (EC > VC > NC), but this trend did not reach statistical significance (p=0.09) (Figure 44D).

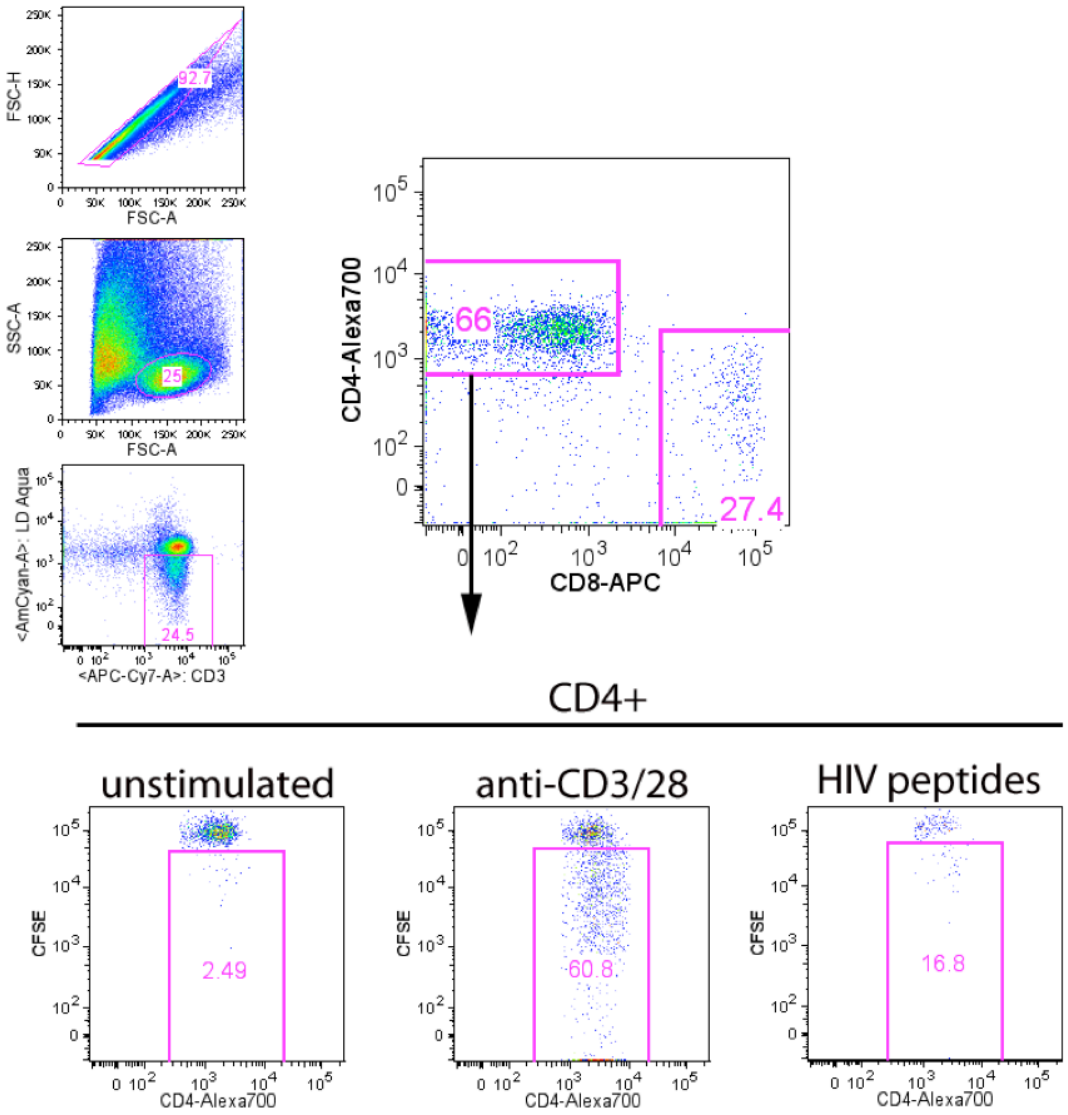


Figure 43. Representative staining of T cell proliferation responses evaluated in the MECC study. Samples were first gated on singlets using FSC-H and FSC-A properties, followed by lymphocyte identification using FSC-A and SSC-A. Live T cells were then identified by gating on CD3+ cells with dim staining of LIVE/DEAD Aqua, and further discriminated based on CD4 and CD8 expression. CFSE dye dilution was used to detect proliferation 6 days post-stimulation with CD3/CD28 beads or HIV peptides. Gating on CD4+ T cells is shown. A parallel approach was used to evaluate CD8+ T cell proliferation. Representative data from IS1959 (HIV+ VC) is used in this example.

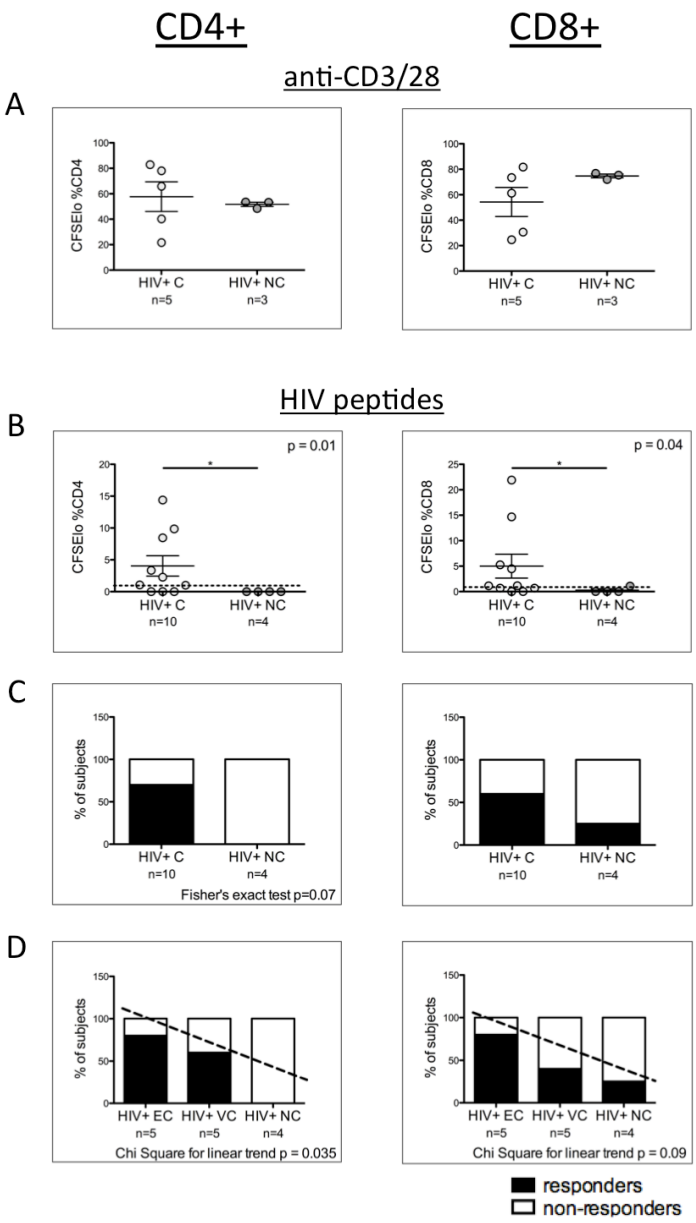


Figure 44. CD4+ and CD8+ T cell proliferation responses in HIV-controllers and non-controllers. (A) Percentages of CD4+ and CD8+ T cells proliferating in response to stimulation with CD3/CD28 beads. (B) Percentages of CD4+ and CD8+ T cells proliferating in response to stimulation with HIV gag peptides. Dashed line indicates background level of response. P-values in upper right corner of graphs were calculated using the Mann-Whitney *U* test. HIV+ C had stronger HIV-specific CD4+ and CD8+ proliferation responses than HIV+ NC. (C) HIV+ C had HIV-specific CD4+ proliferation responses more frequently than HIV+ NC. (C) When the HIV+ C group was broken down based on level of viral control, linear trends were observed in the frequency of HIV-specific CD4+ and CD8+ proliferation responses observed (EC > VC > NC).

7.5 Summary

A distinct chemokine and cytokine profile was identified that distinguished HIV-controllers from non-controllers. The observations of reduced IP-10 and MCP-1 concentrations fit with the hypothesis that HIV-controllers would express low levels of pro-inflammatory mediators than non-controllers. TGFβ, an anti-inflammatory cytokine, was also expressed at a low level in HIV-controllers. However, TGFβ is expressed at high levels by Tregs during HIV infection [371], so low levels of this cytokine may prevent Treg-mediated inhibition of HIV-specific T cell responses. MIP-1α is a pro-inflammatory chemokine, yet it was elevated in HIV-controllers, contrary to the hypothesis. However, MIP-1α may directly block HIV infection through interaction with CCR5 [372].

HIV-controllers had low levels of T cell activation, which corresponded to maintenance of absolute counts of Tregs. These observations were in line with the hypothesis that T cells from HIV-controllers would demonstrate lower levels of activation and elevated Tregs compared to non-controllers.

HIV-specific T cell proliferative capacity was associated with the durability of viral control. This observation is consistent with the literature and with the hypothesis that HIV-controllers would maintain robust HIV-specific T cell responses that are qualitatively distinct from non-controllers.

Chapter 8. Discussion

Understanding natural mechanisms of protection against HIV infection and disease have become key priorities for informing vaccine and microbicide design. The aim of the research presented in this thesis was to characterize possible mechanisms of defence in HESN, who naturally resist infection by HIV, and HIV-controllers, who are HIV-infected but suppress viral replication in the absence of antiretroviral therapy. We focused on addressing the role of immune quiescence in susceptibility to HIV infection and disease progression. Immune quiescence refers to a state of low baseline immune activation, which was previously characterized by reduced gene transcription and reduced baseline production of cytokines by CD4+ T cells from HESN in the Pumwani CSW cohort [336]. **The central hypothesis of this thesis is that immune quiescence protects against HIV infection and disease progression by limiting the pool of activated target CD4+ T cells susceptible to HIV infection.**

8.1 T cell Phenotypes in HIV Susceptibility and Disease

To determine whether immune quiescence could be observed at the level of T cell activation in HESN women of the Pumwani cohort, T cell phenotypes were compared between HESN and HIV-N. The hypothesis for this section was:

1. HESN will demonstrate lower levels of T cell activation and higher levels of regulatory T cells than HIV-N controls.

8.1.1 HESN have reduced T cell activation

A comparison of T cell activation markers between the study groups confirmed our hypothesis that HESN would demonstrate lower levels of T cell activation than HIV-N. Low T cell activation in HESN was marked by reduced frequencies of CD4+ CD69+ (Figure 4A), CD8+ CD69+ (Figure 4B), CD4+ CD38+ T_{CM} (Figure 6) and CD8+ CD95+ T_{EM} (Figure 7) cells.

These data are in line with other studies of T cell activation in HESN. Low frequencies of CD4+ and CD8+ T cells expressing the activation markers HLA-DR, CD38, CD70 and Ki67 were identified in HESN MSM [340]. Studies of serodiscordant couples found reduced expression of CD38 [341], HLA DR and CCR5 [342] on CD4+ T cells from the uninfected partner. Lymphocytes isolated from HESN CSW from Abidjan, Côte d'Ivoire expressed lower levels of CD69 and proinflammatory cytokines and chemokines following allostimulation when compared to controls [343].

Data that conflict with the immune quiescence hypothesis appear in reports of elevated immune activation as a correlate of protection in HESN. Elevated levels of T cell activation [303, 308, 373] and proinflammatory cytokine production [374] have been found in systemic or mucosal samples from multiple cohorts of HESN. It is possible that the disparate findings from the above studies may be reconciled by differences in the site or intensity of HIV exposure, presence of other immunogenic factors or differences in assays used to characterize immune activation.

Maintenance of low levels of T cell activation may protect against HIV infection in HESN by limiting the availability of target cells required for productive HIV replication. HIV can enter quiescent CD4+ T cells [375-379], but the processes of reverse transcription and integration are inefficient [375, 376, 378]. However, subsequent activation can rescue productive infection [375, 378, 380]. An evaluation of cellular susceptibility to HIV infection at early stages of the cell cycle demonstrated that 'resting' cells could be divided into two categories with differential susceptibility. Cells in the G_0/G_{1a} phase of the cell cycle are truly resting, and are resistant to productive HIV infection. In contrast, cells in the G_{1b} phase exhibit higher levels of gene transcription and support productive HIV infection, albeit at lower levels than cells in later stages of the cell cycle [381, 382]. CD69 is expressed prior to entry into the G_{1b} phase, indicating that CD69 expression may be a marker of cellular infectability [381, 382]. Importantly, CD69 also possesses immunoregulatory function, such that it polarizes T cells toward a Treg phenotype [88]. Despite its immunoregulatory role, it is clear that CD69 is expressed along with other cofactors important for HIV replication. As such, the finding of reduced frequencies of CD69+ CD4+ T cells suggests that HESN have fewer cells capable of supporting productive HIV infection. HESN also had reduced CD4+ CD38+ T_{CM} cells. CD38 is expressed during G_{1b} and later phases of the cell cycle [382]. As such, CD38- CD4+ T cells may have entered the G_{1b} phase and be permissive to HIV infection, but if infected, would produce lower levels of virus than cells in later stages of the cell cycle [381].

HESN also had reduced levels of CD8+ CD69+ and CD8+ CD95+ T_{EM} cells than HIV-N. CD95/Fas contains an intracellular death domain that triggers apoptosis through interactions with Fas ligand (FasL). The Fas-FasL pathway is a central mechanism of activation-induced cell death (AICD), which occurs following cellular activation in order to limit the immune response [383]. Indeed, CD95 expression has previously been linked with immune activation and apoptosis in HIV-uninfected women from the Pumwani CSW cohort [384], so reduced frequencies of CD8+ CD95+ T_{EM} cells support to the observation of low levels of activation in HESN. However, CD8+ T cells are not susceptible to HIV infection, and CD8+ T cell activation does not have the same direct implication for HIV susceptibility as CD4+ T cell activation. However, activated CD8+ T cells produce multiple proinflammatory cytokines and chemokines, so reduced levels of CD8+ T cell activation likely contribute to an overall state of immune quiescence in HESN.

The relationship between activated T cells and cellular susceptibility to infection was explored in Chapter 6, and will be discussed in Section 8.4.

8.1.2 HESN have skewed T cell memory profiles

Memory T cell subsets were compared between groups, and HESN were found to have reduced frequencies of CD4+ and CD8+ T_{NAIVE} cell populations relative to HIV-N. This reduction in T_{NAIVE} cells was accompanied by elevated frequencies of CD4+ T_{EM} and CD8+ T_{EFF} cells in HESN (Figure 5). The skewed differentiation of CD4+ and CD8+ T cell

subsets toward a more differentiated phenotype in HESN may have functional implications for the ability of the host to respond to infectious challenges. For instance, the type and quality of memory response elicited during vaccination or infection influences the efficacy and duration of protection [81, 84]. However, aging has previously been associated with loss of naïve CD4+ and CD8+ T cell subsets and elevated frequencies of CD8+ TEFF cells [385]. In agreement with this, age inversely correlated with frequencies of CD4+ and CD8+ TNAIVE cells and positively correlated with CD4+ TEM and CD8+ TEFF cells in the present study. As such, we believe that the observed differences in memory subset distribution are due to differences in age between our study groups.

8.1.3 HESN have elevated regulatory T cells

Due to the capacity of Tregs to suppress cellular activation, we hypothesized that these cells would play a role in maintaining the reduced cellular activation observed in HESN. Consistent with our hypothesis, Tregs were elevated in HESN relative to HIV-N controls (Figure 8). In addition, Treg frequency correlated inversely with several indicators of T cell activation. Specifically, negative correlations were found between Treg frequency and the frequency of CD4+ CD38+ T cells and the expression density (i.e. MFI) of CD38 on CD4+ and CD8+ T cells, CD69 on CD4+ T cells and CD95 on CD8+ T cells (Table 10).

Tregs suppress T cell activation and responses through multiple mechanisms, both indirectly, through production of anti-inflammatory cytokines [70, 73, 74] or interactions

with DC [71], and directly, though direct contact with CD4+ and CD8+ T cells [72, 75, 76]. As such, elevated levels of Tregs in HESN may protect against infection by limiting activation of conventional CD4+ T cells. Tregs may also protect against infection by additional mechanisms. For example, Tregs can directly suppress HIV infection through a cAMP-dependent mechanism [213, 386]. HIV replication is negatively regulated by cAMP due to inhibition of HIV DNA nuclear import, integration and transcription [387, 388], and blockage of HIV long terminal repeat (LTR) activation by NF-κB [389].

Paradoxically, Tregs can also be infected with HIV [199, 205, 206], suggesting that elevated Tregs may actually provide additional target cells for HIV infection. Although X4-tropic viruses may preferentially target Tregs, Tregs are less susceptible to infection with R5-tropic HIV strains than conventional CD4+ T cells [206]. Since mucosal HIV transmission occurs in a CCR5-dependent manner [33], Tregs may not be a major target cell during early transmission events. Furthermore, FOXP3 has been shown to repress HIV LTR transcription in infected cells [390, 391], although one study presented the disparate finding that FOXP3 enhanced HIV LTR activity [392].

8.1.3 Summary of T cell phenotyping study

In this study, the hypothesis that HESN would demonstrate lower levels of T cell activation and higher levels of regulatory T cells than HIV-N controls was confirmed. These findings also support the central hypothesis of this thesis that immune quiescence protects against HIV infection and disease progression by limiting the pool of activated

target CD4+ T cells susceptible to HIV infection, and are consistent with other studies linking resistance to HIV infection with reduced immune activation [272, 336, 339-342].

8.1.4 Limitations and opportunities

A potential limitation of this study is the lack of a definitive Treg-specific biomarker that can be used to identify Tregs accurately, without contamination from the activated CD4+ T cell pool. To date, FOXP3 is the most accurate marker for identification of Tregs, and is commonly used to identify Tregs in the literature. The Treg staining carried out here was performed directly *ex vivo* and PBMC were not stimulated prior to staining. Therefore, we expected the presence of activated T cells in our Treg populations to be low. Furthermore, to address this issue, we confirmed that CD3+ CD4+ CD25+ FOXP3+ cells also express low levels of CD127 and primarily display a memory phenotype, consistent with known properties of Tregs. We further demonstrated the suppressive capacity of these cells in Chapter 4, as will be discussed in section 8.2.

8.2 Regulatory T Cell Function

The finding of elevated Tregs in HESN CSW led us to investigate the suppressive capacity of these cells. Previous studies have identified HIV-specific T cell responses in HESN CSW from the Pumwani cohort but responses are often absent or of low magnitude in HESN [274-285]. As such, we sought to compare Treg function between HIV-N, HESN and HIV+ CSW, and to determine whether Treg depletion could augment weak HIV-specific T cell responses or reveal new responses in HESN. The hypotheses for this section were:

1. HIV-N, HESN and HIV+ will demonstrate comparable increases in T cell responses to CD3/CD28 stimulation following Treg depletion.
2. Depletion of Tregs from HESN and HIV+ CSW will boost pre-existing HIV-specific responses or reveal previously undetectable responses.
3. Amplification of T cell responses following Treg depletion will be strongest in individuals with highest Treg frequencies.

8.2.1 Treg depletion boosts T cell responses to stimulation through CD3/CD28

To compare Treg suppressive capacity between groups, beads coated with anti-CD3 and anti-CD28 were used to stimulate T cells in a non-antigen-specific manner. In accordance with the hypothesis that Treg depletion would augment T cell responses to stimulation with CD3/CD28 beads regardless of HIV status, elevated T cell proliferation (Figure 11A, B) and cytokine production (Figure 14) were observed in the majority of HIV-N, HESN and HIV+ individuals following Treg depletion. However, the degree to which responses were boosted varied widely between individuals. In addition, the effect varied between readouts, such that Treg depletion often boosted T cell proliferation or cytokine production, but not necessarily both. These results are consistent with previous observations of heterogeneity among individuals with respect to Treg suppressive capacity and sensitivity of effector T cells to Treg-mediated suppression of CD3/CD28 responses [211, 228].

8.2.2 Treg depletion boosts and exposes HIV-specific T cell responses

Treg depletion boosted the magnitude of HIV-specific CD4+ and CD8+ T cell proliferation, independent of HIV status (Figure 11C, D). In addition, Treg depletion revealed new HIV-specific CD4+ and CD8+ T cell proliferation responses, but this effect was most evident in the CD8+ T cell compartment, in which proliferation responses were revealed in six individuals with previously undetectable levels of HIV-specific CD8+ T cell proliferation (Figure 12).

Treg depletion had similar effects on cytokine production by HIV-specific T cells as were observed for proliferation. Production of IFNγ, IL-2 and TNFα tended to increase following Treg depletion, but the majority of responses were below the threshold for a positive HIV-specific response (Figure 15). Among the three cytokines measured, IL-2 displayed the largest increase in positive HIV-specific responses following Treg depletion (Figure 16). This preferential increase in IL-2 production was reflected in the results of the cytokine polyfunctionality analysis, which demonstrated that HIV-specific cytokine responses tended to be skewed toward an IL-2+, TNFα+ or IL-2+/TNFα+ phenotype following Treg depletion (Figure 17). In contrast, HIV-specific IFNγ responses were weak or absent in most individuals. These data are consistent with a recent screen of HIV-specific responses in HIV+ women of the Pumwani cohort, which found that the majority of HIV-specific CD8+ T cell responses are IFNγ- [353]. Despite the limited utility of this readout and the fact that HIV-specific IFNγ T cell responses do not correlate with protection against disease progression [393, 394], IFNγ is often used as a proxy measure

of HIV-specific T cell function in immunogenicity studies of HIV vaccine candidates [395]. Future evaluation of vaccine candidates should include multiple readouts of HIV-specific cytokine production and T cell proliferation in vaccinees.

Treg depletion has previously been shown to increase effector T cell responses in HIV-infected patients [190, 202, 216, 225-227]. Specifically, *in vitro* depletion of Tregs enhanced T cell proliferation [190, 216, 226, 227] and production of IFNγ [202, 207, 225-227], IL-2 [226] or TNFα [225, 226] in response to stimulation with HIV antigens, consistent with our data. HIV-specific T cells are important for control of HIV replication. As such, the suppressive effect of Tregs on HIV-specific responses may lead to diminished HIV-specific T cell responses and viral persistence. However, HIV-controllers are capable of mounting robust HIV-specific T cell responses in the presence of normal levels of Tregs [208], but loss of viral control occurs when Tregs are expanded at sites of viral replication [196].

In contrast to studies of Tregs in HIV-infected individuals, only one previous report observed boosted HIV-specific T cell responses following Treg depletion in individuals who were HIV uninfected but had developed HIV-specific T cell responses through prior exposure to HIV. Specifically, that study demonstrated that depletion of Tregs In HIV-exposed uninfected neonates resulted in amplified cytokine production in response to HIV antigen stimulation [320].

The observation that the effects of Treg depletion vary widely between individuals, such that certain effector functions were not boosted at all, is consistent with other studies that have described large variations in Treg suppressive capacity between individuals [226, 227]. One recent report noted HIV-specific CD8+ T cell proliferation responses restricted by protective HLA alleles were resistant to Treg suppression, although Tregs effectively suppressed production of IFNγ by the same cells [149]. In contrast, HIV-specific CD8+ T cells restricted by non-protective HLA alleles in the same individuals were sensitive to Treg-mediated suppression, suggesting that the differences in sensitivity to Treg suppression are attributable to properties of the effector CD8+ T cells, rather than the Tregs themselves. Elevated expression of the inhibitory molecule Tim-3 on the surface of 'non-protective' CD8+ effector T cells rendered the cells sensitive to Treg suppression through ligation of Tim-3 by Galectin-9 expressed by Tregs [149]. Another study described boosting of CMV-specific, but not HIV-specific, T cell responses in HIV-infected individuals [204], providing additional evidence for differential effector T cell sensitivity to Treg mediated suppression.

The observation that Treg depletion tended to preferentially boost HIV-specific IL-2+ and dual-functional IL2+/TNFα+ responses could have implications for vaccine design or administration, as approaches for inducing high-quality responses are currently being investigated [85]. High-quality responses are defined by the effector functions of distinct populations of HIV-specific T cells that are associated with favourable clinical status. For example, HIV-specific CD4+ [396] and CD8+ [126] T cells that produce multiple cytokines

(i.e. polyfunctional cells) are characteristic of responses in HIV-controllers and long-term non-progressors. In addition, HIV-specific CD4+ TCM cells that proliferate and produce IL-2 are characteristic of protective long-lived memory responses [397], and tend to be lost early in infection in viremic patients [398]. Previous studies have identified a wide variety of HIV-specific effector functions that are moderated by Tregs [190, 202, 207, 216, 225-227], but the tendency of Treg depletion to preferentially boost particular types of HIV-specific responses has been largely unexplored. However, one study found that Treg depletion increased the polyfunctional HIV-specific T cell response to a therapeutic vaccine administered to HIV-infected volunteers [399]. The prospect of boosting favourable responses through Treg modulation warrants further investigation.

8.2.3 Frequency and phenotype of ex vivo Tregs have limited predictive value of suppressive capacity

The final hypothesis for this section was that amplification of T cell responses following Treg depletion would be strongest in individuals with the highest Treg frequencies. Consistent with this hypothesis, the increase in CD3/CD28-induced TNFα responses resulting from Treg depletion correlated with *ex vivo* Treg frequency (Table 4.2). However, no other readout demonstrated a comparable correlation. Interestingly, the frequency of CTLA-4+ Tregs correlated with the magnitude of the change in CD4+ IL-2 responses to CD3/CD28 (Table 4.2), suggesting CTLA-4 may be involved in Treg-mediated suppression of IL-2 production. CTLA-4 can mediate Treg suppression in several ways. CTLA-4 and CD28, an important costimulatory receptor expressed by

effector T cells, both bind CD80 and CD86 expressed on APC. CTLA-4 has higher affinity for both ligands, so it outcompetes CD28 for binding, preventing the costimulatory signal required by effector T cells for activation [400, 401]. Binding of CTLA-4 to CD80/86 can induce DCs to produce IDO, which has anti-inflammatory effects [73, 74]. CD80 and CD86 are also expressed on activated CD4+ T cells, so CTLA-4+ Tregs can suppress target cells through direct cell contact, leading to effector T cell suppression [72].

8.2.4 Summary of regulatory T cell function study

The data presented in this study of Treg function in HIV-N, HESN and HIV+ CSW demonstrate that there is no difference between groups in the outcome of Treg depletion when cells are stimulated with non-specific ligation of CD3 and CD28, consistent with the hypothesis that Treg depletion would augment T cell responses, regardless of HIV status. We further confirmed the hypothesis that depletion of Tregs from HESN and HIV+ CSW would boost pre-existing HIV-specific responses or reveal previously undetectable responses. In addition, Treg depletion revealed responses in the HIV-N group, despite their reduced exposure to HIV.

We extended our analyses to explore potential correlates of Treg suppressive capacity in HIV-uninfected and infected women. Contrary to our hypothesis that amplification of T cell responses would be strongest in individuals with highest Treg frequencies, *ex vivo* Treg frequency did not reliably predict the magnitude of the change in response

resulting from Treg depletion. However, there was some evidence that high frequencies of Tregs or CTLA4+ Tregs were associated with suppressive capacity.

8.2.5 Limitations and opportunities

Some interesting trends were identified in this study, but the analyses were limited by low sample numbers. Future studies should expand on these studies by evaluating Treg suppression in more participants and examining additional Treg functional molecules to clarify the relationships between Tregs and T cell responses in HESN.

An unexpected observation was the detection of HIV-specific T cell responses in some HIV-N individuals. Although these individuals are relatively new participants of the Pumwani cohort, the majority of them have been active in sex work for several years (median 7.5). As such, they have likely been exposed to HIV and developed T cell responses in a similar manner to the HESN women. Therefore, future studies should also include low-risk HIV-uninfected individuals as controls for investigating the effects of Treg depletion on HIV-specific responses.

In addition to the limitations associated with participant enrolment into the study, these experiments faced some technical challenges. To definitively demonstrate the suppressive effects of Tregs on effector T cell responses, we attempted to add back the Treg population to Treg-depleted cultures. However, due to limited sample material, we were unable to collect sufficient numbers of pure Tregs from the cell separations.

8.3 Immune Quiescence in the Female Genital Tract Mucosa

The evidence for immune quiescence in peripheral blood from HESN led us to investigate inflammation, T cell activation and Tregs at the FGT, which is the primary site of HIV exposure for the study participants. To this end, we compared the levels of cytokines and chemokines and T cell phenotypes in the FGT mucosa between HIV-N and HESN CSW from the Pumwani cohort. The hypotheses for this section were:

4. Proinflammatory cytokines and chemokines will be reduced in CVL samples from HESN relative to HIV-N.
5. HESN will have lower T cell activation and higher Tregs in CMC compared to HIV-N.
6. Frequencies of activated T cells and Tregs will be elevated in CMC compared to peripheral blood.

8.3.1 HESN express reduced levels of inflammatory cytokines in the FGT

A panel of cytokines and chemokines were assayed in CVL samples to evaluate the inflammatory microenvironment in the FGT of HESN and HIV-N women. Consistent with the hypothesis, IL-1α and IL-8 were found at lower concentrations in HESN women (Figure 18A). In addition, fewer HESN women had detectable levels of IL-1α than HIV-N women (Figure 18B). These findings differ from a recent study that did not find any difference between study groups in mucosal levels of IL-1α or IL-8 in CVL [338]. However, a larger study confirmed our observation of reduced IL-1α and IL-8 in HESN CVL [365].

IL-1α is a pro-inflammatory cytokine that targets multiple cell types. In its secreted form, it is involved in recruitment of cells such as neutrophils [402], which are key players in the acute inflammatory response [45]. Binding of IL-1α/β to IL-1R on target cells leads to activation of NF-κB, which promotes cellular activation and has a wide variety of downstream pro-inflammatory consequences [403]. In addition to its pro-inflammatory effects, IL-1α can stimulate HIV gene expression, leading to increased viral replication [404, 405]. Accordingly, high plasma levels of IL-1α during acute HIV infection are associated with rapid CD4+ T cell loss and rapid disease progression [406].

IL-8/CXCL8 is a proinflammatory chemokine, which primarily attracts neutrophils resulting in establishment of an inflammatory environment [407]. IL-8 is upregulated during HIV infection and is associated with viral shedding [408]. Moreover, IL-8 can directly stimulate HIV replication [409] and has been associated with increased cellular susceptibility to HIV infection in cervical explant models [410].

The varying effects of microbicide candidates on cytokine and chemokine expression in mucosal tissue exemplify how mucosal inflammation can affect HIV susceptibility. Nonoxyl-9 is a non-ionic surfactant that, despite demonstrating anti-HIV activity *in vitro*, led to increases in HIV transmission when tested as a microbicide candidate in a phase 3 clinical trial [411]. Subsequent mechanistic studies demonstrated that nonoxyl-9 induced release of IL-1α/β from cells in the FGT. IL-1α/β expression led to activation of

NF-κB and initiation of a proinflammatory cascade, which included production of IL-8 [412]. In contrast, the use of glycerol monolaurate (GML), an anti-inflammatory compound, has been shown to prevent SIV infection when used as a microbicide [335]. The protective effects of GML have been linked with reduced infiltration of activated target cells due to reduced production of proinflammatory chemokines, including MIP-3α and IL-8. In addition, production of IL-8 in control animals was associated with epithelial thinning and disruption, which could further promote HIV infection [335].

Taken together, these observations suggest that low levels of IL-1α and IL-8 in the FGT of HESN may play a protective role by reducing mucosal infiltration of activated target cells and substrates for HIV replication.

In contrast to our findings, uninfected partners of discordant couples have previously been shown to produce elevated levels of IL-1α and IL-8 (and other cytokines) upon stimulation with TLR ligands [261]. However, our study differs from the previous report in two ways. First, the previous study did not report levels of IL-1α or IL-8 at baseline, i.e. before stimulation with TLR ligands. Second, in the previous study, the evaluation of cytokine production was conducted on cells isolated from peripheral blood, which can be expected to differ from mucosal samples. As such, the results from the previous study and our own work are not directly comparable.

8.3.2 Evidence of differential T cell phenotypes in CMC of HESN and HIV-N

T cell phenotypes were evaluated in CMC of HESN and HIV-N to test the hypothesis that HESN would have lower T cell activation and higher Tregs in CMC compared to HIV-N. Contrary to this hypothesis, HESN and HIV-N tended to have comparable levels of CD4+ and CD8+ T cell activation, as indicated by expression of CD69, CD38 and HLA DR (Figures 20 and 21). However, CD4+ CCR5+ T cells were lower in HESN than HIV-N, although the observed difference was not statistically significant (Figure 28).

CCR5 is expressed on activated T cells and is also the HIV coreceptor for R5-tropic viruses, which almost exclusively mediate HIV transmissions [32], regardless of the route of transmission [413]. The pivotal role of CCR5 in susceptibility to HIV infection is illustrated by the CCR5Δ32 polymorphism, which results in the generation of a non-functional receptor that does not permit HIV to enter the cell and confers protection against infection [34-36]. Moreover, inhibition of CCR5 with a modified version of RANTES results in protection of macaques from SHIV challenge [414]. A recent study from the Pumwani cohort described a subset of cervical T_H17 cells that coexpressed multiple markers of HIV susceptibility, including CCR5, $\alpha4\beta7$, CD69 and IFNγ. This CD4+ T cell subset preferentially bound HIV gp120 *in vitro*, and was almost completely depleted in CMC samples from HIV-infected women [105]. Reduced frequencies of CCR5+ CD4+ T cells in CMC from HESN may therefore be associated with a reduction in this highly HIV-susceptible subset. Thus, although our sample size was small, the observed reduction in CD4+ CCR5+ cells in CMC of HESN warrants further investigation.

Levels of Tregs in CMC did not differ significantly between groups (Figure 22A). However, the median FOXP3+ CD4+ cell frequencies were higher in HESN than HIV-N (6.7% and 3.1%, respectively). In addition, HESN had more CD103+ CD4+ FOXP3+ CD25+ T cells than HIV-N (Figure 22C). CD103 ($\alpha E\beta 7$ integrin) is a mucosal homing molecule that binds to E-cadherin thereby mediating mucosal trafficking and retention [364]. Expression of CD103 by activated Tregs has also been associated with elevated suppressive activity and protection from colitis in mice [415]. Elevated expression of this marker on Tregs could thus signify enhanced homing of activated Tregs to the FGT in HESN.

Memory subset distribution of CD4+ FOXP3+ CD25- T cells was also found to differ between HIV-N and HESN, such that HESN had fewer T_{EM} and more T_{CM} CD4+ FOXP3+ CD25- cells than HIV-N (Figure 27). The functional relevance of this observation is not known, as T_{CM} and T_{EM} Tregs have been shown to have comparable suppressive capacity [416]. The major phenotypic difference between these subsets is differential expression of CCR7, which is present on T_{CM} but absent on T_{EM}. CCR7 is involved in homing to lymph nodes, so expression of this molecule on CD4+ FOXP3+ CD25- cells in HESN may suggest elevated recirculation of this subset [417]. However, seminal fluid has also been shown to upregulate expression of the CCR7 ligand CCL19 (MIP3β) in the FGT of mice, which was associated with Treg accumulation [418]. Although this association has not yet been demonstrated in humans, increased Treg accumulation due

to exposure to seminal fluid has clear relevance in a CSW population. The impact of sex work on expression of these key chemotactic mediators should be considered in future studies.

8.3.3 CMC are enriched for activated T cells and Tregs

Collection of matched PBMC from study participants facilitated comparison of T cell phenotypes between systemic and mucosal compartments. We hypothesized that frequencies of activated T cells and Tregs would be elevated in CMC compared to PBMC samples.

Consistent with the hypothesis, several subsets of activated CD4+ and CD8+ T cells were elevated in CMC relative to PBMC. In particular, CD4+ cell subsets expressing CD69 and HLA DR were enriched in CMC, but CD38+ CD4+ T cells did not differ between the two compartments (Figure 22). CD38+ cells constituted the majority of activated CD4+ T cells in PBMC, such that the two compartments did not differ in the size of the total activated CD4+ T cell pool, despite the enrichment of other activated T cell subsets in CMC. However, high levels of CD38+ CD4+ cells in PBMC may be due to high numbers of CD4+ T_{NAIVE} cells [92], which were elevated in PBMC (Figure 27). Markers of T cell memory were assessed on a separate panel from T cell activation markers, so CD38 could not be assessed on individual memory subsets to confirm this hypothesis. In addition to conventional markers of T cell activation, a trend toward elevated CD4+ CCR5+ T cells in CMC relative to PBMC was observed (Figure 29), indicating trafficking of HIV target cells

to the mucosa. The total pool of activated CD8+ T cells was significantly elevated in CMC relative to PBMC. This enrichment of activated CD8+ T cells in the mucosa was marked by elevated CD69+ and HLA DR+ subsets (Figure 23). Our data are consistent with the results of two recent reports of higher levels of activated T cells in CMC than PBMC [105, 419].

Comparisons of Treg subsets between CMC and PBMC confirmed our hypothesis that Tregs would be enriched in mucosal samples. CD4+ FOXP3+ cells were elevated in CMC, but expression of CD25 on these cells was lower (Figure 26, B), in line with previous reports [200, 363]. The homing of CD4+ FOXP3+ cells may be due to expression of CD103, which was also elevated in CMC samples (Figure 26C). A greater frequency of bulk CD4+ T cells expressing CTLA-4 was also observed in CMC compared to PBMC (Figure 26C), which may reflect the elevated FOXP3+ population, which tended to express CTLA-4. A similar trend was observed in the CD4+ FOXP3+ CD25+ T cell subset, suggesting elevated suppressive capacity of mucosal-resident Tregs due to the suppressive activity of CTLA-4 [71, 73, 74, 420].

8.3.4 Summary of mucosal immunology study

In this study, the hypothesis that proinflammatory cytokines and chemokines would be reduced in CVL from HESN relative to HIV-N was confirmed. Low levels of IL-1α and IL-8 in CVL from HESN marked this reduction in proinflammatory mediators. Reduced

expression of proinflammatory mediators may prevent infiltration of activated target cells to the site of HIV exposure, reducing the risk of infection.

We were unable to confirm the hypothesis that HESN would have reduced T cell activation and elevated Tregs in CMC compared to HIV-N. The observed trends toward reduced CD4+ CCR5+ and elevated CD4+ FOXP3+ cells in CMC from HESN favour this hypothesis, and suggest that further characterization of T cell phenotypes in CMC of HESN is important.

Finally, we confirmed the hypothesis that frequencies of activated T cells and Tregs would be elevated in CMC compared to PBMC samples. However, high levels of CD4+ CD38+ cells in PBMC confounded our analysis. Future studies should include markers of T cell memory in panels with CD38 to account for CD38 expression by T_{NAIVE} cells.

8.3.5 Limitations and opportunities

Our analysis of cytokines and chemokines in CVL would have been strengthened by comparison of matched plasma samples from study participants. While we were able to detect differences between HESN and HIV-N in expression of IL-1α and IL-8 in CVL, a more complete picture of the inflammatory milieu would involve evaluation of the gradients of cytokine and chemokine expression between the mucosal and peripheral compartments [421].

Low sample numbers limited the evaluation of T cell phenotypes in CMC, such that we were not sufficiently powered to detect differences between groups. This was due to the challenges of working with CMC specimens, which are problematic for analysis by flow cytometry due to low lymphocyte yields and interference by cervical mucus. However, understanding T cell biology at the site of HIV exposure in HESN could provide valuable information on the correlates of protection. This is emphasized by the preliminary data presented here, which demonstrate some differences between HIV-N and HESN, even with low sample numbers. Protocols for analyzing CMC specimens are continually evolving and improving, so future studies should continue to examine T cell phenotypes in CMC samples from HESN.

Our analysis of possible confounding variables was limited by the clinical information available on the samples. A more complete analysis of possible confounders would include additional information on infections, such as HSV2 status, and information about contraceptive usage.

8.4 Immune Quiescence and Cellular Susceptibility to Infection in vitro

HIV preferentially establishes productive infection in activated T cells, which readily support viral replication due to the availability of host substrates required by the virus [102, 331-334]. Due to the reduced levels of T cell activation observed in HESN in the Pumwani CSW cohort, we reasoned that unstimulated PBMC from HESN women would not support HIV replication as readily as those from HIV-N, which contain greater

numbers of activated T cells. As such, we sought to compare the infectability of unstimulated PBMC from HESN and HIV-N from the Pumwani CSW cohort. We further addressed the role of target cell activation on susceptibility to infection. The hypotheses for this section were:

1. Unstimulated PBMC from HESN will demonstrate reduced susceptibility to *in vitro* infection relative to HIV-N, but stimulation of PBMC with PHA prior to infection will abrogate differences between groups.
2. Elevated levels of T cell activation *ex vivo* will be associated with increased susceptibility to *in vitro* infection of unstimulated PBMC.
3. Activated CD4+ T cells will be preferentially infected by HIV.

8.4.1 Unstimulated PBMC from HESN demonstrate reduced susceptibility to HIV infection in vitro

Unstimulated and PHA-stimulated PBMC from HESN and HIV-N were infected with a primary HIV isolate, HIV_{ML1956}. Virus levels were quantified in supernatants and percentages of HIV p24+ CD4+ T cells were determined by flow cytometry.

Infection frequency, which is the percentage of replicate wells that demonstrated productive infection, was compared between HESN and HIV-N. Consistent with the hypothesis, unstimulated PBMC from HESN demonstrated lower infection frequencies than HIV-N on day 9 post-inoculation (Figure 30A). In accordance with this finding, fewer HIV p24+ CD4+ T cells were observed in HESN compared to HIV-N (Figure 33). Reduced

susceptibility to infection was only evident at low MOIs, suggesting that inoculation of cells with high viral titres overcomes barriers to infection. On day 16 post-inoculation, no differences in infection frequency were detectable between groups (Figure 30A). Since cells were only inoculated once with virus (on day 0), this suggests that cells from HESN were infected upon inoculation, but establishment of productive viral replication was delayed. Indeed, resting T cells can be infected with HIV, but produce minimal amounts of virus [102, 375-377, 381, 422]. The increase in productive viral replication may have occurred due to increases in activated target cells resulting from HIV-driven activation of resting T cells [423, 424] or increases in cellular activation or coreceptor expression resulting from prolonged cell culture. The delayed kinetics of viral replication may have implications for susceptibility to HIV infection *in vivo*. If low numbers of resting cells are infected in the FGT of HESN upon exposure to HIV, but the infected cells do not immediately produce virus, a window of opportunity may exist in which innate or adaptive immune mechanisms can clear the low level infection before dissemination occurs.

PHA stimulation of PBMC prior to inoculation with virus enhanced cellular infectivity in both HESN and HIV-N. However, contrary to the hypothesis that stimulation of PBMC with PHA prior to infection will abrogate differences between groups, PHA-stimulated PBMC from HESN demonstrated lower infection frequencies than HIV-N on day 6 post-inoculation (Figure 30B). These data suggest that PHA stimulation does not consistently rescue productive infection of PBMC at early time points. This could be due to varying

effects of PHA on T cell activation between individuals or the presence of additional factors that limit HIV infection, such as β-chemokines [254] or HIV restriction factors [425-427]. Indeed, resistance of PHA-stimulated PBMC from some HESN to infection has been linked to increased β-chemokine production [428].

In order to determine whether infected cells from HESN produce reduced levels of virus, HIV p24 produced by infected cultures was quantified in supernatants. The amount of HIV p24 produced by productively infected cells was comparable between groups (Figure 31), indicating that once infected, cells from HESN can support HIV replication as readily as cells from HIV-N.

These results are consistent with a previous study that demonstrated reduced *in vitro* HIV infectability of unstimulated cells from HESN partners of HIV-infected individuals [342]. In contrast, several studies have demonstrated comparable infectability of PHA-stimulated cells from HESN and control groups [266, 339, 429, 430]. However, examples of reduced cellular susceptibility to infection in PHA-activated PBMC from HESN have also been reported [428, 431-434]. The mechanisms controlling infectability of PHA-activated cells varied between individuals, and included CD8+ T cell suppression of infection [432], β-chemokine activity [428, 433] and reduced CCR5 expression resulting from CCR5 polymorphisms [433], further emphasizing the importance of CCR5 in infection susceptibility.

The finding that PHA-stimulated cells from HESN had lower infection on day 6 post-inoculation is in contrast to a previous evaluation of HIV infectability of PHA-stimulated cells from HESN conducted in the Pumwani cohort [266]. However, the previous study quantified levels of HIV p24 produced in culture, but did not evaluate infection frequency. We demonstrated that the amount of HIV p24 produced by productively infected cells was comparable between groups, consistent with the previous study [266]. In addition, different study participants and HIV isolates were included in the two studies, so the results are not directly comparable.

8.4.2 Immune quiescence is associated with resistance to HIV infection in vitro

T cell phenotypes were analyzed in unstimulated PBMC prior to inoculation with virus to address the hypothesis that elevated levels of T cell activation *ex vivo* would be associated with increased susceptibility to *in vitro* infection. Consistent with this hypothesis, individuals with detectable levels of HIV replication by day 9 post-infection had higher levels of CD4+ CD69+ T cells and lower levels of Tregs *ex vivo* than those with no detectable infection by day 9 (Figure 32). In line with these observations, lower levels of HLA DR+ and CCR5+ CD4+ T cells in HESN partners of HIV-infected individuals were previously associated with reduced susceptibility of unstimulated cells to *in vitro* HIV infection [342]. In contrast, a separate study suggested that reduced cellular susceptibility to infection was associated with elevated expression of markers of immune activation, including CD69 [431]. However, in that study, cells were stimulated before infection, and immune activation was only measured after stimulation, rather

than at baseline. As such, immune activation actually represents responsiveness to stimulation in that study, preventing any direct comparisons of our results.

We previously described reduced frequencies of CD4+ CD69+ T cells and elevated Tregs in HESN compared to HIV-N, as was presented in Chapter 3 and our published data [435]. The associations between these T cell phenotypes and reduced susceptibility to HIV infection *in vitro* provide further support for immune quiescence as a mechanism of protection in HESN.

8.4.3 Activated CD4+ T cells are enriched in HIV p24+ cell population

Finally, the phenotypes of *in vitro* infected CD4+ T cells were assessed by flow cytometry. We hypothesized that activated CD4+ T cells would be preferentially infected by HIV. Consistent with this, the HIV p24+ CD4+ T cell population contained higher levels of activated T cells than the HIV p24- CD4+ T cell population (Figure 34A). Despite this enrichment of activated cells within the infected CD4+ T cell population, a large proportion of infected cells did not appear to be activated, based on expression of CD69 or HLA DR; a more comprehensive panel aimed at assessing T cell activation, including markers such as CD38, CD95, $\alpha 4\beta 7$, Ki67 and CCR5, could be utilized in the future to further address this finding. Furthermore, although activated cells are more readily infected, resting cells have also been shown to produce virus, albeit at lower levels than their activated counterparts [102, 331, 436]. An evaluation of gene expression in so-called 'resting' cells that support viral replication revealed that they express genes

involved in transcription, RNA processing and vesicular transport, which may provide sufficient metabolic energy for viral replication, despite the fact that these cells do not express classical activation markers [423]. These data indicate that evaluation of activation markers alone may not be sufficient to detect subtle differences in cellular activation that affect the capacity to sustain viral replication.

Tregs were also enriched within the HIV p24+ CD4+ T cell population (Figure 34C). Indeed, Tregs can be infected with HIV [199, 205, 206]. The Treg population may also include activated T cells due to the phenotyping strategy. Due to complications of co-staining HIV p24 and FOXP3, Tregs were defined by high expression of CD25 and low expression of CD127.

We also compared the phenotypes of infected CD4+ T cells between high and low MOIs. A higher proportion of HIV+ p24+ CD4+ T cells had an activated phenotype Infection when cells were infected with a low MOI than when they were infected with a high MOI (Figure 34B, D). These data suggest that at low doses of virus, activated cells are preferentially targeted, but at higher virus doses, infection is also established in resting cells due to the relative abundance of these cells as targets. This is supported by previous reports on the role of target cell availability in establishment of productive infection in non-human primate models of infection [102, 331, 437].

8.4.4 Summary of cellular infectability study

The analysis of PBMC susceptibility to infection favoured the hypothesis that unstimulated PBMC from HESN would demonstrate reduced susceptibility to *in vitro* infection relative to HIV-N, consistent with a previous report [342]. This reduced susceptibility was marked by reduced infection frequencies early in infection and reduced frequencies of infected cells in HESN. However, reduced infection frequencies were also observed in PHA-stimulated cells at the early timepoint, contrary to our hypothesis that stimulation of PBMC with PHA prior to infection would abrogate differences between groups. Differences in infection frequencies were not observed later in infection, suggesting delayed establishment of viral replication in cells from HESN.

Elevated levels of T cell activation *ex vivo* were associated with increased susceptibility to *in vitro* infection, consistent with our hypothesis. Specifically, individuals without detectable levels of virus replication on day 9 post-inoculation had lower levels of CD4+ CD69+ T cells and elevated levels of Tregs than those with detectable levels of virus production on day 9.

The final hypothesis addressed in this study was that HIV would preferentially infect activated CD4+ T cells. In support of this hypothesis, infected CD4+ T cells were enriched for cells expressing the activation markers CD69 and HLA DR. Infected cells were also enriched for Tregs, although the Treg population likely contained a significant

proportion of activated cells due to the limited utility of CD25 and CD127 as definitive Treg markers.

8.4.5 Limitations and opportunities

An unexpected finding of this study was that PHA-stimulated PBMC from HESN demonstrated reduced infectability at the early timepoint. This is intriguing, as our data suggests that low infectability is a result of decreased activation, which should be abgrogated by PHA stimulation. To address this issue, evaluation of infectability of PHA-stimulated PBMC should be performed on all participants, rather than just a subset as was done in our study. Furthermore, T cell activation should be measured *ex vivo* and after PHA stimulation, to determine whether the effect of PHA stimulation on T cell activation varies between groups. In addition, the effects of stimulation should be assessed by titration of PHA, as suboptimal activation may contribute to the differences observed between groups. An evaluation of β-chemokine production and restriction factor expression would complement our analysis of T cell activation when evaluating correlates of reduced cellular susceptibility to infection.

An interesting finding was that a large proportion of infected cells appeared to be resting, based on lack of expression of activation markers. While HIV is known to infect resting cells, this may also be due to the limitations of our panel for detecting activated cells. As such, future studies should evaluate additional markers of T cell activation on infected cells. The use of more sensitive readouts for T cell activation, such as

phosphorylation of signalling proteins or expression of transcription factors may also prove useful in discriminating between cells that only appear to be resting and those that are truly quiescent. The question of relative Treg infectability is also an important unresolved issue. Future studies should focus on expanding Treg panels and optimizing co-staining of FOXP3 and HIV p24 to address this matter.

Finally, our use of HIV_{ML1956} as the infecting isolate was based on the assumption that it is the most relevant strain to what is circulating in the community. However, this strain has not been compared to circulating strains since its isolation. Future studies should expand our observations by testing infectability with other primary isolates as well as R5- and X4-tropic lab adapted strains of HIV.

8.5 Correlates of Protection in HIV-controllers

Our studies in HESN suggested that low levels of T cell activation and inflammation and elevated Treg activity are associated with protection from HIV infection. We wanted to further these analyses to examine how these parameters affected disease progression following infection. HIV-controllers (HIV+ C) represent a heterogeneous population unified by the common ability to suppress HIV replication in the absence of antiretroviral therapy, and are thus a good model of natural protection from disease progression. While multiple mechanisms for containment of viral replication have been described, no single mechanism accounts for all cases of HIV control. Plasma markers of

inflammation, T cell phenotypes and HIV-specific T cell responses were evaluated in HIV-controllers from Manitoba. The hypotheses for this section were:

1. HIV-controllers will express lower levels of pro-inflammatory cytokines and chemokines than non-controllers.
2. T cells from HIV-controllers will demonstrate lower levels of activation and elevated Tregs compared to non-controllers.
3. HIV-controllers will maintain robust HIV-specific T cell responses that are qualitatively distinct from non-controllers.

8.5.1 HIV+ C are characterized by a distinct cytokine and chemokine profile

We hypothesized that HIV+ C would express lower levels of pro-inflammatory cytokines and chemokines than non-controllers (HIV+ NC). Consistent with this hypothesis, HIV+ C were characterized by low levels of IP-10 and MCP-1. However, HIV+ C also expressed lower levels of the anti-inflammatory cytokine TGFβ and elevated levels of the pro-inflammatory chemokine MIP-1α (Figure 35).

MIP-1α is a natural ligand for the HIV coreceptor CCR5. Elevated levels of this chemokine directly block HIV infection through competitive binding of CCR5 [372], and resistance to HIV infection among HESN individuals has been associated with MIP-1α activity [294, 428]. MIP-1α secretion is reduced during HIV infection, but CD4+ T cells from LTNP maintain high expression [438], and cells from HIV-controllers have been shown to secrete high levels of MIP-1α relative to non-controllers [129, 198]. These

findings are consistent with our own observations of elevated MIP-1α in HIV+ C. In addition to blockage of HIV entry, MIP-1α activity is associated with suppression of HIV replication at post-entry steps in the viral life cycle, independent of coreceptor tropism [439, 440].

In contrast to elevated levels of MIP-1α, we observed reduced levels of IP-10 and MCP-1 in HIV+ C relative to HIV+ NC (Figure 35). IP-10 and MCP-1 attract activated T cells and macrophages to sites of inflammation [441, 442], thereby recruiting target cells for HIV replication [443]. Both IP-10 and MCP-1 have previously been observed to increase following HIV infection [421, 444-448], and IP-10 has been correlated to HAART treatment failure [447]. IP-10 has also been observed to stimulate HIV replication in monocyte-derived macrophages and PBMC [449], demonstrating an additional mechanism by which this chemokine may influence disease progression. MCP-1 also acts on CD4+ T cells, causing them to upregulate expression of CXCR4, thus rendering them more susceptible to HIV infection with X4-tropic viral variants [450]. Taken together, these findings suggest that the lower levels of proinflammatory chemokines observed in HIV+ C serve to limit infiltration and infection of activated target cells at sites of viral replication.

In contrast to the proinflammatory chemokines discussed above, TGFβ is an anti-inflammatory cytokine. We found reduced TGFβ concentrations in HIV+ C (Figure 35). This data is consistent with studies demonstrating reduced levels of TGFβ in lymphoid

tissues of LTNP [215] and higher HIV-specific CD4+ TGFβ+ responses in individuals with progressive disease [224]. Although elevated levels of TGFβ could arise from multiple cell types, Tregs may express high levels of this cytokine during HIV infection [371]. Importantly, the anti-inflammatory effects of TGFβ negatively impact the magnitude and quality of antigen-specific T cell responses. Thus, maintenance of low levels of TGFβ may be one mechanism by which HIV+ C are able to mount robust HIV-specific T cell responses resulting in viral control.

8.5.2 HIV+ C have reduced T cell activation

In addition to measuring inflammatory mediators in plasma, immune activation was evaluated at the cellular level. Consistent with the hypothesis that T cells from HIV+ C would demonstrate lower levels of T cell activation, HIV+ C had reduced frequencies of activated CD4+ and CD8+ T cells, which correlated inversely with CD4 count and positively with viral load (Figure 37). These data are in line with previous studies that found reduced T cell activation in HIV-controllers [138, 196, 212, 355, 368].

Contrary to the hypothesis that HIV+ C would have elevated Tregs, no differences were observed between the two study groups in terms of Tregs as a percentage of CD4+ T cells, which is in contrast to larger cohort studies describing a relative expansion of Tregs within the CD4+ T cell compartment in progressive disease [193, 196, 197, 208]. However, consistent with other studies [196, 197, 211], our data showed lower absolute Tregs in patients with advanced disease, with relative preservation of absolute Tregs in

HIV+ C (Figure 38). Absolute counts of Tregs inversely correlated with frequencies of activated T cells, suggesting that the loss of this suppressive cell type may contribute to the excessive immune activation that characterizes progressive disease. However, it important to note that loss of Tregs and increased immune activation may both be independently driven by other factors related to HIV replication and CD4+ T cell loss.

8.5.3 HIV+ C mount robust HIV-specific T cell proliferation responses

Lastly, we evaluated HIV-specific T cell responses in a subset of patients. Due to limited sample material, we focused our analysis on bulk gag-directed CD4+ and CD8+ T cell responses, and evaluated quality of response as indicated by dual-cytokine secretion and proliferation. We hypothesized that HIV+ C would maintain robust HIV-specific T cell responses that were qualitatively distinct from HIV+ NC.

HIV+ C were not identified by a particular cytokine expression pattern, although they tended to demonstrate IL-2 responses more often (CD4+ responses, Figure 41B) or of higher magnitude (CD8+ responses, Figure 42A) than HIV+ NC. However, there was a striking difference between study groups in terms of HIV-specific proliferation responses. Both CD4+ and CD8+ T cells from HIV+ C demonstrated higher magnitudes of HIV-specific proliferation than HIV+ NC (Figure 44B). Interestingly, when the HIV+ C population was broken down into elite controllers and viremic controllers, a linear trend was observed in which elite controllers were most likely to respond to stimulation by T cell proliferation, followed by viremic controllers and finally non-controllers (Figure

44D), suggesting that the level of viral containment is directly related to the propensity to mount an HIV-specific proliferative T cell response. These data are consistent with previous studies identifying proliferative T cell responses as an important mechanism of protection in non-progressive HIV disease [126, 349-352, 354, 451], and confirm our hypothesis that HIV-specific responses in HIV+ C would be qualitatively distinct from those in HIV+ NC.

8.5.4 Summary of HIV-controller Study

This study focused on evaluating immune activation, inflammation and T cell responses in the HIV-controller model to determine whether mechanisms of protection against infection are also protective against disease progression. The observations of reduced IP-10 and MCP-1 supported the hypothesis that HIV+ C would express lower levels of pro-inflammatory cytokines and chemokines than HIV+ NC. However, the findings of reduced TGFβ and elevated MIP-1α did not support an anti-inflammatory profile. Differential expression of these factors likely contributes to delayed disease progression by allowing robust HIV-specific T cell responses to control viral replication and by offering direct anti-HIV activity, respectively. The hypothesis that HIV+ C would demonstrate lower levels of T cell activation relative to HIV+ NC was confirmed in this study, as demonstrated by lower levels of CD38+ HLA DR+ T cells in HIV+ C. Absolute numbers of Tregs were lower in HIV+ NC, in line with the hypothesis that Tregs would be elevated in HIV+ C. However, Treg frequencies (as a percentage of CD4+ T cells) were comparable between groups, suggesting that loss of Tregs parallels the loss of other

CD4+ T cells in HIV infection. Finally, we confirmed our final hypothesis that HIV+ C would maintain robust HIV-specific T cell responses that were qualitatively distinct from HIV-NC. Specifically, HIV-specific T cell proliferation responses were directly related to the durability of viral control.

It is widely thought that control of viral replication is a consequence of robust HIV-specific T cell responses, and our data is in agreement with this view. We further propose that maintenance of low levels of immune activation contributes to viral control by reducing substrates available for HIV replication and preventing exhaustion of HIV-specific T cells.

8.5.5 Limitations and opportunities

The analyses of T cell phenotypes and HIV-specific T cell responses were performed on cryopreserved samples from MECC. Although cryopreservation can impact expression of phenotypic markers [452] and negatively impact detection of antigen-specific responses, this was done to minimize inter-experiment variation. In our hands, fresh and frozen samples demonstrated minimal differences in the phenotypic parameters included in our panels.

Although we were able to identify some protective aspects of cytokine and chemokine expression, T cell phenotypes and T cell response quality, we were limited by small sample sizes for some assays. As such, some differences between groups may have been

overlooked. This is particularly relevant for the evaluation of HIV-specific T cell cytokine and proliferation responses. Specifically, dual cytokine producing cells (IFNγ+/IL2+) were rare events in our analyses of HIV-specific responses. As such, we were not sufficiently powered to detect differences in polyfunctional T cells between groups.

8.6 Major Findings and Models of Protection

In this thesis, we investigated mechanisms of immune defence against HIV in HESN and HIV-controllers. We identified several correlates of protection, which contribute to our understanding of reduced susceptibility to HIV infection and disease progression.

8.6.1 Immune quiescence and protection against infection

A previous study from the Fowke lab showed that HESN were characterized by low baseline levels of gene expression in CD4+ T cells, suggesting a state of immune quiescence. In particular, genes involved in T cell receptor signalling and HIV replication were among those expressed at lower levels in HESN [336]. Unstimulated PBMC from HESN also produced lower levels of proinflammatory cytokines and chemokines than controls. Differences between groups were abrogated by stimulation, indicating that while baseline activation is lower in HESN, responses to antigen are normal [336]. Thus, immune quiescence is not immunosuppression. A screen of differentially expressed mucosal proteins between HESN and HIV-N identified the overexpression of anti-inflammatory serpin B anti-proteases in HESN [272, 338], providing further support for reduced baseline immune activation as a mechanism of protection.

The studies presented in this thesis further our understanding of immune quiescence in HESN. We found that HESN had reduced levels of activated T cells and elevated levels of Tregs in PBMC. Tregs are a potential driver of the immune quiescent state, as Tregs are known to suppress T cell activation. We demonstrated that Tregs suppress HIV-specific T cell responses in HESN, as was evident by enhanced responses upon depletion of Tregs. Further evidence for immune quiescence was provided by our mucosal studies, which identified lower levels of the inflammatory cytokines and chemokines IL-1α and IL-8 in CVL samples from HESN. Reduced levels of proinflammatory mediators may protect from infection by reducing infiltration of activated HIV target cells to the FGT. We linked immune quiescence with reduced susceptibility of unstimulated CD4+ T cells to *in vitro* HIV infection in HESN by demonstrating that lack of infection was associated with reduced levels of activated CD4+ T cells and elevated frequencies of Tregs.

Taken together, these data were used to frame a model of reduced susceptibility to infection (Figure 45). In susceptible individuals, HIV exposure leads to penetration of the mucosal barriers and establishment of infection in a small focus of resting and activated CD4+ target cells. Inflammation resulting from infection drives infiltration of activated target cells, which fuel propagation of infection and dissemination to lymphoid tissues. Once dissemination occurs, the infection cannot be cleared. In contrast, in HESN, innate molecules in the FGT mucosa may limit establishment of the initial focus of infection through direct anti-viral activity and maintenance of mucosal integrity [271, 272]. While

it is likely that some viral particles are able to penetrate the epithelial barriers and gain access to target cells in the submucosa, elevated levels of Tregs maintain low levels of T cell activation, leading to reduced availability of activated CD4+ target cells (Chapter 3) [336, 435]. Consequently, HIV establishes infection in resting T cells, due to the relative abundance of these targets. Propagation of infection by resting T cells is minimal, and reduced expression of proinflammatory cytokines and chemokines (Chapter 5) [365] prevents infiltration of activated target cells to fuel infection. As such, productive infection is reduced in HESN (Chapter 6), allowing innate or adaptive cells, such as NK [255-259] or HIV-specific CTL [274-285], opportunity to clear the low-level infection and prevent dissemination.

This model is supported by studies of mucosal SIV transmission in non-human primates (NHP) [103]. In acute infection of rhesus macaques, a small founder population of infected cells is established in the submucosa within the first few days following viral challenge [331, 437]. This founder population is comprised of mainly resting CD4+ T cells, as these cells outnumber activated cells [102]. Local expansion of the founder population occurs through low-level viral replication in resting cells [437]. However, the infection is fuelled by an influx of activated target cells in the inflammatory infiltrate resulting from the innate response to HIV [335]. As such, SIV exploits the inflammatory immune response to gain access to additional substrates and target cells for viral replication and dissemination of infection. Interestingly, use of the anti-inflammatory compound glycerol monolaurate blunts the inflammatory response, preventing

infiltration of activated target cells, and resulting in protection from SIV infection [335]. In the SIV model of infection, SIV-specific CD8+ T cells are detectable by the second week of infection. However, infection is well established by this time, such that the ratio of HIV-specific CD8+ T cells to infected target cells is too low to prevent further viral replication and dissemination [142]. Nonetheless, mucosal HIV-specific T cells present at the time of inoculation have been shown to be protective against SHIV challenge [143]. We propose that in HESN, mucosal HIV-specific T cells, which have been previously identified in HESN from the Pumwani cohort [278], are present early enough to clear the initial focus of infection prior to expansion and dissemination.

8.6.2 Protection against disease progression

Correlates of protection against disease progression were investigated in HIV-controllers. We found that HIV control was associated with low levels of proinflammatory chemokines, reduced frequencies of activated T cells and maintenance of absolute Treg counts, consistent with the immune quiescence model. Reduced levels of anti-inflammatory TGFβ and elevated levels of proinflammatory MIP-1α also contributed to HIV control through alternate mechanisms. Importantly, HIV control was strongly associated with robust HIV-specific T cell proliferation responses that were lost in progressive disease.

The data collected on HIV-controllers can also be built into a model of protection from disease progression in HIV-infected individuals (Figure 46). In this model, HIV-controllers

contain viral replication through robust HIV-specific proliferative T cell responses in an environment of low inflammation and T cell activation. In this context, low activation may serve to limit substrates and target cells available for HIV replication, and would further prevent exhaustion of HIV-specific T cells. Maintenance of absolute Treg counts may contribute to controlling aberrant T cell activation, reducing the pool of activated target cells available for viral propagation. Furthermore, reduced levels of IP-10 and MCP-1 may prevent recruitment of activated CD4+ target cells to sites of viral replication, while elevated levels of MIP-1α serve to directly inhibit infection of target cells by HIV. Future studies should evaluate the levels of these chemokines in mucosal tissues in HIV-controllers and non-controllers to confirm their effects on recruitment of target cells to sites of viral replication.

8.6.3 Revisiting the central hypothesis

The central hypothesis of this thesis is that immune quiescence protects against HIV infection and disease progression by limiting the pool of activated target CD4+ T cells susceptible to HIV infection.

In HESN, immune quiescence was marked by low frequencies of activated T cells, high frequencies of Tregs and low levels of proinflammatory mediators in the FGT mucosa. Infection of unstimulated cells *in vitro* demonstrated that HIV preferentially infects activated cells, and reduced T cell activation is associated with cellular resistance to infection. These data suggest a mechanism whereby immune quiescence protects

against infection by limiting the activated target CD4+ T cell pool, in support of the central hypothesis.

HIV-controllers had low levels of proinflammatory mediators, low frequencies of activated T cells and normal levels of absolute Tregs. These findings provide insight into mechanisms of viral control and also lend support to our observations of the protective effects of immune quiescence in another natural model of protection. The observed low levels of inflammation and T cell activation in HIV-controllers demonstrates that immune quiescence is protective not only prior to exposure, but also following infection with HIV, consistent with the central hypothesis. HIV-controllers also had elevated MIP-1α and reduced TGFβ, which contribute to protection by direct mechanisms other than immune quiescence. In addition, HIV-controllers demonstrated robust HIV-specific T cell proliferation responses, emphasizing the importance of T cells in control of HIV replication.

8.7 Contributions to the Field of HIV Immunology Research

The first study presented in this thesis described reduced levels of activated T cells and elevated levels of Tregs in PBMC from HESN, implicating Tregs as a potential driver of immune quiescence [435]. That study was the first to report that Tregs are associated with reduced susceptibility to HIV infection in HESN, and supported previous reports of reduced T cell activation markers in other HESN cohorts [340-342].

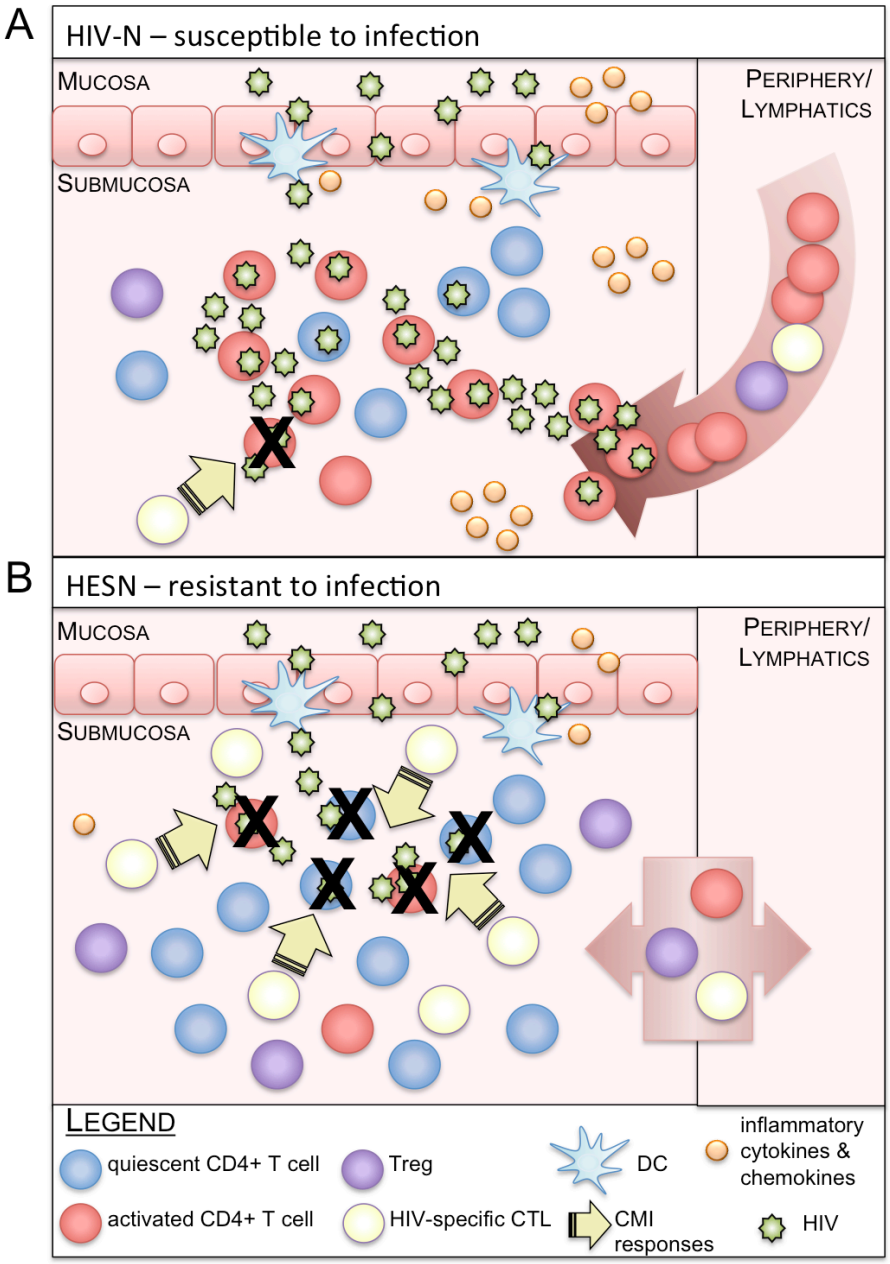


Figure 45. Model of protection from HIV infection. (A) In HIV-susceptible individuals, infection is established in resting and activated CD4+ target cells. Inflammation resulting from infection drives infiltration of activated target cells, which fuel propagation and dissemination of infection. (B) In HESN, elevated levels of Tregs maintain low levels of T cell activation, leading to reduced availability of activated CD4+ target cells. HIV establishes infection in resting T cells, which support low levels of virus replication. Reduced expression of pro-inflammatory cytokines and chemokines (e.g. IL-1α, IL-8) prevents infiltration of activated target cells to fuel infection. Productive infection is therefore reduced in HESN, allowing HIV-specific CTL sufficient opportunity to clear the low-level infection and prevent dissemination.

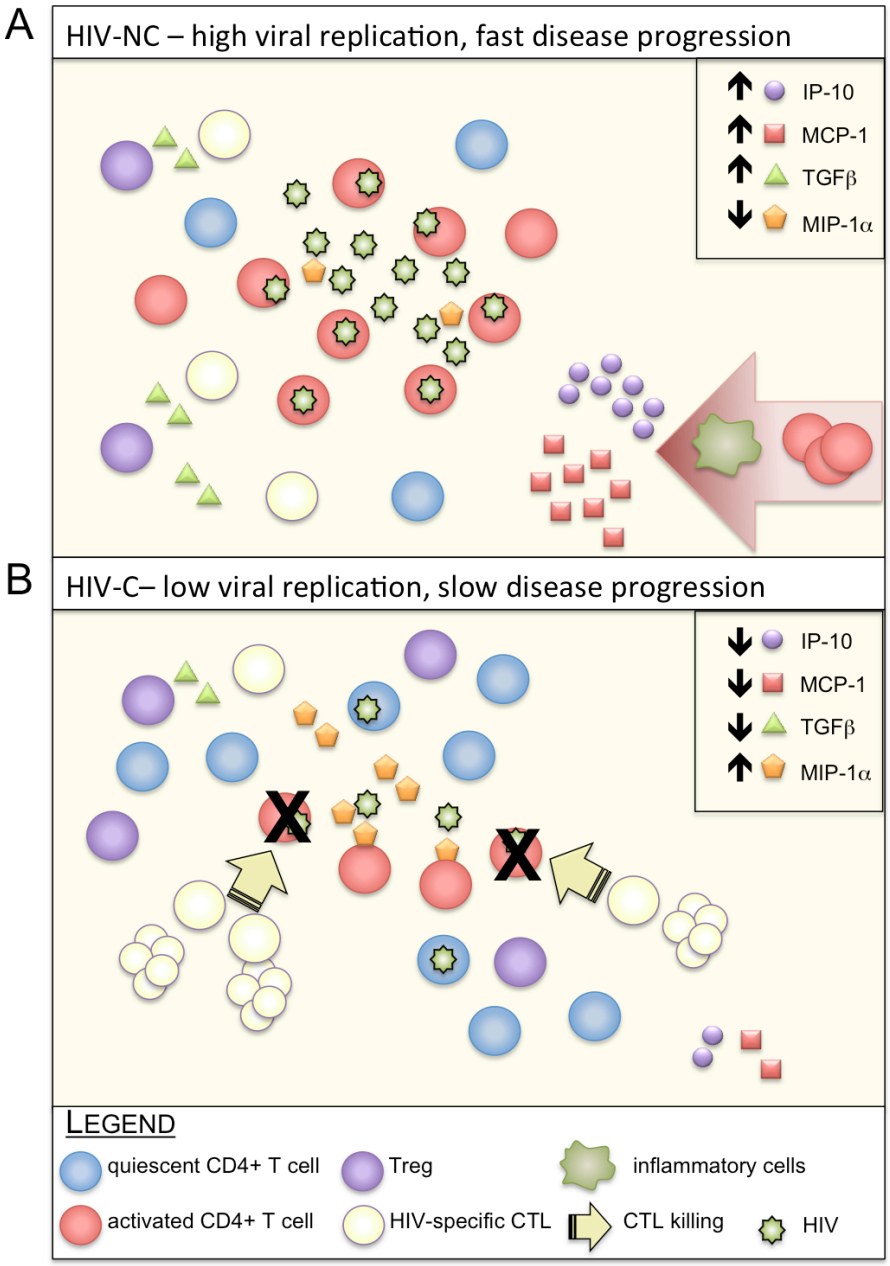


Figure 46. Model of protection from disease progression. (A) In HIV+ NC, activated CD4+ target cells fuel virus replication. HIV-specific CTL have limited functional capacity to clear infected cells. High levels of TGFβ add to this dysfunction. High levels of pro-inflammatory chemokines attract inflammatory and activated target cells to the site of viral replication, driving viral propagation and disease progression. (B) In HIV+ C, maintenance of absolute Tregs contributes to controlling T cell activation, reducing the pool of activated target cells available for viral propagation. HIV-specific CTL with high proliferative capacity contain HIV replication. Furthermore, reduced levels of chemokines (e.g. IP-10, MCP-1) prevent recruitment of activated CD4+ target cells, while elevated levels of MIP-1α serve to directly inhibit infection of target cells by HIV.

We next evaluated Treg function in HESN, and demonstrated for the first time that depletion of Tregs *in vitro* reveals uncharacterized HIV-specific T cell responses in HESN and HIV-N CSW. One previous report observed boosted HIV-specific T cell responses following Treg depletions in HIV-exposed uninfected neonates [320].

The mucosal immunology study identified lower levels of the inflammatory cytokines and chemokines IL-1α and IL-8 in CVL samples from HESN. Inflammation in the FGT in HESN has only been addressed in a few studies [265, 272, 338]. Together with another report from the Fowke lab [365], this is the first characterization of reduced IL-1α and IL-8 in CVL from HESN.

The *in vitro* infection study was the second study [342] to show that unstimulated cells from HESN had reduced susceptibility to infection, which was associated with reduced frequencies of activated CD4+ T cells. We furthered these observations to show for the first time that reduced susceptibility to infection is associated with elevated Tregs *ex vivo*. Moreover, we demonstrated that once cells from the different study groups were infected, they produced similar amounts of virus.

The HIV-controller study identified a distinct plasma cytokine and chemokine profile, reduced frequencies of activated T cells, maintenance of absolute Treg counts and robust HIV-specific T cell proliferation as correlates of HIV control [453]. The functions of the cytokines discussed have been studied in the context of HIV disease. Nonetheless

differential expression of plasma levels of those mediators was a novel finding. Our data confirmed previous studies of reduced T cell activation [138, 196, 212, 355, 368], maintenance of absolute Tregs [196, 197, 211] and robust proliferative responses [126, 349-352, 354, 451] in HIV-controllers.

8.8 Concluding Remarks and Future Directions

Based on the findings presented in this thesis, there are several future directions for this project. The observation of elevated Tregs in HESN leads us to question what is driving this phenotype. Several factors may contribute to Treg differentiation and expansion. Characterization of the cytokine and cellular microenvironment, transcription factors, and host genetics in HESN will shed light on this issue. The interactions between IRF-1 and Tregs are of particular interest, as IRF-1 negatively regulates Treg development, and the expression and responsiveness of the IRF-1 protein are reduced in HESN from the Pumwani cohort.

Further characterization of how Tregs affect the quality of responses could have implications for prophylactic or therapeutic vaccine design. The relative contributions of Treg surface proteins (e.g. Galectin-9, CTLA-4, CD39) and anti-inflammatory cytokines (IL-10, TGFβ), to suppression of immune activation by Tregs should be considered in future work.

It would also be beneficial to expand studies of immune quiescence in the genital mucosa. We observed reduced inflammatory mediators in HESN, but our data on cellular phenotypes in CMC were limited. As such, future projects should focus on characterizing cellular populations in the FGT.

The observations of reduced cellular susceptibility to infection in HESN also provide some direction for future research questions. It will be important to confirm the observed associations using other viral isolates, and examining the role of coreceptor tropism in differential susceptibility to *in vitro* infection. Elucidating the steps of the HIV replication cycle at which infection is impeded could provide valuable information for designing interventions that block infection in the early stages.

On a larger scale, an important consideration is how we can use the data we collected to inform design of interventions that prevent HIV infection. Our data suggests that inducing immune quiescence at the site of HIV exposure will reduce the number of activated target cells, thereby preventing infection or limiting it to small foci of infected resting target cells, which can be cleared by mucosal HIV-specific T cells or innate mechanisms such as NK cells. A practical method of inducing immune quiescence in the mucosa is a microbicide that incorporates anti-inflammatory mediators into the formulation. A successful microbicide may also incorporate antiretroviral compounds, as such candidates have demonstrated moderate efficacy in recent trials. The success of this intervention may depend on the presence of mucosal HIV-specific T cells at the time

of exposure, so that low-level infections can be subverted before viral propagation and dissemination. As such, development of mucosal HIV vaccines is critical.

Development of such microbicide and vaccine candidates will certainly be a challenge. We know that protection from infection is complex, as is HIV itself. It is therefore unreasonable to expect successful interventions to be simple. However, I believe that these are realistic goals that will be achieved through systematic evaluation of protective correlates of natural protection, confirmation of putative correlates in multiple cohorts and innovative approaches to integrating this knowledge into the product pipeline. Looking to the future, realization of these goals will help to relieve the burden of HIV/AIDS on the health and socio-economic wellbeing of millions of people currently affected by the epidemic, and many more in future generations.

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Chapter 10. Appendices

10.1 Abbreviations

AIDS	acquired Immunodeficiency syndrome
APC	antigen presenting cell (*cell type*)
APC	allophycocyanin (*fluorochrome*)
ART	antiretroviral therapy
ARV	antiretroviral drugs
BV	bacterial vaginosis
cAMP	cyclic adenosine 3',5' monophosphate
CD	cluster of differentiation
CFSE	carboxyfluorescein diacetate succinimidyl ester
CLR	c-type lectin receptor
CMC	cervical mononuclear cell
CRF	circulating recombinant form
CSW	commercial sex worker
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen 4
CVL	cervicovaginal lavage
DC	dendritic cell
DC-SIGN	dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
EC	elite controller
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
FGT	female genital tract
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
FOXP3	forkhead box P3
GALT	gut-associated lymphoid tissue
GI	gastrointestinal
GITR	glucocorticoid-induced TNF-receptor-related protein
GML	glycerol monolaurate
HAART	highly active antiretroviral therapy
HESN	highly-exposed seronegative
HIV	human immunodeficiency virus
HIV+ C	HIV-controller
HIV-N	HIV negative
HLA	human leukocyte antigen
ICS	intracellular cytokine staining

IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN	interferon
IL	interleukin
IL-1ra	interleukin-1 receptor agonist
IP-10	interferon gamma-induced protein 10
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
IRF-1	interferon regulatory factor 1
IU	international units
KIR	killer immunoglobulin-like receptor
LAG3	lymphocyte activation gene 3
LC	Langerhans cell
LN	lymph node
LPS	lipopolysaccharide
LTNP	long term non-progressor
LTR	long terminal repeat
MCP	monocyte chemotactic protein
MDC	macrophage-derived chemokine
mDC	myeloid dendritic cell
MECC	Manitoba Elite Controller Cohort
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MOI	multiplicity of infection
MSM	men who have sex with men
NC	non-controller
NHP	non-human primate
NK	natural killer cell
NKT	natural killer T cell
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PD-1	programmed death 1
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PE-TR	phycoerythrin Texas Red
PFA	paraformaldehyde
PHA	phytohemagglutinin
PRR	pattern recognition receptor
RANTES	regulated upon activation normal T cell expressed and secreted
RM	rhesus macaque
RNA	ribonucleic acid
SA-HRP	streptavidin horseradish peroxidase
sCD40L	soluble CD40 ligand

SHIV	simian human immunodeficiency virus
sIL-2R	soluble interleukin-2 receptor
SIV	simian immunodeficiency virus
SLPI	secretor leukocyte protease inhibitor
SM	sooty mangabey
ssRNA	single stranded ribonucleic acid
STI	sexually transmitted infection
$TCID_{50}$	50% tissue culture infective dose
T_{CM}	central memory T cell
TCR	T cell receptor
T_{EFF}	terminally-differentiated effector T cell
T_{EM}	effector memory T cell
TGF	transforming growth factor
T_H	T helper cell
Tim-3	T-cell immunoglobulin domain and mucin domain 3
TLR	Toll-like receptor
TMB	tetramethyl benzidine
TNF	tumor necrosis factor
Treg	regulatory T cell
TRIM	tripartite motif protein
VC	viremic controller

10.2 Gag peptide pool

Peptides were generated using the HIV gag sequences listed below and pooled for HIV-specific stimulations.

MGARASVLSGGKLDA
SVLSGGKLDAWEKIR
GKLDAWEKIRLR
LDAWEKIRLRPGGKK
KIRLRPGGKKKYRLK
PGGKKKYRLKHLVWA
KYRLKHLVWASREL
KHLVWASRELERFAL
ASRELERFALNPGLL
ERFALNPGLLETSEG
NPGLLETSEGCQQII
ETSEGCQQIIGQL
EGCQQIIGQLQPAL
CQQIIGQLQPAL
QIIGQLQPALQ
IIGQLQPALQTGTEEL
PALQTGTEELRSLY
TGTEELRSLYNTVA
ELRSLYNTVATLYCV
YNTVATLYCVHQKI
ATLYCVHQKIEVK
YCVHQKIEVKDTKEA
KIEVKDTKEALDKI
KDTKEALDKIEEEQNK
LDKIEEEQNKSQQK
EEEQNKSQQKTQQAA
KSQQKTQQAAADTGN
TQQAAADTGNSSQV
AADTGNSSQVSQNY
GNSSQVSQNYPIV
SQVSQNYPIVQNL
SQNYPIVQNLQGQMV
IVQNLQGQMVHQAI
LQGQMVHQAISPRTL
VHQAISPRTLNAWVK
SPRTLNAWVKVIEEK
NAWVKVIEEKAF
WVKVIEEKAFSPEVI
EEKAFSPEVIPMFSA
SPEVIPMFSALSEGA
PMFSALSEGATPQDL
LSEGATPQDLNTML
ATPQDLNTMLNTV
PQDLNTMLNTVGGH
NTMLNTVGGHQAAM
NTVGGHQAAMQMLK
GHQAAMQMLKDTI
AAMQMLKDTINEEAA
LKDTINEEAAEWDRL
NEEAAEWDRLHPVHA
EWDRLHPVHAGPI
RLHPVHAGPIPPGQM
HAGPIPPGQMREPR
IPPGQMREPRGSDIA
MREPRGSDIAGTTSTL
SDIAGTTSTLQEQI
GTTSTLQEQIGWM
STLQEQIGWMTSNPPI
SNPPIPVGEIYKRWI
PVGEIYKRWIILGL
IYKRWIILGLNKIVR
IILGLNKIVRMYSPV
NKIVRMYSPVSILDI
MYSPVSILDIRQGPK
SILDIRQGPKEPFR
IRQGPKEPFRDYVDR
KEPFRDYVDRFFKTL
DYVDRFFKTLRAEQA
FFKTLRAEQATQEVK
RAEQATQEVKNWM
EQATQEVKNWMTETL
EVKNWMTETLLVQNA
MTETLLVQNANPDCK
LVQNANPDCKTILKA
NPDCKTILKALGPGA
TILKALGPGATLEEM
LGPGATLEEMMTA
GATLEEMMTACQGV
EEMMTACQGVGGPSH
ACQGVGGPSHKARVL
GGPSHKARVLAEAM
HKARVLAEAMSQV
RVLAEAMSQVTNAAI
AMSQVTNAAIMMQR
VTNAAIMMQRGNFK
AIMMQRGNFKGQRRI
RGNFKGQRRIVKCF
RIVKCFNCGKEGHIA
FNCGKEGHIARNCRA
EGHIARNCRAPRKK
ARNCRAPRKKGCWK
RAPRKKGCWKCGK
RKKGCWKCGKEGHQM
WKCGKEGHQMKDCTER
GHQMKDCTERQANFL
DCTERQANFLGKIW
RQANFLGKIWPSNK
FLGKIWPSNKGR
GKIWPSNKGRPGNFL
SNKGRPGNFLQSR
GRPGNFLQSRPEPTA
FLQSRPEPTAPPA
SRPEPTAPPAESFGF
TAPPAESFGFGEETT
ESFGFGEETTPSPK
FGEETTPSPKQEQK
TTPSPKQEQKDK
PSPKQEQKDKEPPLA
EQKDKEPPLASLKSL
EPPLASLKSLFGNDPL
LKSLFGNDPLSQ

10.3 Publications

Publications arising from this thesis work

Card CM, Rutherford WJ, Ramdahin S, Yao XJ, Kimani M, Wachihi C, Kimani J, Ball TB, Plummer FA and Fowke KR. Reduced cellular susceptibility to *in vitro* HIV-1 infection in HIV-exposed seronegative (HESN) Kenyan sex workers is associated with T cell immune quiescence. *Submitted to AIDS.*

Card CM, Keynan Y, Lajoie J, Bell CP, Dawood M, Pindera C, Becker M, Kasper K and Fowke KR. HIV-controllers are distinguished by chemokine expression profile and HIV-specific T cell proliferative potential. J Acquir Immune Defic Syndr 2012, *in press.*

Schellenberg JJ, **Card CM**, Ball TB, Mungai JN, Irungu E, Kimani J, Jaoko W, Wachihi C, Fowke KR and Plummer FA. Bacterial vaginosis, HIV serostatus and T cell subset distribution in a cohort of East African commercial sex workers: Retrospective analysis. AIDS 2012, 26(3): 387-93.

Fowke KR, **Card CM** and Kaul R. The immune system and resisting HIV infection. In: Pancino G, Silvestri G, Fowke KR, eds. Models of Protection against HIV/SIV: Avoiding AIDS in Humans and Monkeys. Academic Press, 2011; 211-229.

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Keynan Y*, **Card CM***, McLaren PJ, Dawood MR, Kasper K and Fowke KR. The role of regulatory T cells in chronic and acute viral infections. Clin Infect Dis 2008; 46(7): 1046-52. (*=equal contribution)

Other Publications

Juno JA, Tuff J, Choi R, **Card C,** Kimani J, Wachihi C, Koesters-Kiazyk S, Ball TB, Farquhar C, Plummer FA, John-Stewart G, Luo M and Fowke KR. The Role of G Protein Gene GNB3 C825T Polymorphism in HIV-1 Acquisition, Progression and Immune Activation. Retrovirology 2012, 9(1): *epub ahead of print.*

McKinnon LR*, **Card CM*** and the CIHR International Infectious Disease and Global Health Training Program. HIV vaccine efficacy trials: a brief history, and options for going forward. AIDS Rev 2010; 12(4): 209-17. (*=equal contribution)

Keynan Y, **Card CM**, Ball TB, Li Y, Plummer FA and Fowke KR. Cellular immune responses to recurring influenza strains have limited boosting ability and limited cross-reactivity to other strains. Clin Micro Infect 2010; 16(8): 1179-86.