Investigation of Immune Quiescence: Assessing the Role of Regulatory T cells and their link with IRF-1 in HIV-Exposed Sero-Negative Individuals

By

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Abstract

Recent research of a cohort of HIV exposed sero-negative (HESN) female commercial sex workers in Nairobi has revealed an Immune Quiescent phenotype; characterized by reduced T cell activation and higher regulatory T cells (Tregs) in peripheral blood. HESN women also express lower levels of interferon regulatory factor-1 (IRF-1), a critical regulator known to negatively impact Treg development in mice. In this study, we analyzed the functional capacity of Tregs by an *in vitro* depletion assay and measured functionality by flow cytometry. Data showed Tregs suppressed CD4+ and CD8+ proliferation responses. We characterized the link between Tregs and IRF-1 in HESN and observed an inverse correlation between IRF-1 expression and Treg proportions. We also established reduced expression of IRF-1 in Tregs of healthy donors by flow cytometry. In a separate study, flow cytometric analysis of high-risk sex-workers revealed that CTLA-4 expression in memory CD4+cells, not Treg frequency, was associated with HIV seroconversion.

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Chapter 1 **Introduction**

1.1 HIV Pandemic

It has been more than three decades since the Human Immunodeficiency Virus (HIV) was described as the agent that results in Acquired Immunodeficiency Syndrome (AIDS) yet it has remained the cause of one of the major global epidemics, with over 36 million people living with HIV and 2 million newly infected people in 2014 [1]. The Sub-Saharan region of Africa contains much of the people living with HIV (25.8 million). However, in the last several years significant progress in HIV treatment, improved quality of services and targeting of key populations has resulted in the hopes of eradication of the AIDS epidemic [1].

1.2 HIV Treatment and Prevention

1.2.1 Anti-Retroviral Therapy

Anti-retroviral therapy (ART) is the most effective treatment method to reduce or delay HIV-induced mortality. It involves the combination of anti-viral drugs to slow the replication of HIV to undetectable levels and delay progression to AIDS [2]. ART is characterized by a cocktail of drug inhibitors that target HIV binding, fusion, HIV reverse-transcriptase, integrase and protease [2]. For example, the WHO recommends a first-line combination therapy of Tenofovir, Efaviren and Lamivudine to treat HIV infection. Recently, it was shown that early initiation of ART, especially in high-risk populations,

results in decreased transmission of HIV [3]. This strategy of expanding ART is now being utilized globally to curb the transmission of HIV.

1.2.2 Vaccines and Microbicides

While ART remains an effective method to combat HIV post-acquisition, more effective HIV prevention approaches are needed. HIV vaccine studies have mostly been unsuccessful [4–6], with one vaccine trial showing a modest protective effect; a vaccine efficacy of 31.2% [7]. Similarly, microbicides, which are substances that are applied vaginally or rectally that protect against bacteria and viruses, have been shown to have limited or no protective effect against HIV [8]. A vaginal gel microbicide containing Tenofovir, an anti-retroviral drug, showed a 39% reduction in HIV acquisition [9]. Vaccines and microbicide strategies will continue to be an intense area of interest in the years to come.

1.3 Natural Protection from HIV

1.3.1 Genetic and Immune Correlates of Protection

It is well understood that there are several genetic and immunological factors that influence HIV susceptibility and progression to AIDS. One of the best characterized genetic correlate of HIV protection is homozygosity for the CCR5Δ32 gene, found mostly in Caucasian populations [10]. A 32 base-pair deletion in the CCR5 gene results in the absence of CCR5 expression and the inability of HIV gp120 to fuse to the cell membrane [10].

Additionally, highly polymorphic Human Leukocyte Antigen (HLA) alleles like HLA-B57 and HLA-B27 have been associated with control of HIV and delayed disease progression to AIDS because of the broad cross-reactivity to HIV epitopes they present [11]. Elite-Controllers and Long-Term non-progressors (LTNP); HIV-infected individuals who exhibit complete or partial control of HIV in the absence of ART, have been shown to be enriched with these protective HLA-B57 and HLA-B27 alleles [10]. At the cellular level, studies of HIV- exposed sero-negative cohorts from Amsterdam, Central Africa and West Africa have shown that low T cell activation is a significant immune correlate for reduced susceptibility to HIV [12–14] (discussed further in 1.3.3).

1.3.2 HIV-Exposed Sero-negative (HESN)

HIV-Exposed Sero-negative (HESN) individuals are people who are persistently exposed to HIV yet remain infection-free; effectively epidemiologically "resistant" to HIV. Several HESN cohorts have been identified globally; discordant couples, commercial sex workers (CSW), men who have sex with men (MSM), injection drug users (IDU), infants born to HIV-infected mothers and hemophiliacs [15–21]. Studying the genetics, immunology and environments of these HESN cohorts has shed significant light onto the mechanisms of protection from HIV [22].

One of the best characterized HESN cohorts is the Pumwani CSW cohort in Nairobi, Kenya. Since 1984, our research group has been following this cohort of female CSW studying sexually transmitted infections and in 1996, identified a group (~10%) among them as

intensely exposed yet HIV-free by serology and PCR [23]. HESN women of the Pumwani cohort are epidemiologically defined as HESN if they remain infection free for seven or more years while engaging in sex work. HIV susceptible women (HIV-S), are women who are engaged in sex work for less than three years and are presumed susceptible to HIV, serve as the control group to the HESN women. Only a small proportion of HIV-S women become HESN, the vast majority of HIV-S eventually become infected with HIV. Studies of HESN women have revealed genetic correlates of protection through specific HLA alleles and polymorphisms of the Interferon Regulator Factor-1 (IRF-1) gene, which is involved in regulating the innate anti-viral response [24–27]. HESN also exhibit HIV-specific CD8 T cell responses and qualitatively distinct CD4+ T cell responses compared to HIV-S, suggesting that protection is in part mediated by the cell-mediated immune response [28,29]. Thus, HIV protection in HESN may be mediated by factors such as genetics and innate and adaptive immunologic factors [30].

1.3.3 Immune Quiescence

Recently, a promising model called Immune Quiescence (IQ) was proposed by our lab to explain of the mechanism of protection in HESN women from the Pumwani CSW cohort [30]. Immunological studies showed that HESN women had low T cell activation, downregulation of genes (particularly genes involved in cellular activation) in CD4+ T cells and reduced pro-inflammatory cytokines in the periphery and genital mucosa compared to HIV-susceptible controls [30–32]. Additionally, they exhibited elevated immuno-suppressive T regulatory cells (Tregs) in the periphery [31]. HESN also demonstrated lower expression of

pro-inflammatory chemokines MIG and IP-10 and increased anti-inflammatory serpin anti-proteases in the genital tract. Serpins inhibit serine proteases that are secreted by immune cells, which function by triggering pro-inflammatory functions such as complement activation and secretion of pro-inflammatory cytokines [32,33]. The IQ state is not immunosuppression, as HESN have responded comparably to controls following *in vitro* stimulation with flu peptides [30]. This evidence taken together, points to an overall reduced baseline immune activation state or Immune Quiescence [34]. How might IQ result in protection from HIV? It is well-characterized that HIV infects activated cells more efficiently and preferentially than resting or quiescent cells [35,36]. Infection of quiescent cells is possible but inefficient due to the lack of necessary host factors used by HIV [37,38]. The model suggests that in HIV susceptibility, more activated cells are present than quiescent cells, therefore, HIV can efficiently infect activated cells resulting in the establishment of a productive infection. In HIV resistance, there are more quiescent cells than activated cells. HIV can infect the limited pool of activated CD4+ T cells, but the host innate and cell-mediated immune response is sufficient to target the infected cell and clear the infection [34]. (Figure 1). What are the potential drivers of IQ? Immuno-suppressive Tregs, which are elevated in HESN, may be playing a role in reducing overall immune activation by directly suppressing T cell activation. An important regulator of the human immune response called Interferon Regulatory Factor-1 (IRF-1), which is differentially expressed in HESN, is also potentially a driver of IQ. Persistent exposure to allo-antigens through sex-work and elevated anti-inflammatory anti-proteases may also contribute to IQ. Research is ongoing to further characterize IQ and to elucidate if it is an appropriate model of protection against HIV.

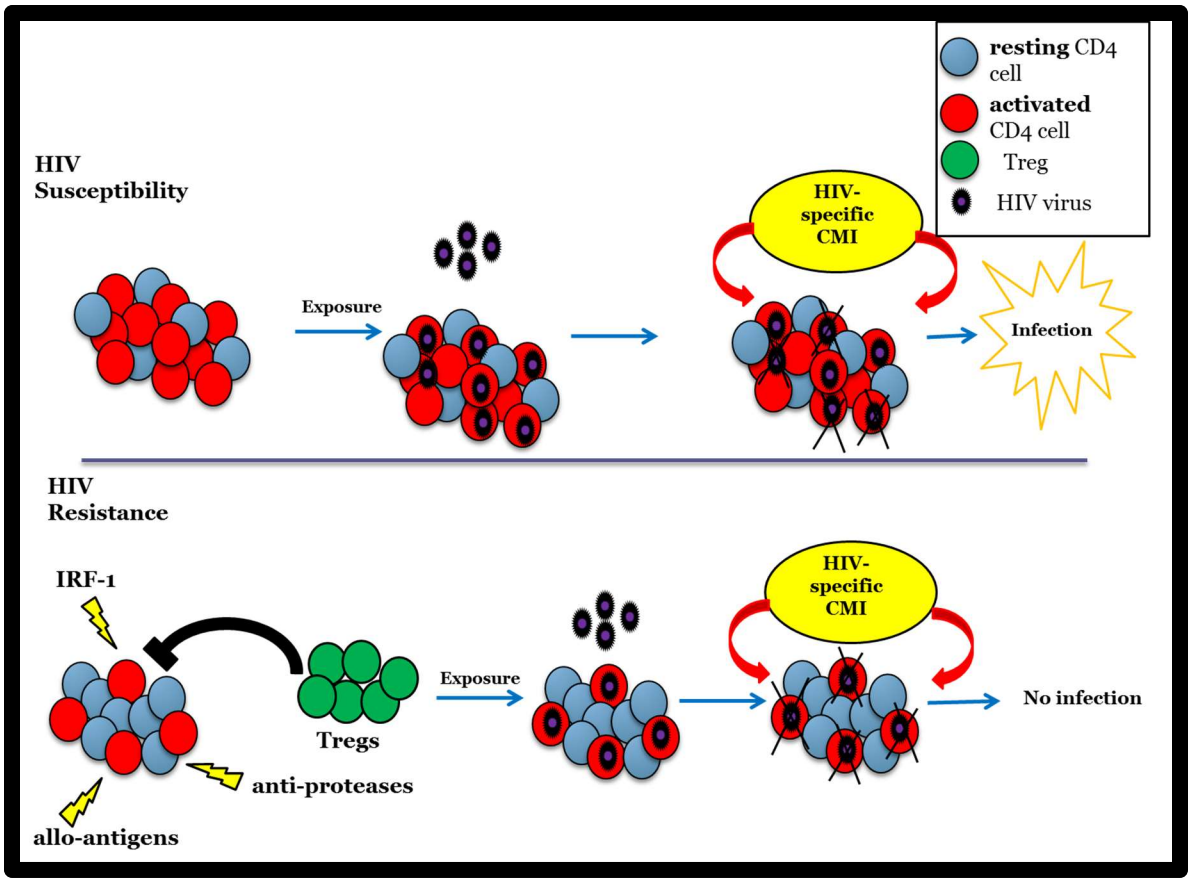

Figure 1: Mechanism of the Immune Quiescence Model of Resistance to HIV. HIV preferentially infects activated cells. The model predicts that in IQ, fewer activated cells and more quiescent cells are present. HIV can infect the limited pool of activated cells, however, the host HIV-specific cell-mediated immune response (CMI) can remove the infected cell, resulting in the prevention of HIV infection. Factors such as differential regulation of IRF-1, elevated Treg proportions, exposure to allo-antigens from sex-work, increased anti-proteases may be driving the Immune Quiescent phenotype in HESN women.

IQ has been observed in other HESN groups. HESN cohorts from the Central African Republic, Netherlands and the Ivory Coast were shown to have reduced T cell activation [12–14]. IQ has also been characterized in a non-human primate SIV model. Rhesus macaques that were vaginally challenged with glycerol monolaurate, an anti-microbial compound, showed reduced production of pro-inflammatory cytokines in the genital mucosa and protection from SIV infection [39].

1.3.4 Immune Activation and Susceptibility to HIV Infection

Immune activation, characterized by the proliferation of activated immune cells and secretion of pro-inflammatory cytokines, has been shown to impact both HIV susceptibility and progression. Persistent immune activation is one of the hallmarks of HIV pathogenesis and a strong predictor of progression to AIDS, even a better predictor than viral load [40,41]. More interestingly, increased immune activation is associated with increased susceptibility to HIV infection, which has been shown in non-human primate models, human *in vitro* models and cohort studies [35,42–44]. Elevated immune activation leading to enhanced HIV susceptibility has also been implicated at the vaginal mucosa. The application of a vaginal gel containing nonoxynol-9, which causes the disruption of the vaginal epithelium and increased immune activation of the vaginal mucosa, lead to an increased risk of HIV infection [45]. Furthermore, the Tenofovir microbicide gel was shown to be less effective due to pre-existing innate immune activation [46]. Bacterial vaginosis (BV) caused by excessive bacterial infection and inflammation in the vaginal tract also was associated with increased risk of HIV acquisition [47]. These studies suggest that increased immune activation at the

initial site of HIV contact may promote the recruitment of target cells creating an environment conducive to the infection, propagation, and dissemination of HIV.

1.4 Human Immune System

1.4.1 Innate Immune System

The innate immune system is the first-line defense against pathogens. It is present innately and activated within minutes post-exposure [48]. Structural barriers, such as the skin and epithelial surfaces at the respiratory, digestive, urinary, and reproductive tracts, serve as a physical barrier between the pathogen and the host [48]. Pattern Recognition Receptors (PRR), located intracellularly or on the surface of the many host cells, detect conserved components or pathogen-associated molecular patterns (PAMPs), resulting in the uptake and destruction of the pathogen [48]. Binding of PRR also results in the initiation of the inflammatory response through the secretion of molecules called cytokines, which help recruit phagocytic cells to the site of pathogen [48]. Phagocytes such as neutrophils and macrophages traffic to the site, engulfing the pathogen, leading to its degradation [48]. Natural Killer (NK) cells also play a role by recognizing infected cells and inducing apoptotic cell death [48]. Lastly, Dendritic cells (DC) are one of the more important players of the innate immune response. They are spread throughout various tissues and organs, contain a wide variety of PRR, are phagocytic and secrete many different cytokines [48]. DC are also involved in linking the innate and adaptive responses by presenting pathogen-

peptides to T cells and providing the necessary secondary signal, activating T cells and B cells and initiating the adaptive immune response [48].

1.4.2 Adaptive Immune System

The adaptive immune system is the specific, long-lasting immune response against pathogens [48]. It is activated a few days after the initial encounter of antigen. Activated antigen-presenting cells such as macrophages, DC, or B cells present the pathogen-peptide MHC-II complex to the T cell receptor (TcR), initiating the activation of the T Helper Cell (Th cell) [48]. This results in the clonal expansion of pathogen-specific Th cells, CTL and B cells into effector cells that can target the pathogen for destruction [48]. A pool of long-lasting memory T and B cells develop and remain ready to respond to the secondary encounter of the pathogen [48]. Memory T and B cells are primed to respond and quickly proliferate after secondary exposure—resulting in a robust adaptive immune response[48].

1.4.3 T cells and B cells

T lymphocytes or T cells develop in the thymus and are functionally defined by the antigen-specific TcR on the surface of the cell [48]. There are several subsets of T cells; Th cells, Cytotoxic T Lymphocytes (CTL), T regulatory cells, NKTs and $\gamma\delta$ T cells [48]. The Th cell plays an essential role in assisting the activation of other immune cells through direct

interactions and secretion of cytokines. Th cells are CD4+ and differentiate into several subsets resulting in diverse immune responses. CTL are CD8+ cells that target virally infected or tumor cells in the cell-mediated immune response. CTL recognize infected cells by a MHC-I –TcR interaction and destroy the infected cell by apoptosis [48].

B Lymphocytes, or B cells, develop in the bone marrow and are functionally defined by the antigen-specific B cell receptor (BcR) on its surface. Pathogen-antigen binding to the BcR results in the activation and differentiation into Plasma B cells, which produce circulating soluble antibodies that bind and neutralize the pathogen [48]. This is known as the humoral adaptive immune response. This type of response is important in targeting extracellular pathogens like bacteria [48].

1.5 Regulatory T cells

1.5.1 History, Description, and Function

T regulatory cells (Tregs) are central regulators of the human immune system and are essential in maintaining peripheral tolerance and immune homeostasis [49]. Tregs function not only to remove auto-reactive T cells but also to dampen uncontrolled immune responses. They were originally identified in the 1970s as suppressor T cells [50]. The discovery began with the observations that the neonatal thymectomy of normal mice (at day 3 after birth) and adult thymectomy of normal rats followed by X-ray irradiation resulted in systemic autoimmunity [51,52]. Later it was shown that adoptive transfer of CD4+ T cells resulted in the inhibition of auto-immunity – indicating that a CD4+ T cell

population was involved in immune tolerance [53,54]. Further studies would show that these cells expressed CD25 and the FOXP3 transcription factor [55,56].These CD4+CD25+FOXP3+ cells came to be defined as Regulatory T cells. Today, it is well understood that Tregs play an essential role in regulating the immune system and have an important impact on autoimmunity, allergy, acute and chronic viral infections, and cancer.

T regulatory cells make up 3-10% of peripheral CD4+ T cells. They are suppressive in nature; restricting activation, proliferation and effector functions of CD4 and CD8 T cells, NKs, B cells and APCs [57]. Tregs up-regulate CD25, the α-chain of IL-2 receptor, and rely on IL-2 for its maintenance and survival [58]. They express TcRs that are antigen-specific, however, can function in an antigen-independent manner [49].The most distinctive characteristic of Tregs is the expression of the transcription factor FOXP3 (fork-head box P3), which directs differentiation and controls the Treg suppressive program [59–61]. In humans, FOXP3+ Tregs are a heterogeneous population unlike in mice, which have distinct homogenous population of FOXP3 expressing Tregs [57]. They can be divided into two major groups; natural Tregs, which develop in the thymus and induced Tregs, which develop in the periphery from naïve T cells following antigenic stimulation [62] (Figure 2). Natural Tregs, which make up the majority of FOXP3+ Tregs in the periphery, are the main players in peripheral immune tolerance. Induced Tregs are mainly associated with mucosal sites such as the gastrointestinal tract [62]. Other induced Tregs such as Tr1 cells and Th3 cells have been identified, however, these Tregs lack FOXP3 expression [63]. Natural Tregs develop in the thymus from a CD4+CD8- precursor following a high avidity TcR interaction with self-antigen and stimulation of IL-2 (via CD25 receptor) resulting in the induction of FOXP3

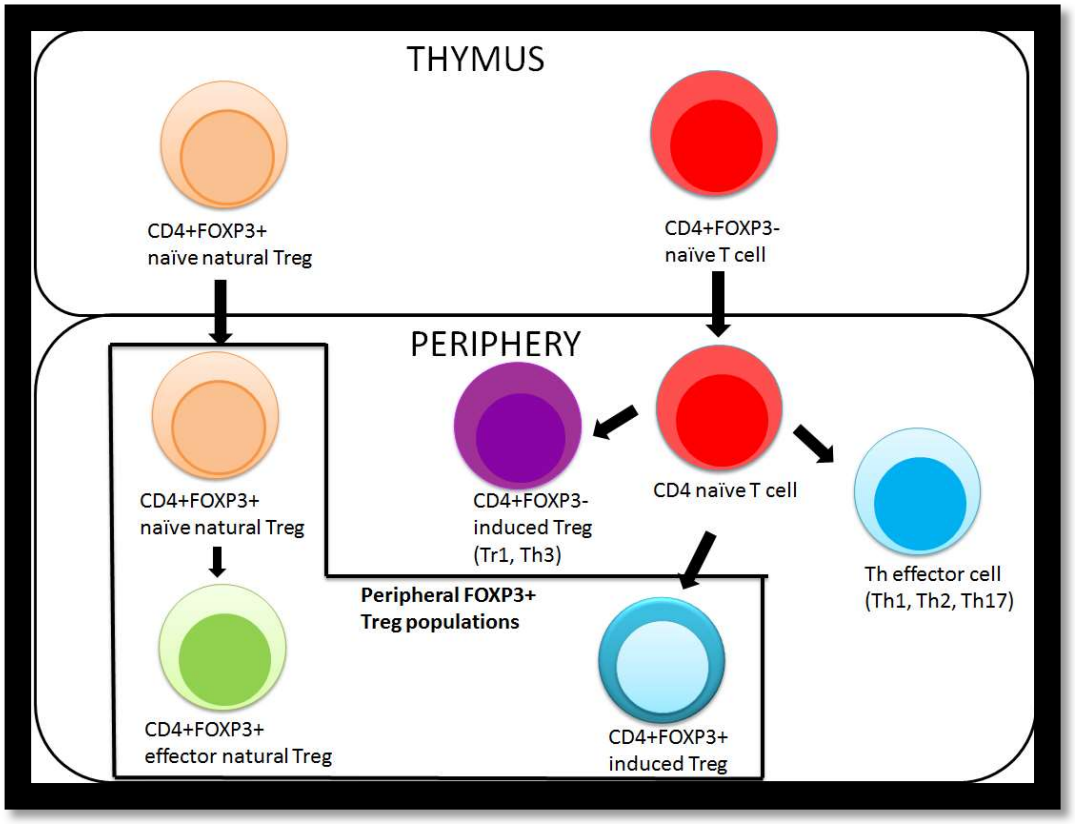

Figure 2: Regulatory T cell development in the thymus and periphery. Natural Tregs develop from the thymus through interactions with self-antigen. Induced Tregs develop from CD4+ naïve T cells in the periphery. FOXP3-negative induced Tregs, Tr1 and Th3, differentiate from CD4+ naïve T cells at mucosal sites. Peripheral FOXP3+ Treg populations include naïve natural Tregs, effector natural Tregs and induced Tregs.

expression [64]. Conversely, induced Tregs develop in the periphery following TcR stimulation in the presence of TGF-β, which directly induces the up-regulation of FOXP3 [65].

While Tregs make up only a fraction of the peripheral CD4+ T cell population, they are exceptionally potent suppressive cells. They possess a diverse TcR repertoire compared to conventional T cells and respond robustly to self-antigen and a wide variety of antigens [49,66]. Treg suppression mechanisms can be broken down into 2 categories; contact-independent (1) and contact-dependent mechanisms (2-4) (Figure 3). (1) Tregs can secrete inhibitory anti-inflammatory cytokines such as TGF-β, IL-10, targeting cells in a contact-independent manner [67]. (2) Tregs can directly destroy target cells by cytolysis via perforin and granzyme interaction [68].(3) Tregs can disrupt the metabolism of target cells by depriving them of the essential IL-2 through CD25. They can also disrupt metabolism by directly transferring inhibitory cyclic adenosine mono-phosphate (cAMP) into target cells through gap junctions [68]. Another metabolic disruption mechanism is mediated by co-expression of CD39 and CD73 – which deplete extracellular ATP, creating an anti-inflammatory environment. (4) Tregs can inhibit DC function through CTLA-4, preventing DC maturation and subsequent activation of effector cells [68]. Natural Tregs suppress target cells via contact dependent mechanisms while induced Tregs use inhibitory cytokines IL-10 and TGF-β [69].

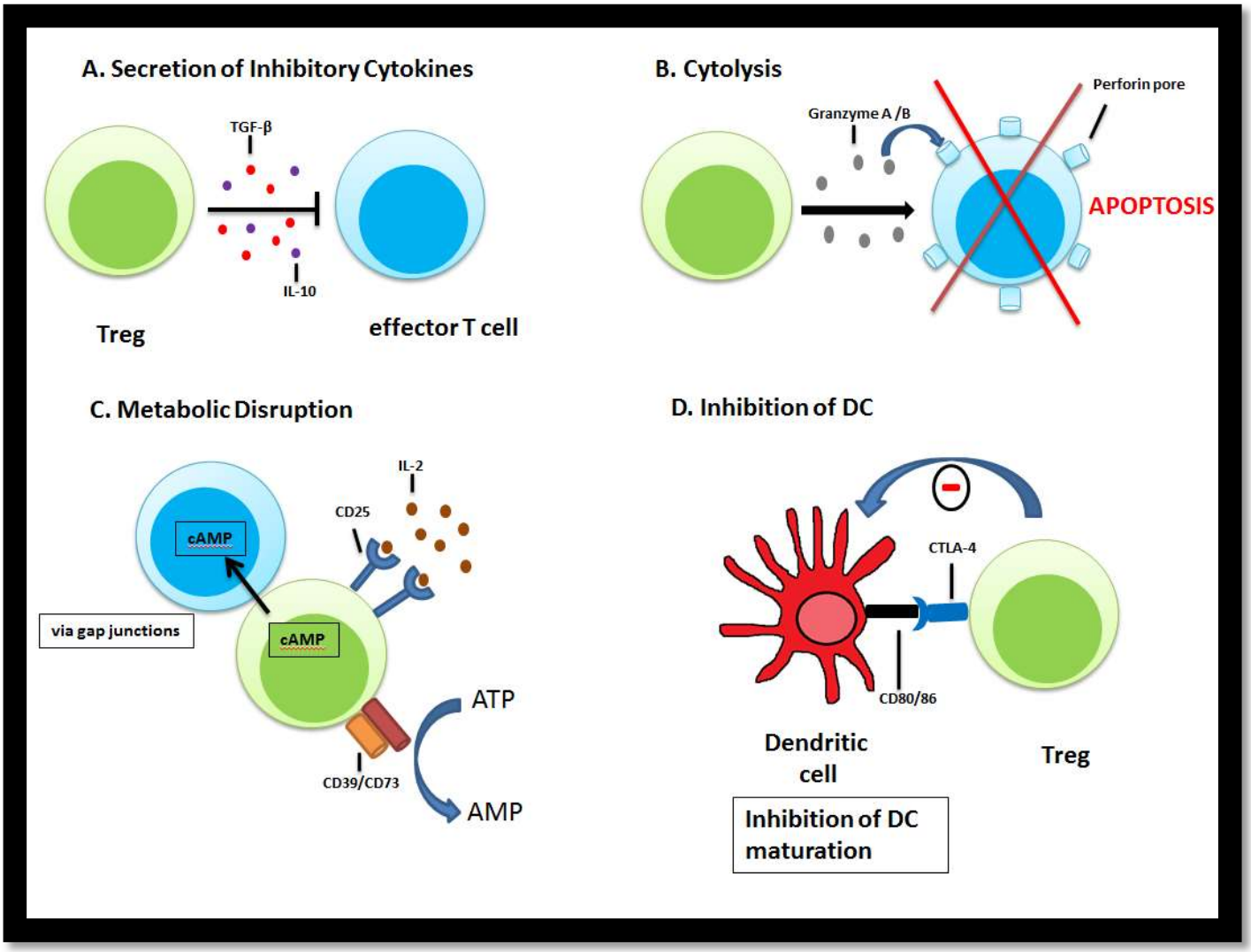

Figure 3: Regulatory T cell Suppression Mechanisms. Tregs can function via contact-dependent and contact-independent suppression mechanisms. Tregs can function by contact-independent suppression through the secretion of inhibitory cytokines TGF-β and IL-10 **(A)**. Contact-dependent suppression mechanisms include cytolysis of target cells via perforin and granzyme**(B)**, metabolic disruption through IL-2 deprivation, extracellular ATP depletion and delivery of anti-inflammatory cAMP via gap junctions **(C)** and inhibition of DC maturation via CTLA-4 **(D)**. (Adapted from Vignali *et al*).

One of the major hurdles in T regulatory cell research is the lack of definite identification markers. Conventional Tregs are characterized CD4+CD25+FOXP3+CD127-, however this description is not definitive as some activated CD4+ T cells are included in this definition [70]. Recently, the use of a combination of naive and memory markers; CD45RA, CD45RO and functional markers such as CTLA-4, CD39, have shown a new direction in identifying Tregs. An important study demonstrated that Tregs can be further delineated into naïve CD45RA+FOXP3low and effector CD45RA-FOXP3high Treg subsets – thus separating them from activated FOXP3+ non-Treg population [71]. The distinction between natural Tregs and induced Tregs remains elusive, though some data has suggested that a transcription factor called Helios may differentiate natural Tregs from induced Tregs [72].Tregs are most commonly defined as CD3+CD4+CD25+FOXP3+, which is the definition used in this study.

1.5.2 FOXP3

FOXP3 (forkhead box P3) is a member of the forkhead family of transcription factors. It is considered the master regulator of Tregs and is essential for their development and suppressive function [59–61]. Mice (Scurvy mice) and humans that lack a functional FOXP3 gene develop a wide range of autoimmune and lymphoproliferative diseases [73–75]. The mutation of the human FOXP3 gene results in the development of early infancy systemic autoimmune disease, IPEX (Immune dysregulation, polyendrocrinopathy, enteropathy, X-linked syndrome) [75].The FOXP3 transcription factor impacts the expression of various genes, primarily enhancing genes involved in suppression. Induced expression of FOXP3 in

CD4+CD25- T cells results in the acquisition of suppressive capacity [59,60]. It functions as an homo-oligomer, interacting with other factors such as NFAT (nuclear factor of activated T cells), RUNX1 (runt-related transcription factor 1), HAT (histone acetyl-transferase), HDAC (histone deacetyl-transferase) and NF-κB to influence the activation and repression of target genes [49]. FOXP3 in cooperation with NFAT, transcriptionally activates the expression of suppression-associated factors like CTLA-4 and GITR, while repressing proliferative and inflammatory factors such as IL-2, IFN-γ and IL-4 [76].

Several signaling pathways influence FOXP3 expression. NFAT and AP1 (activator protein 1) induced in TcR signaling, can bind to the FOXP3 promoter and enhance FOXP3 expression [77]. Another TcR signal transducer molecule PKC- θ (protein kinase C- θ), was found to stimulate FOXP3 promoter activity [78]. Some evidence suggests that CD28-costimulation is necessary for FOXP3 expression and initiation of Treg differentiation, however, this seems to be the case for only natural Tregs and not induced Tregs [79]. FOXP3 expression is also influenced by cytokine-mediated signals. STAT-5, induced from IL-2 signaling, plays an essential role in regulating FOXP3 expression by binding to its locus and inducing its expression [80]. TGF-β-induction of TIEG1 (TGF-β Inducible Early Gene-1) in cooperation with ITCH (itchy E3 ubiquitin protein ligase) can also induce FOXP3 expression [81]. Another TGF-β signaling molecule, Smad3, was shown to co-operate with NFAT by binding to the FOXP3 locus, enhancing its expression [82]. Retinoic acid, a vitamin A metabolite, has been shown to induce the expression of FOXP3 by the phosphorylation of Smad-3 in mucosally-generated induced Tregs [83]. FOXP3 expression can also be inhibited by cytokine signals such as IL-4-induced factors GATA-3, STAT-6 and the interferon-induced IRF-1, by binding to the FOXP3 promoter and repressing its transcription [84–86].

FOXP3 expression can also be modulated epigenetically by DNA methylation of CpG motifs and histone modifications [87]. It is well understood that DNA is accessible and expressible with the demethylation of CpG motifs and histone molecules and conversely, is inaccessible with methylation. There are three important sites of epigenetic regulation of FOXP3; the FOXP3 promoter, and two highly-conserved non-coding sequences, CNS-1 and CNS-2 [88]. Studies have shown that CpG motifs in the FOXP3 promoter are almost exclusively de-methylated in Tregs and only weakly methylated in resting conventional T cells [87]. Tregs also contain more acetylated histones than conventional T cells in the FOXP3 locus [88]. The CNS-1consists of TGF-β-sensitive enhancer element and is the binding site for FOXP3 enhancers Smad-3 and NFAT [82]. Studies have shown increased histone acetylation of CNS-1 in Tregs compared to conventional T cells[82]. The CNS-2 region has been characterized as the Treg-specific demethylated region, TSDR [88]. This CpG-rich site is the most significant site of epigenetic regulation of FOXP3. This site is demethylated in Tregs and fully methylated in conventional T cells[89–92]. Interestingly, natural Tregs have this region completely demethlylated while induced Tregs and activated T cells show incomplete demethylation, which may explain the transient FOXP3 expression in these cells [88,90]. Multiple studies have indicated that natural Tregs are more stable and long lasting in the periphery compared to induced Tregs. This suggests that the TSDR region is the most significant region that influences the stability of FOXP3 expression [93] (Figure 4).

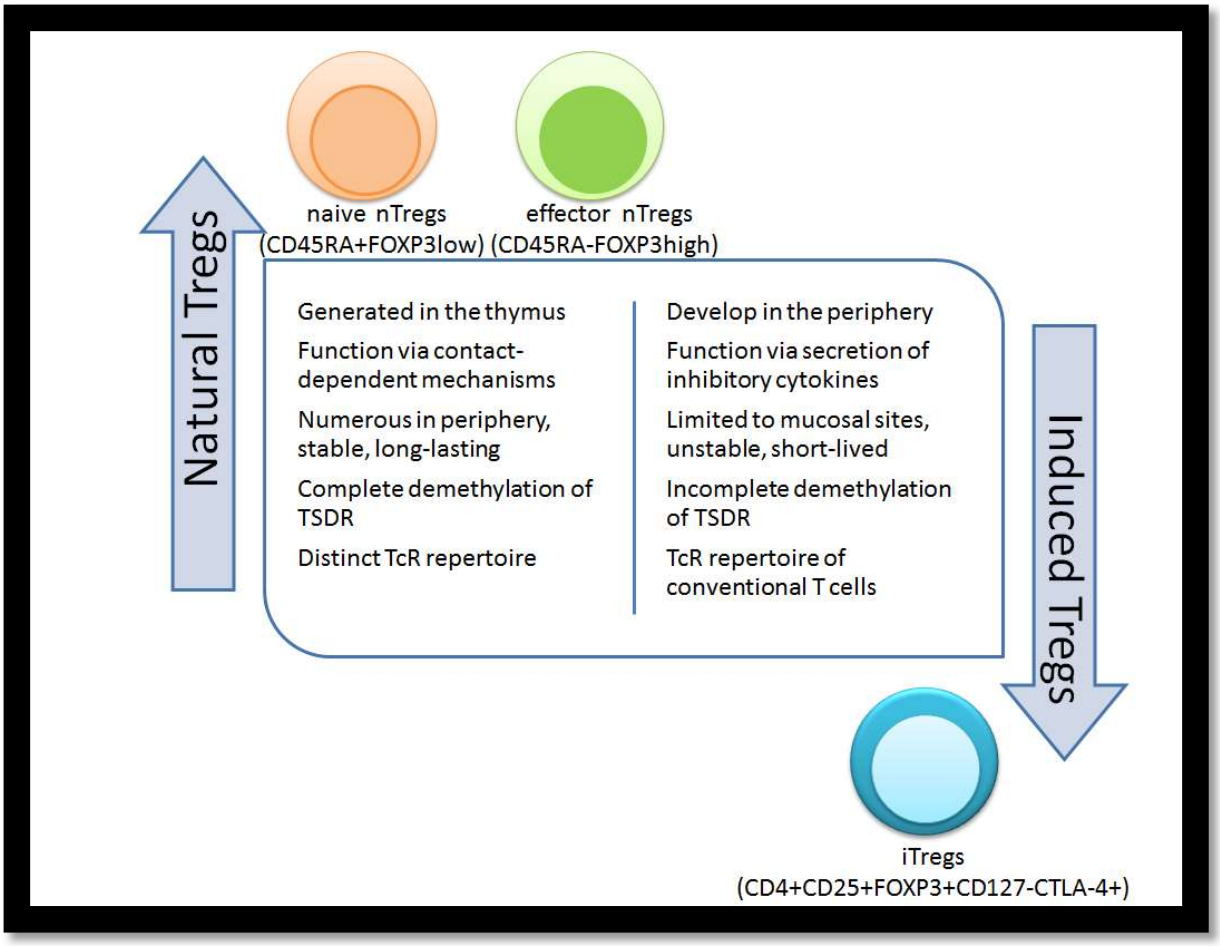

Figure 4: Comparison of natural and induced Regulatory T cells in the human immune system. Natural Tregs are stable, numerous in the periphery and develop in the thymus following interaction with self-antigen. Natural Tregs function by contact-dependent mechanisms. Induced Tregs are unstable, short-lived and develop in the periphery from naïve T cells at mucosal sites. Natural Tregs have a completely demethylated TSDR region of FOXP3, while induced Tregs have incomplete demethylation of the TSDR region.

1.5.3 Tregs in HIV Infection

Since systemic immune activation and establishment of HIV-specific T cell responses are consequences of HIV pathogenesis, Tregs would seem to be essential regulators of an established HIV infection. Moreover, Tregs are CD4+ T cells and express HIV co-receptors CCR5 and CXCR4, thus, can serve as efficient targets for HIV infection [94,95]. Several studies have revealed their benefits in limiting systemic immune activation and their role in weakening protective HIV-specific T cell responses. However, the overall effect of Tregs in a progressive HIV infection is unclear [96].

Data on the role of Tregs in HIV infected patients has been contradictory due to factors such as study differences in Treg localization (periphery or tissues), stage of HIV disease (acute or chronic), treatment condition (untreated or ART-treated), quantification method (frequency or absolute numbers) and Treg definition (conventional or naïve/effector) [96]. Thus, having a clear Treg definition and considering the environmental context of the HIV infection is essential in elucidating the role of Tregs. Data from acute HIV infection is limited, increased or decreased frequency of Tregs (compared to healthy controls) has been reported [96-98]. In untreated chronic HIV infection, most studies have reported increased frequency of peripheral Tregs and an inverse relationship with CD4 count [96]. This increase of Tregs can be attributed to the increased expansion of Tregs in the thymus and periphery in response to persistent immune activation [96]. However, analyzing Tregs as percentage of overall CD3+ T cells, and not declining CD4+ T cells, reveal that absolute numbers of Tregs decline over a progressive HIV infection [98]. Some reports have indicated

that ART decreases or normalizes Tregs to levels comparable to healthy controls [98–101]. ART-treated patients had lower Treg frequencies compared to chronic untreated controls and treatment interruption resulted in increased Treg frequencies [101,102]. Analysis of Tregs in LTNP and elite-controllers revealed decreased Tregs or comparable levels to healthy donors [96,98,101,103–105]. Furthermore, patients undergoing ART treatment showed an inverse correlation between T cell activation and Treg proportions [104,106]. Chevalier *et al* have hypothesized that this data taken together suggests that Tregs may be sufficient in controlling T cell activation in HIV-infected patients undergoing ART but not effective in limiting systemic immune activation in untreated patients due to elevated HIV levels [96]. Examination of *ex vivo* HIV-specific T cell responses in ART-treated patients has revealed an inverse correlation between Tregs and HIV-specific CD8+ T cell responses [96,102]. Hence, the effect of Tregs in patients undergoing ART may be two-fold; Tregs limit generalized immune activation and they also suppress protective HIV-specific CD8+ T cell responses [96].

Recent reports have identified a specific CD39+ Treg subset that is elevated during HIV infection [101,107,108]. The studies showed that this CD39+ Treg population in HIV infected patients was inversely correlated with CD4+ count and positively correlated with viral load and T cell activation [90,96,101,107,108]. Additionally, a CD39 gene polymorphism causing CD39 downregulation has been shown to be associated with delayed progression to AIDS [107]. This data implicates CD39+ Tregs as the critical subset responsible for suppressing protective HIV-specific responses during HIV infection and thus, may serve as a potential therapeutic target to boost protective HIV-specific responses and slow HIV progression [96].

Tregs may play a role in susceptibility to HIV infection. As described previously in our IQ studies [31,34], Tregs may have a protective role by suppressing the overall immune activation, thus decreasing the number of efficient targets for HIV [34]. CD4+CD25+FOXP3+ Tregs were elevated in HESN women and associated with reduced CD4+/CD8+ T cell activation [31]. This finding was supported in HIV-exposed uninfected neonates, who exhibited high levels of Tregs and low CD4+/CD8+ T cell activation in cord blood [109]. Furthermore, a recent study of a high-risk HESN population found that increased Treg frequency, not HIV-specific responses, was associated with protection from HIV infection [110].

Murine studies have characterized a link between the critical transcriptional regulator IRF-1and FOXP3. Murine studies have also shown that IRF-1 impacts Treg development and function. Based on these studies, we sought to examine the relationship between IRF-1 and Tregs in HESN women of the Pumwani CSW cohort.

1.6 Interferon Regulatory Factor-1 (IRF-1)

1.6.1 Interferon Regulatory Factors (IRFs)

The interferon regulatory factor (IRF) family of transcription factors are central regulators of genes that regulate various biological processes such as cell-division, cell-differentiation and the immune response [111]. IRF's are characterized by a unique helix-turn-helix DNA binding motif at the N-terminus and a variable regulatory domain at the C-terminus. The

regulatory domain breaks down IRF's into ones that act as transcriptional-activators (IRF-3, IRF-5, IRF-6, IRF-7, IRF-9), and others that can act as both transcriptional-activators and repressors (IRF-1, IRF-2, IRF-4, IRF-8)[111]. IRF's can interact with different transcription factors and other IRF's and bind to ISRE (Interferon Stimulated Response Element) in DNA. IRF's respond primarily to interferons (IFNs) that are produced in the host anti-viral response. Interferons can be classified into Type I IFNs, such as IFN-α, IFN-β, and Type II IFNs, like IFN-γ [111]. Viral infections induce the secretion of interferons and the initiation of the interferon response via the JAK-STAT signaling pathway, resulting in the activation of the IRF network of transcription factors [111]. These IRFs, which can act as activators or repressors, can turn on hundreds of ISGs (interferon stimulated genes) involved in the anti-viral response. IRFs also have roles in other biological processes like cell growth and death, hematopoietic differentiation and immune-modulation [111].

1.6.2. Function and Regulation of IRF-1

IRF-1 has been shown to be a critical regulator of genes involved in innate and adaptive immune responses, cell division, carcinogenesis and immune cell differentiation and function. IRF-1 is expressed at low basal levels in almost all nucleated cells and is up-regulated in response to stimuli such as TNF-α, IL-1, IL-12 and Type I/II interferons secreted during viral infection [112]. IRF-1 is structurally similar to its homologue IRF-2 (62% homology), and acts antagonistically to IRF-2 [112]. IRF-1 is essential for the development of an effective anti-viral response [113,114]. Mice with IRF-1 null mutations in mouse embryonic fibroblasts (MEFs) showed impaired anti-viral responses against encephalomyocarditis

virus (EMCV)[113,114]. Other data showed that IRF-1 knockout mice have enhanced West Nile virus replication and decreased anti-viral responses [115]. IRF-1 also seems to be important in macrophage and NK cell function. Mice lacking IRF-1 have decreased cytotoxicity of macrophages and NK cells and inhibited production of inflammatory IL-12 and IL-15 [113,116]. Furthermore, IRF-1 has been implicated as a central regulator of class I and II MHC expression, hence, affecting antigen-presentation [117].

The impact of IRF-1 seems to extend beyond the innate immune response. Studies have shown that IRF-1 plays an important role in Th1/Th2 differentiation. IRF-1 is essential for the establishment of the Th1 response and its impairment results in the Th2 response [116,118]. IRF-1 may also impact autoimmunity as IRF-1 knockout mice displayed reduced incidence and severity of autoimmune diseases [119]. IRF-1 has been shown to affect both myeloid and lymphoid differentiation [120]. Murine studies have illustrated a clear impact of IRF-1 on CD8+ T cells and Tregs. Mice lacking IRF-1 displayed the inability to develop mature CD8+ T cells due to impaired selection in the thymus [121]. Research has shown that IRF-1 affects the development and function of Tregs. Fragale *et al* showed that IRF-1 negatively regulates Treg development and function by binding to the IRF-E binding element in the FOXP3 promoter and repressing its expression [84]. The IRF-1 -/- mice had increased CD4+CD25+ Tregs and were more functionally suppressive than wild-type Tregs [84]. Another study has shown the presence of two ISRE sites at the intronic region of the FOXP3 locus, which can be bound by IRF-1, leading to repression [122]. There is also evidence that IRF-1 indirectly effects Treg development by modulating dendritic cells [123]. IRF-1-/- mice had inhibited DC maturation and skew differentiation to plasmacytoid DC over

conventional DC [123]. DC from IRF-1-/- mice were tolerogenic and produced Treg differentiation favoring cytokines IL-10 and TGF-β [123]. Furthermore, resistin, a hormone secreted by adipocytes, suppresses IRF-1 expression by disrupting DC function and inducing expansion of Tregs [124].

1.6.3. IRF-1 in HIV replication, Latency & Pathogenesis

The HIV virus relies on host cellular factors for the transcription, translation, and assembly of functional virions. IRF-1 is an essential host factor utilized by HIV, particularly in the early stages of HIV infection [119]. HIV encodes for a regulatory protein called Tat, which binds to the trans-activation response (TAR) element of the 5' Long Terminal Repeat (LTR) of HIV, enhancing the viral transcription of HIV. In early HIV infection, Tat is absent, or in very low levels, so HIV utilizes host IRF-1 to activate and enhance HIV transcription [125]. An HIV mutant with a deletion of the IRF-1 binding site in the HIV LTR led to reduced HIV replication in monocyte-derived DCs (MDDCs) [126]. In *Jurkat* cells, increasing IRF-1 expression correlated with increased HIV transcription, even with little or no Tat presence [127]. Other studies have shown that IRF-1 is upregulated in early HIV infection before Tat expression in cell types such as macrophages, MDDCs, cell lines and stimulated primary cells [125–128]. These data point to the importance of host IRF-1 in activating and enhancing HIV transcription during early HIV infection in the absence of Tat [125]. Furthermore, IRF-1 forms a complex with NF-κB and binds to the enhancer region of HIV LTR; this interaction is essential for efficient transcription of the HIV LTR [129].

HIV latency is an essential characteristic of the HIV pathogenesis and an important evasion strategy from the host immune response. HIV latency can be described as the persistence of replication-competent HIV provirus in resting CD4+ cells [119]. This low level HIV replication can be due to the lack of viral Tat and reduced expression of host factors such as IRF-1 and NF-κB in resting cells [130]. Studies have shown that stimulation of target cells with mitogens or CD3-CD28 anti-bodies can reactivate latent proviruses by up-regulating IRF-1 and NF-κB expression through T cell activation [125,130]. Other reports have indicated the role of IRF-2 and IRF-8, which are inhibitors of IRF-1, in maintaining HIV latency [125]. IRF-2 competes with IRF-1 for the binding site while IRF-8 complexes with IRF-1, preventing its action [125-126]. IRF-8 was shown to inhibit the formation of the IRF-1-Tat complex and implicated in the transcriptional repression of the ISRE element in the HIV promoter [125,126,131].

Interestingly, the Tat interaction with IRF-1 seems to impact HIV pathogenesis. Initially, extracellular Tat secreted by infected cells can attach to target cells and stimulate IRF-1 expression via T cell activation, creating conditions favorable for efficient HIV replication [132]. Later, Tat is internalized by the target cell and forms a complex with IRF-1, preventing its interaction with STAT-1, resulting in impaired stimulation of genes involved in the IRF-1-directed anti-viral response [133]. Thus, Tat modulates IRF-1 expression to promote infection and later to evade the host anti-viral response [132,133].

1.6.4. Protective role of IRF-1 in HESN Individuals

One of the strongest correlates of protection in HESN women from Pumwani CSW cohort is the polymorphisms within the IRF-1 locus [26,27]. Three polymorphisms; two SNPs located at

619 (A>C), 6516 (G>T) regions, and the 179 microsatellite allele were associated with reduced susceptibility to HIV infection [21]. These protective genotypes resulted in reduced basal expression of IRF-1 and reduced responsiveness to exogenous IFN-γ stimulation of IRF-1 in PBMC [27]. PBMC from HESN with protective genotypes showed decreased HIV-LTR trans-activation when infected with the VSV-G pseudotyped HIV virus [125,134]. Furthermore, study of HESN showed that there was no association of protective IRF-1 genotypes with disease progression, viral load or CD4 count; Sivro *et al* suggest that this indicates that protection in HESN individuals is mediated during the early stages of HIV infection [119,125,135]. Recent studies suggest that HESN women have differential epigenetic regulation of IRF-1 compared to HIV-S women [136]. IFN-γ stimulation of PBMC in HESN resulted in a robust but transient IRF-1 expression compared to continuous robust expression of IRF-1 in HIV-susceptible women [136]. This robust but transient IRF-1 expression phenotype in HESN may provide protection by striking a balance between supporting the activation of host IRF-1-directed antiviral responses while limiting IRF-1 transactivation of the HIV-LTR, thus preventing the establishment of a productive HIV infection [125,136,137].

1.7 Rational and Hypotheses

The overall goal of this project is to clarify the mechanisms that drive Immune Quiescence. CD4+CD25+FOXP3+ Tregs were shown to be elevated in HESN, however, their functionality hasn't been confirmed. Additionally, the factors driving IQ have not yet been addressed, particularly the impact of the critical regulator IRF-1 on Tregs. In this study, the relationship between IRF-1 and Tregs in HESN subjects was examined. In a separate study,

the role of Tregs in HIV susceptibility and seroconversion was characterized in a high-risk non-HESN population.

The project has three components. (1) Analysis of Treg function though *in vitro* experiments (Chapter 3). (2) Clarification of the relationship between FOXP3, IRF-1 and Tregs in HESN and in human cells (Chapter 4). (3) Assessing the role of Tregs in HIV seroconversion (Chapter 5).

(1) Analysis of T regulatory cell Function

A previous study showed that HESN women of the Pumwani CSW cohort have increased CD4+CD25+FOXP3+ Tregs compared to HIV-susceptible controls. Although CD4+CD25+FOXP3+ Tregs have been shown to be suppressive in many studies, we were interested in confirming their suppressive function as a matter of proof of concept.

Hypothesis **CD4+CD25+FOXP3+ Tregs are *suppressive* in function**

(2) Assessment of the relationship between FOXP3, IRF-1 and Tregs in HESN

Murine studies have shown that IRF-1 is a negative regulator of FOXP3 and Tregs. Previous research of the Pumwani CSW cohort has shown HESN women have higher Treg numbers in the peripheral blood compared to HIV susceptible women and have lower baseline expression of IRF-1. The link between reduced IRF-1 expression in HESN women and

elevated T regulatory cells has yet to be established. Furthermore, the relationship between IRF-1, FOXP3 and Tregs in human cells has not been characterized.

Hypotheses

- **Reduced IRF-1 expression drives increased FOXP3 expression and thus higher frequency of Tregs in HESN individuals**
- **Tregs have reduced IRF-1 expression compared to other cell types**
- **Increasing FOXP3 expression in Tregs is associated with reduced IRF-1 expression**

(3) Assessing the role of Tregs in HIV sero-conversion

A recent study of HIV pre-seroconversion samples found that HIV seroconversion was associated with increased activated CD4 and CD8 CD38+CD69+HLA-DR+ T cells. The proportion of Tregs in pre-seroconversion samples is undetermined.

Hypothesis **Decreased Treg frequency is associated with HIV seroconversion**

Global Hypothesis: CD4+CD25+FOXP3+ Tregs are functionally suppressive and contribute to the reduction of overall immune activation, resulting in IQ in HESN women; conversely, decreased Treg frequency results in elevated immune activation and increased susceptibility to HIV infection. This frequency of Tregs is driven by the expression of negative regulator of FOXP3, IRF-1.

Chapter 2 **Materials and Methods**

2.1 General Reagents

2.1.1. Solutions

PBS Solution: 8g NaCl (137mM) 0.2 g KCl (2.7mM) 1.44g Na_2HPO_4 (10mM) 0.24g KH_2PO_4 (1.8mM) dissolved in 1L ddH2O. Solution was autoclaved or filter-sterilized and stored at 4°C.

PBS + 2% FCS: ("FACS buffer") PBS solution with 2% heat-inactivated fetal calf serum (FCS) –heated at 56°C for 30min, stored at 4°C

R-10 media: RPMI-1640 Media (Hyclone/Sigma-Aldrich) with 10% FCS and 1% Penicillin/Streptomycin. Stored at 4°C.

Freezing Media: 90% FCS solution with 10% dimethylsulfoxide DMSO (Sigma-Aldrich)

Mitomycin C: 2 mg lyophilized Mitomycin C (Sigma-Aldrich) was dissolved in 4ml of sterile PBS(0.5mg/mL). Covered with aluminum and stored at 4°C.

2.1.2. Stimulation and Differentiation molecules

CD3/CD28Dynabeads (Invitrogen)

Used to activate T cells in Treg depletion experiment. Added to cell suspension at 1:1 bead to cell ratio. (4×10^7 beads/ml)

CEF Peptide Pool

Peptide mixture of CMV, EBV, Flu peptides (CEF, 32 peptides/pool, 5 µg/ml/peptide; AnaSpec). Used to stimulate cells in Treg depletion experiments.

Mitomycin C-treated allo-pool of cells

We used to allo-stimulate cells in Treg depletion experiments. Prepared by treating PBMC from 5 different donors with 50µg/ml mitomycin C for 2 hours at 37°C and pooling cells together. Cells were resuspended in freezing media at 5 million cells/ml and stored long-term at -120°C. For allo-stimulations, allo-cells were thawed, resuspended in R-10 media and combined with responder cells at 3:1 allo-pool to responder cell ratio. Mitomycin-C inhibits DNA synthesis of allopool cells, this is necessary to measure effector responses of responder cells only.

Purified CD3 Antibody (Invitrogen)

Coated wells at 5µg/ml to stimulate cells for Treg differentiation. One hundred µl of purified CD3 antibody was added to each well and incubated at 37°C for 4 hours. Following incubation, the mixture was aspirated out and rinsed once with PBS. The culture plate was kept in the incubator until use.

Purified CD28 Antibody (BD):

Added1ng CD28 (0.5μg/ml) to CD24+CD25- cells to stimulate Treg differentiation

Recombinant Human TGF-β1 (Peprotech):

Prepared by suspending in 50ul 10mM citric acid solution (pH=3.0) and diluted to 950ul with PBS solution containing 0.1% BSA. 2ng of TGF-β1 was added to cell suspension at 2ng/ml.

Recombinant IL-2 (Invitrogen)

Added to Treg differentiation cell suspension at 50U/ml.

2.2 Study Samples

2.2.1 Samples

Blood samples for Treg functional and differentiation studies were obtained by from healthy donors of the department of Medical Microbiology in the University of Manitoba. Blood was drawn from donors intravenously into heparinized vacutainers.

Analysis of relationship between Treg proportions and IRF-1 genotypes/IRF-1 expression were based on previous data obtained from HESN subjects of the Pumwani CSW cohort. Cryopreserved PBMC samples from the Pumwani CSW cohort were used for the HIV-seroconversion study (described in 5.4.1). Ethics approval was obtained from research

ethics boards at the University of Manitoba and University of Nairobi/Kenyatta National Hospital and written informed consent was obtained from all participants.

2.2.2 PBMC Isolation from Whole Blood

Peripheral Blood Mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation. Whole blood in heparinized vacutainers were obtained from healthy donors and centrifuged at 1500 rpm for 10 minutes. The upper plasma layer was discarded. Blood was then diluted with equal volume of PBS+2% FCS and subsequently layered over Ficoll and centrifuged at 1400 rpm (with brake off) for 25 minutes. The developed white blood cell layer was collected into a new tube and diluted with PBS+2% FCS. The tube was then centrifuged for 10 minutes at 1600 rpm and washed once with R-10 media. An aliquot of cell suspension was stained with trypan blue and visualized under a hemocytometer to determine cell count and viability. The cell pellet was then resuspended with R-10 media to the desired cell concentration. Cells were then placed in a 37°C+5% CO_2 incubator for cell growth and survival. For long term storage, cells were resuspended in freezing media (at 10 million cells/ml) and transferred into cryovials. The cryovials were then transferred to an alcohol-containing freezing container (Mr. Frosty- Invitrogen) at -80°C overnight and then moved to -120°C for long-term storage. Frozen cells were thawed quickly in a 37° water bath, washed/resuspended in cold R-10 media and rested for 3 hours prior to staining in a 37°C/5% CO_2 incubator.

2.3 Flow Cytometry

2.3.1 Surface Staining

One million PBMC were transferred to a 5ml FACS tube. Cells were washed 1X with FACS buffer and centrifuged at 1600 rpm for 10 minutes. Meanwhile, a master-mix of surface stain antibodies in FACS buffer was prepared. After wash was complete, the supernatant was dumped and cell pellet was resuspended in residual buffer by "scraping" the tube. Surface stain antibodies from the master-mix were then added to the tube and incubated for 30 minutes at 4°C in the dark. The tubes were washed once with FACS buffer and centrifuged at 1600 rpm for 10 minutes. The supernatant was removed and the cell pellet was resuspended in 300ul FACS buffer. The surface stained cells were ready for intracellular staining or could be fixed with fixation buffer (BD) and analyzed on a flow cytometer.

2.3.2 Intracellular Staining (FOXP3, CTLA-4, Ki67)

After surface staining, the cells were washed 1X with FACS buffer and centrifuged at 1600rpm for 10 minutes. The cell pellet was resuspended in 250µl Fixation/Permeabilization solution (ebioscience) and incubated at 4°C for 30 minutes in the dark. The cells were then washed once with 1X Permeabilization buffer (Perm Buffer-ebioscience) and centrifuged at 1600 rpm for 10 minutes. After removing the supernatant, 100µl blocking solution (1µl mouse IgG, 2µl Rat Serum, 6µl FCS, 81µl Perm Buffer) was

added to the cells, followed by incubation at room temperature for 15 minutes, and covered with aluminum foil. The cells were then spun at 1600rpm for 8 minutes and the blocking solution was discarded. Following blocking, a master-mix of intracellular antibodies in Perm Buffer was prepared and added to the cells. The mixture was incubated for 1 hour at 4°C in the dark. The tubes were then washed 2X with Perm Buffer and resuspended in 300ul FACS buffer. The surface and intracellular stained cells were then analyzed by flow cytometry.

2.3.3 IRF-1 Staining

Due to the lack of a commercially available fluorochrome-attached IRF-1 antibody, we utilized a primary rabbit anti-IRF-1antibody (Santa Cruz sc-497 or abcamab26109) and a secondary goat anti-rabbit alexa-fluor488 conjugate antibody (Invitrogen). IRF-1 staining using this system involved the same intracellular staining procedure outlined above, with an additional staining round. After intracellular staining with anti-IRF-1 antibody (and other intracellular antibodies) for 1 hour at 4°C, the cells were washed 2X with Perm Buffer and centrifuged at 1600 rpm for 10 minutes. After the supernatant was removed, the cells were mixed with the secondary goat anti-rabbit alex-fluor488 conjugate antibody and incubated for 30 minutes at 4°C in the dark. The cells were then washed 3X with Perm Buffer and resuspended with 300ul FACS buffer. The cells were then analyzed on a LSRII flow cytometer.

2.3.4 LIVE/DEAD cell staining

When using frozen PBMC samples, cells were stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen) prior to surface staining. After thawing of frozen PBMC and resuspension in R-10 media, the cells were incubated with100µl LIVE/DEAD aqua dye in PBS solution (1µl aqua dye in 99µl PBS; 100µl total mixture) for 30 minutes at 4°C in the dark. The cells were then washed 1X with PBS and subsequently surface/intracellular stained as outlined above. The LIVE/DEAD stain binds to dead cells and not to live cells.

2.3.5 Proliferation Staining Protocol

For the Treg functional experiments (Chapter 3), cells were stained with a proliferation dye (eFlour670- eBioscience) to quantify the proportion of proliferating cells after stimulation. As cells proliferate, expression of eFlour670 is decreased and can be visualized by flow cytometry. Cells were washed 2X with PBS to remove serum and resuspended in 500µl PBS. An equal volume of eFlour670 dye (500µl) was mixed with the cell suspension by vortexing. This mixture was then incubated for 10 minutes at 37°C in the dark. The labelling was stopped by adding R-10 media (5X the volume) and placing on ice for 5min. The labelled cells were washed 3X with R-10 media to remove any unbound dye.

2.3.6 Compensation

For multi-color flow cytometry experiments, compensation controls are necessary to correct for the spectral overlap of different fluorochromes. Compensation involves determining the fluorescence intensity of each fluorochrome by itself and then using an algorithm (BD software) to remove the overlap of fluorescence. We used BD compensation beads for our flowcytometry experiments. A negative control unstained tube was prepared by adding one drop of negative control compbeads (beads that can't bind to antibodies) to a FACS tube. The negative control is necessary to establish background fluorescence. Compensation tubes for individual antibodies were prepared by adding one drop of vortexed positive compbeads (anti-mouse or anti-rat) into 400µl PBS -- 100µl of which was transferred to a separate FACS tube. One µl of the antibody of interest was mixed with the 100ul of compbeads and incubated at room temperature for 5 minutes. The antibody-compbead mixture was diluted with 400µl PBS and was ready to be run on the flow cytometer for compensation (stored at 4°C in the dark until use).

For the LIVE/DEAD stain andeFlour670 proliferation stain, compbeads can't be used to make compensation controls. The positive compensation control was prepared for the LIVE/DEAD stain by killing cells by treatment of cells with 50µl DMSO followed by incubation for 15 minutes. The cells were washed with 1X PBS and stained for dead cells with LIVE/DEAD stain to form the positive control. LIVE/DEAD-stained non-DMSO-treated "living cells" were used as the negative control. For the eFlour670 stain, a tube was prepared with a mixture of eFlour670-stained and unstained cells, for the positive and

negative compensation controls, respectively. Additionally, compbeads cannot be used with the goat secondary anti-rabbit alexa-fluor488 conjugate antibody so a single-stain was prepared with positive anti-mouse compbeads and a different mouse antibody that stains in the same FITC channel—anti-Ki67-FITC.

2.3.7 Data Acquisition and Analysis

Flow cytometry data was obtained from stained cells by acquiring on a LSRII flow cytometer (BD). Forward Scatter (FSC) and Side Scatter parameters were utilized to determine the lymphocyte population. Data was acquired by recording 100,000 events in the lymphocyte gate. Data was further analyzed on Flowjo software (Tree Star).

2.4 Regulatory T cell Function Experiments

2.4.1 Treg depletion

Whole blood was obtained from a healthy donor and isolated for PBMC. CD4+CD25+FOXP3+ Tregs were depleted from donor PBMC by staining with the Treg depletion Panel (Table 1) followed by the FACS sorting of the CD25highCD127low population using BD FACSCanto-II. Treg depletion was confirmed by staining with the Treg Panel (Table 1) and analyzed by flow cytometry.

2.4.2 Stimulation and Assessing Proliferation by Flow cytometry

Depleted and non-depleted fractions were centrifuged at 1600rpm for 10 minutes and stained with the eFlour670 proliferation dye (described in 2.3.5). Stained cells were centrifuged and resuspended in R-10 media at 2 x 10^6 cells/ml. Five-hundred µl of cell suspension (equal to 1 x 10^6 cells) were transferred to wells of a cell culture plate. Depleted and non-depleted fractions were unstimulated, stimulated with CD3/28 dynabeads (50ul dynabeads added to well), CEF peptides (5ug/ml) or stimulated via co-culture with mitomycin C-treated allo-cells (500ul of 6 x 10^6 cells/ml allo-cells added to well – 3:1 ratio). Cells were cultured at 37°C for 6 days. After 6 days, CD4 and CD8 proliferating cells were phenotyped by staining with the T cell Proliferation Panel (Table 1) and analyzed by flow cytometry.

2.4.3 Treg function Flow Cytometry Panels

Table 1 Flow Cytometry panels used to assess T Regulatory Cell function

	Panels					
	Treg Depletion		Treg		T cell Proliferation	
Fluorochrome	Marker	Source	Marker	Source	Marker	Source
Pacific Blue	CD3	BD	CD3	BD	--	--
APC-H7	CD4	BD	CD4	BD	CD3	BD
PE	CD25	BD	CD25	BD	--	--
PE-Cy7	CD127	BD	CD127	BD	--	--
AlexaFluor 700	--	--	--	--	CD4	BD
PE-Texas Red (ECD)	--	--	--	--	CD8	BD
PE-Cy-5	--	--	--	--	--	--
APC	--	--	FOXP3	eBioscience	Proliferation	eBioscience
FITC	--	--	--	--	--	--
Amcyan	--	--	LIVE/DEAD	invitrogen	LIVE/DEAD	invitrogen

Antibody Volume Used per Test: CD3-Pacific Blue: 1µl, CD4 APC-H7: 1µl, CD25-PE: 10µl, CD127-PE-Cy7: 5µl, FOXP3-APC: 5µl, CD3-APC-H7: 5µl, CD4-AlexaFluor700: 5µl, CD8-PE-TexasRed: 1µl, LIVE/DEAD dye: 1µl.

2.5 Treg-IRF-1 Expression Experiments

2.5.1 Determination of IRF-1 Expression in CD4+ T cells

Frozen PBMC obtained from healthy donors were thawed in a 37°C water bath, washed with R-10 media, rested for 3 hours at 37°C (outlined in 2.2.2) and resuspended in R-10 at 2 x 10^6 cells/ml. Five-hundred µl of cells (equal to 1 x 10^6 cell) were transferred to FACS

tubes. The FACS tubes were stained first for LIVE/DEAD, followed by surface staining for CD3, CD4, CD25, CD127, CD45RA and intracellular staining for IRF-1 and FOXP3 (Table 2). IRF-1 MFI was quantified in bulk CD4+ cells, conventional CD4+CD25+FOXP3+ Tregs, and naïve and effector Treg subsets by flow cytometry.

2.5.2 IRF-1 Expression Flow Cytometry Panel

Table 2 IRF-1 expression flow cytometry panel used to assess IRF-1 expression in CD4+ and Treg subsets

Fluorochrome	IRF-1 Expression Panel	
	Marker	Source
Pacific Blue	CD3	BD
APC-H7	CD4	BD
PE	CD25	BD
PE-Cy7	CD127	BD
AlexaFluor 700	CD45RA	BD
PE-Texas Red (ECD)	--	--
PE-Cy-5	--	--
APC	FOXP3	eBioscience
FITC	IRF-1	invitrogen
Amcyan	LIVE/DEAD	invitrogen

Antibody Volume Used per Test: CD3-Pacific Blue: 1µl, CD4 APC-H7:1µl, CD25-PE: 10µl, CD127-PE-Cy7: 5µl, FOXP3-APC: 5µl, CD45RA-Alexa700: 5µl, IRF-1: 1µl, LIVE/DEAD dye: 1µl.

2.6 Treg Differentiation, FOXP3-IRF-1 Kinetics Experiments

2.6.1 CD4+CD25- Isolation from PBMC

Whole blood was obtained from a healthy donor and PBMC were isolated. A cell suspension of 50 million cells/ml was prepared and 1ml of cells was transferred a FACS tube. Fifty µl of the CD4+ T cell enrichment cocktail (EasySep™ Human CD4+ T Cell Enrichment Kit— Stemcell) was added to the FACS tube and mixed thoroughly. After a 10 minute incubation period, 100µl of EasySep magnetic particles were added to the FACS tube and incubated at room temperature for 5 minutes. The volume was then topped up to 2.5mL with FACS wash. The FACS tube was then placed into the Stemcell magnet for 5 min. The supernatant was then poured off into a fresh FACS tube, this was the CD4+ enriched fraction. The FACS tube was then centrifuged at 1500 rpm for 7 minutes and resuspended in 100µl FACS wash. Fifty µl of the Easysep CD25 Positive Selection Cocktail (Stemcell) was added to the tube and incubated for 15 minutes. Twenty-five µl of the EasySep magnetic particles were mixed into the tube and topped up to 2.5mL with FACS wash. The tube was placed into the Stemcell magnet for 10 minutes. The supernatant was then poured off into a fresh FACS tube, this was the purified CD4+CD25- fraction. The cells were then centrifuged at 1500rpm for 7 minutes and resuspended in R-10 media.

2.6.2. *in vitro* Treg Induction Assay

Purified CD3 antibodies were coated to culture plate wells. A 1mL mixture of 5µg/ml CD3 antibody was prepared by diluting 10µl stock CD3 antibody in 990µl PBS (1/100 dilution). One-hundred µl of CD3 antibody was added to each well and incubated at 37°C for 4 hours. Following incubation, the mixture was aspirated out and rinsed once with PBS. The culture plate was kept in the incubator until use.

The CD4+CD25- cells were resuspended to a concentration of 2 x 10^6 cells/ml with R-10 media. 500µl of the cell suspension was added to the CD3-coated culture well. Additionally, 1µl of purified CD28 antibody and 4µl of 1/10 diluted TGF-β1 (2ng/mL concentration) were mixed with the CD4+CD25- cells. In one condition, recombinant IL-2 was added at a concentration of 0.3ng/mL). The cell culture plate was incubated at 37°C for 6 days. Aliquots of cells were obtained after DAY1, DAY3, Day 6 and stained for LIVE/DEAD and surface (CD3, CD4, CD25) and intracellular (FOXP3 and IRF-1) markers to determine the kinetics of IRF-1 and FOXP3 expression in Treg differentiation (Table 3).

2.6.3. Treg Induction Flow Cytometry Panel

Table 3 Treg induction flow Cytometry panel used to assess FOXP3 and IRF-1 kinetics in Treg differentiation

Fluorochrome	Treg Induction Panel	
	Marker	Source
Pacific Blue	CD3	BD
APC-H7	CD4	BD
PE	CD25	BD
PE-Cy7	--	--
AlexaFluor 700	--	--
PE-Texas Red (ECD)	--	--
PE-Cy-5	--	--
APC	FOXP3	eBioscience
FITC	IRF-1	invitrogen
Amcyan	LIVE/DEAD	invitrogen

Antibody Volume Used per Test:CD3-Pacific Blue: 1µl, CD4 APC-H7:1µl, CD25-PE: 10µl, FOXP3-APC: 5µl, IRF-1: 1µl, LIVE/DEAD dye: 1µl.

2.7 Treg Seroconversion Study

2.7.1. Samples

PBMC samples were obtained from non-HESN HIV-negative CSW from Pumwani district in Nairobi, Kenya. Control samples were acquired from non-HESN CSW and case samples were acquired from non-HESN CSW that became infected with HIV (after sample was

acquired). Ethics approval was obtained from research ethics boards at the University of Manitoba and University of Nairobi/Kenyatta National Hospital.

2.7.2. Treg Immuno-phenotyping

Frozen pre-seroconversion (case) and non-seroconversion (control) PBMC samples were thawed, LIVE/DEAD stained, followed by surface (CD3, CD4, CD25, CD127, CD45RA, CCR7) and intracellular (CTLA-4,FOXP3 and Ki67) staining (Table 4). Treg percentages, phenotype, proliferation and CD4+ memory subsets were assessed by flow cytometry.

2.7.3. Treg Seroconverion Panel

Table 4 Treg Seroconversion Flow Cytometry Panel used to assess Treg percentages, phenotype, and CD4+ memory subsets in HIV seroconversion

Fluorochrome	Treg Seroconversion Panel	
	Marker	Source
Pacific Blue	CD3	BD
APC-H7	CD4	BD
PE	CD25	BD
PE-Cy7	CD127	BD
AlexaFluor 700	CD45RA	BD
PE-Texas Red (ECD)	CCR7	BD
PE-Cy-5	CTLA-4	BD
APC	FOXP3	eBioscience
FITC	Ki67	eBioscience
Amcyan	LIVE/DEAD	invitrogen

Antibody Volume Used per Test: CD3-Pacific Blue: 1µl, CD4 APC-H7:1µl, CD25-PE: 10µl, CD127-PE-Cy7: 5µl, CD45RA-Alexa700: 5µl, CCR7-PE-TexasRed: 2.5µl Ki67-FITC: 2.5µl, CTLA-4-PE-Cy5: 5µl, FOXP3-APC: 5µl, LIVE/DEAD dye: 1µl.

2.8 Statistical Analysis

Statistical tests - Kruskal-Wallace (Ch4), Friedman and Dunn post-hoc (Ch4), Spearman Correlation (Ch4), Mann-Whitney U (Ch5) - were performed on Graphpad PRISM 6 software. Differences between compared groups were considered significant if p-value was equal to or less than 0.05.

Chapter 3 **Regulatory T cell Function**

3.1 Rational

A previous study of the Pumwani CSW revealed that HESN women have elevated CD4+CD25+FOXP3+ regulatory T cells in peripheral blood compared to HIV-susceptible women. Murine and *in vitro* models have shown that CD4+CD25+FOXP3+ T cells are suppressive in function. We sought to show the suppressive nature of Tregs through the *in vitro* depletion of CD4+CD25+FOXP3+ in healthy donors.

3.2 Hypothesis

1. **CD4+CD25+FOXP3+ Tregs are *suppressive* in function**
    a. Depletion of CD4+CD25+FOXP3+ Tregs will result in increased CD4 and CD8 effector T cell responses

3.3 Objective

1. Deplete CD4+CD25+FOXP3+ Tregs from healthy donor PBMC, stimulate depleted fraction with CEF peptides, CD3/28 dynbeads and allo-cells and assess the T effector cell proliferation response

3.4 Results

3.4.1. Treg depletion

CD4+CD25+FOXP3+ Tregs were depleted from PBMC by the removal of the CD25+CD127low population by flow sorting. We were able to achieve efficient depletion of CD4+CD25+FOXP3+ Tregs (94% depletion efficiency) (Figure 5).

3.4.2. Effect of Treg Depletion on T effector responses

Treg-depleted and non-depleted fractions were stimulated for 6 days and assessed for CD4 and CD8 T cell proliferation by flow cytometry. Cell proliferation was quantified by measuring expression of eFlour670 proliferation dye – which is decreased as cells undergo proliferation. We expected that Treg depletion from PBMC will result in increased T cell proliferation following stimulation with CEF peptides, CD3/28 Dynabeads and allo-cells – indicating the suppressive nature of Tregs. CEF peptides are generally weak stimulation agents that elicit memory responses to CMV, EBV and Flu antigens. Allostimulation with allo-cells (mixture of PBMC from different donors) elicit intermediate host-graft responses against non-self MHC. CD3/28 dynabeads are strong stimulation molecules that crosslink CD3, CD28 receptors and initiate T cell activation.

Preliminary analysis of one healthy donor revealed increased CD4 and CD8 T cell proliferation following allo-stimulation of the Treg-depleted fraction compared to the non-

depleted fraction (Figure 6). The intensity of the response was quantified by measuring the ratio between the baseline unstimulated response and the stimulated response, which we characterized as the fold change. In the CD4+ T cell compartment, the donor showed an allo-stimulation response fold change of 42 in the Treg-depleted fraction while the non-depleted fraction had a fold change of 25. For CD8+ T cells, the difference was more pronounced; the Treg-depleted fraction had a fold change response of 45 while the non-depleted had a fold change response of 14 (Figure 7).

3.4.3. Summary

The preliminary finding that *in vitro* depletion of CD4+CD25+FOXP3+ Tregs results in increased CD4+ and CD8+ T cell responses indicates that CD4+CD25+FOXP3+ are suppressive in function. However, a definitive conclusion requires data from more samples.

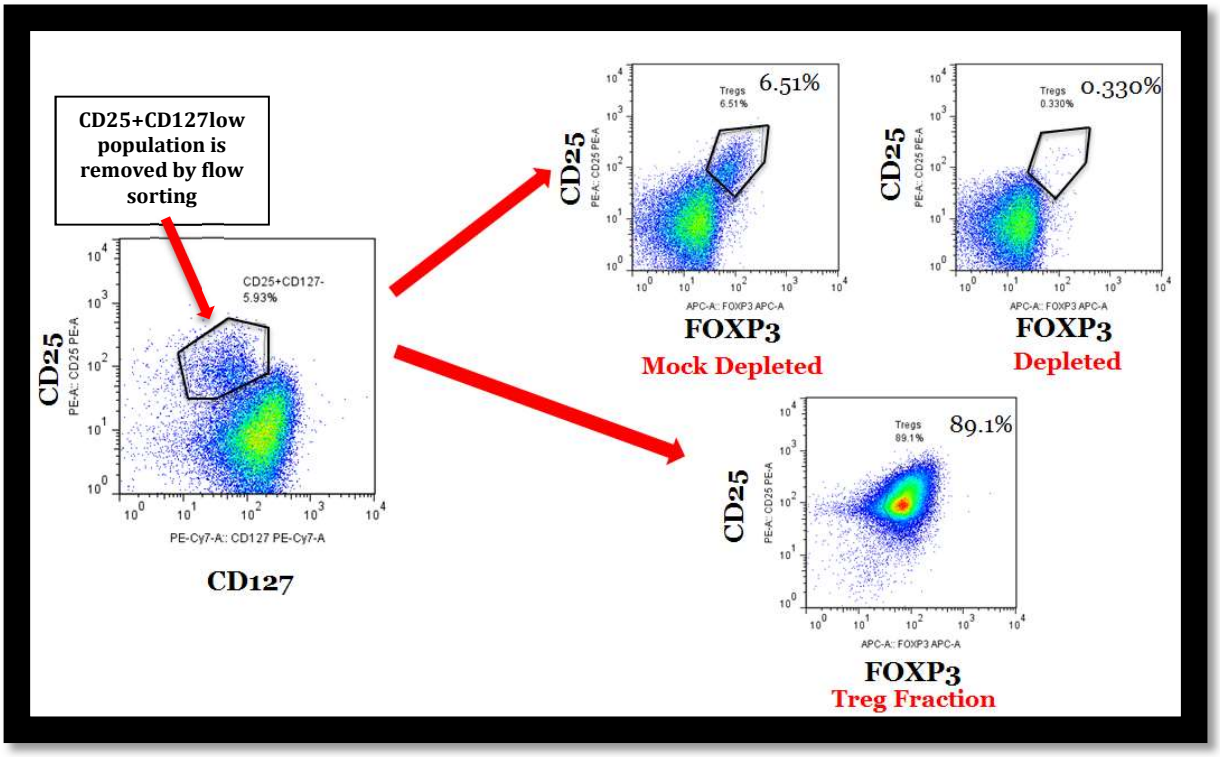

Figure 5: Schematic diagram of in vitro Treg depletion by flow sorting. PBMC were first stained with anti-CD25 and anti-CD127 Tregs followed by the removal of CD25+CD127- population in a BD FACSCanto II flow cytometer. To confirm depletion, Treg-depleted and isolated Treg fractions were stained with Treg panel antibodies and quantified by flow cytometry. CD4+CD25+FOXP3+ Tregs were depleted from PBMC at an efficiency of 94%.

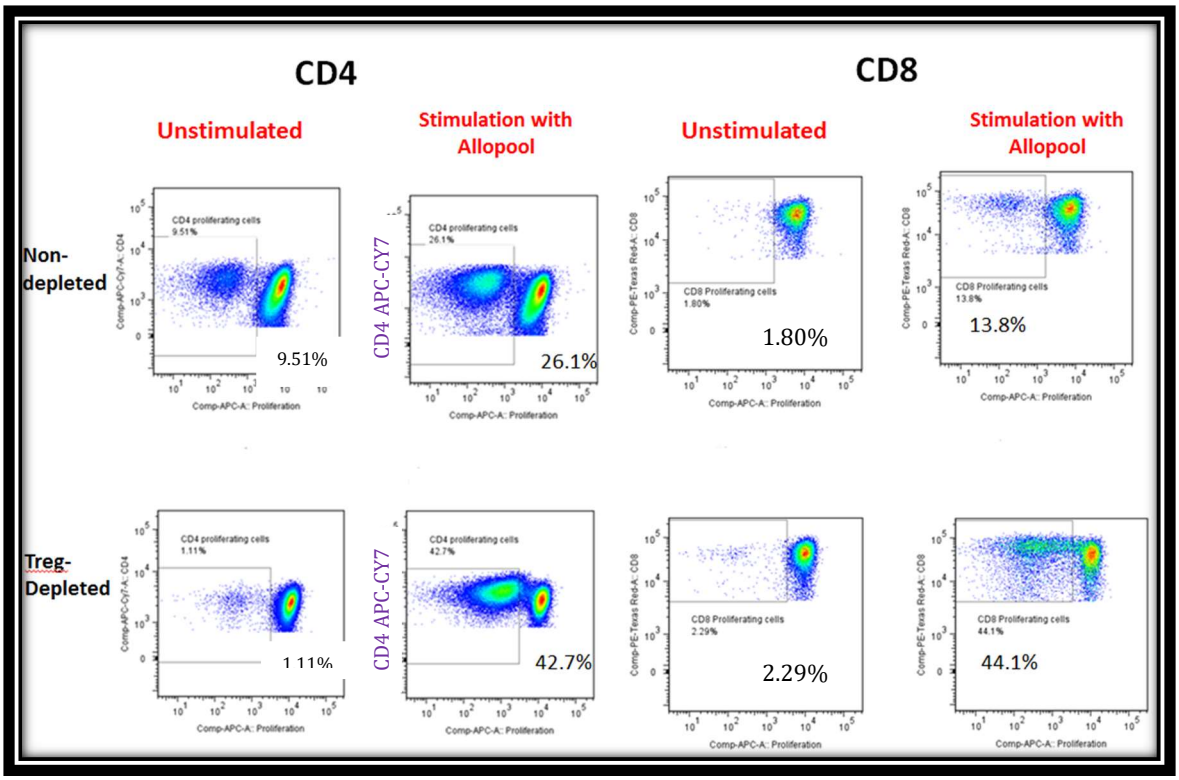

Figure 6: Flow Cytometry plots of CD4+ and CD8+ T cell proliferation responses of Non-depleted and Treg-depleted fractions of healthy donor 215. Treg-depleted and Non-depleted PBMC fractions were stained with eFlour670 proliferation dye and co-cultured with mitomycin C-treated allo-cells at 37°C for 6 days. Proliferating cells display decreasing expression of eFlour670 dye. Donor 215 showed increased CD4 and CD8 T cell proliferation following allo-stimulation in the Treg-depleted fraction compared to the non-depleted fraction.

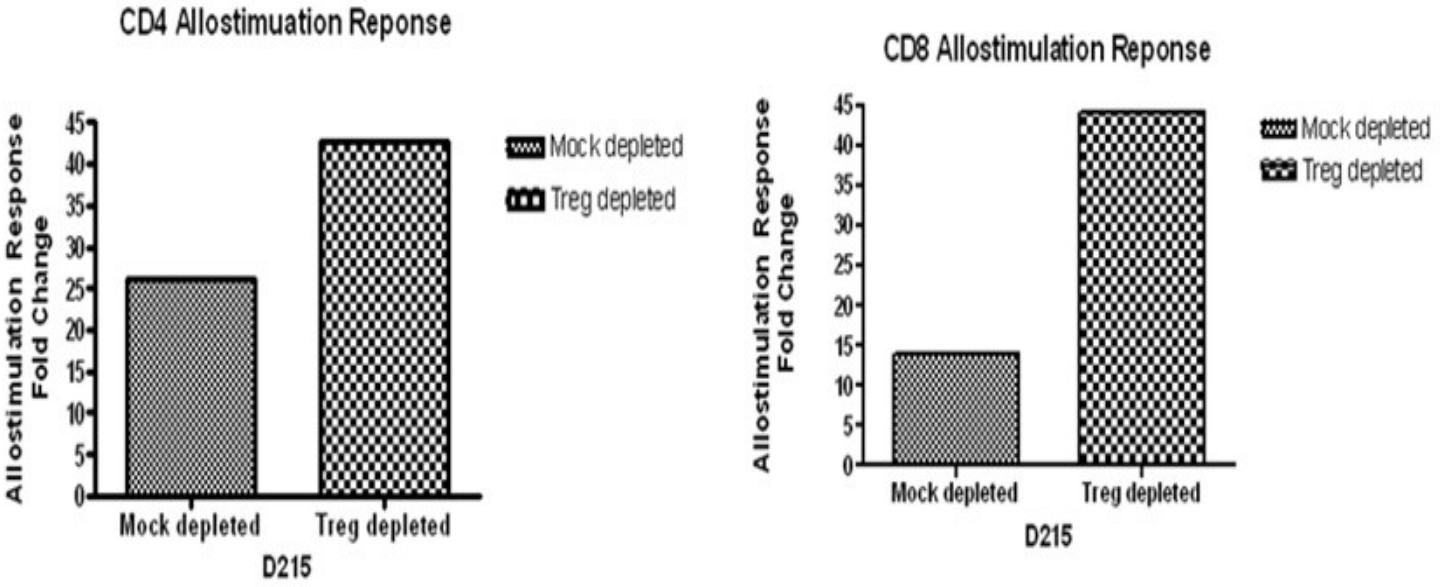

Figure 7: Graphical representation of CD4 and CD8 T cell allo-stimulation responses of mock-depleted (non-depleted) and Treg-depleted fractions of healthy donor 215. Donor 215 showed increased CD4+ and CD8+ allo-stimulation responses in Treg-depleted fraction compared to non-depleted fraction.

Chapter 4 **Relationship between IRF-1, FOXP3 and Tregs in HESN and human primary cells**

4.1 Rational

Murine studies have shown that IRF-1 negatively regulates Treg development and function by directly repressing transcriptional expression of FOXP3 and indirectly by modulating DCs to favor Treg differentiation. Research of HESN has shown higher Treg numbers in the peripheral blood compared to HIV susceptible women. HESN women have also been shown to have lower baseline expression of IRF-1. These findings of reduced expression of IRF-1 and elevated T regulatory cells were characterized in separate studies. No attempts have been made to determine if reduced IRF-1 expression is driving the increased Treg numbers in HESN subjects. Furthermore, the relationship between IRF-1, FOXP3 and Tregs in human cells has not been characterized.

4.2 Hypotheses

1. **Reduced IRF-1 expression drives increased FOXP3 expression and thus higher frequency of Tregs in HESN individuals**
2. **Tregs have reduced IRF-1 expression compared to other cell types**
3. **Increasing FOXP3 expression in Tregs is associated with reduced IRF-1 expression**

4.3 Objectives

1. Define the relationship between IRF-1 and Tregs in HESN women
2. Analyze the expression of IRF-1 in Tregs
3. Characterize the relationship between IRF-1, FOXP3 and Tregs through an *in vitro* Treg induction assay

4.4 Results

4.4.1. The Relationship between IRF-1 and Tregs in HESN

Previous data from the Pumwani CSW cohort revealed that HESN have protective polymorphisms in the IRF-1 locus that result in reduced IRF-1 expression. Other reports indicated that HESN have significantly more Tregs than non-HESN women. Using existing genotype and Treg frequency data of HESN, we sought to determine if there was an association between the IRF-1 genotype and Treg proportions. There are three types of polymorphisms observed in HESN; protective haplotypes (619AA, 179179, 6516GG), neutral haplotypes (619AC, 179XXX, 6516GT) and haplotypes associated with increased susceptibility (619CC, 179XXX, 6516TT). We hypothesized that the protective IRF-1 genotypes would be associated with increased proportions of Tregs. We analyzed the association between IRF-1 genotypes and Treg proportions using a Kruskal-Wallis (ANOVA) statistical test. The data showed no association between the IRF-1 genotype and Treg frequency. (Figure 8).

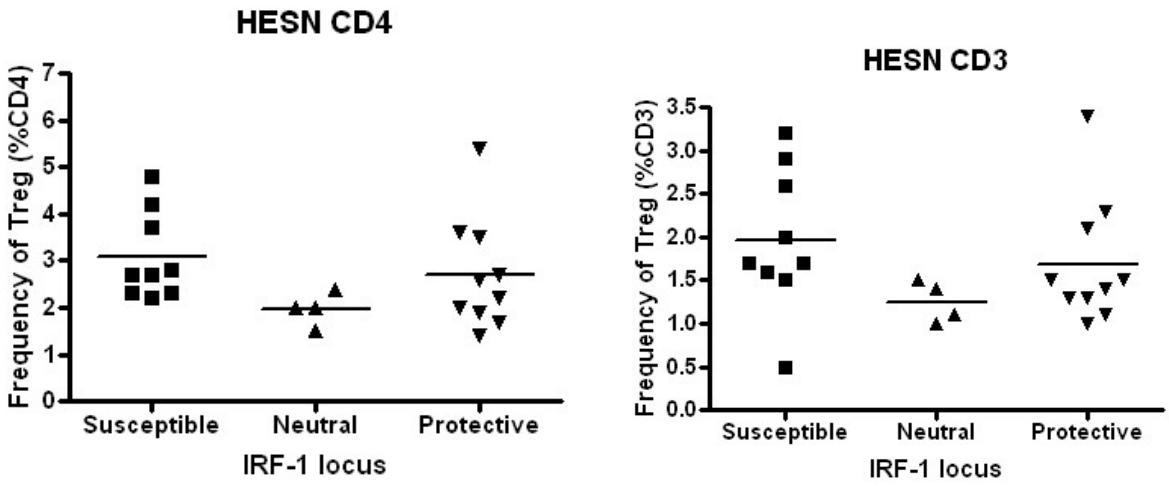

Figure 8: Graphical representation of the association between susceptible, neutral and protective IRF-1 genotypes with Treg frequency (%CD3 and %CD4) in HESN women. No association between IRF-1 genotype and Treg Frequency was observed in HESN.

However, not all HESN exhibit the protective IRF-1 genotype – HESN can also regulate IRF-1 expression epigenetically different than HIV-S women. So, we sought to further clarify the link between IRF-1 and Tregs in HESN by analyzing the relationship between IRF-1 expression and Treg proportions in HESN. Using whole PBMC IRF-1 expression data from HESN individuals (from a previous study), an analysis was performed to determine if IRF-1 MFI correlated with Treg frequency. Data was evaluated using a Spearman non-parametric correlation test. Interestingly, IRF-1 MFI inversely correlated with Treg frequency (Tregs%CD4 $p<0.0001$, $r= -0.7626$, Tregs%CD3 $p=0.0002$, $r= -0.7450$) (Figure 9).

4.4.2. IRF-1 Expression in Tregs

Murine studies have definitively shown that IRF-1 is a negative regulator of FOXP3 and Tregs, however, the role of IRF-1 in Tregs in human cells is unclear. We hypothesized that since IRF-1 is a negative regulator of FOXP3 in mice, IRF-1 expression would be decreased in Tregs compared to other CD4+ T cells.

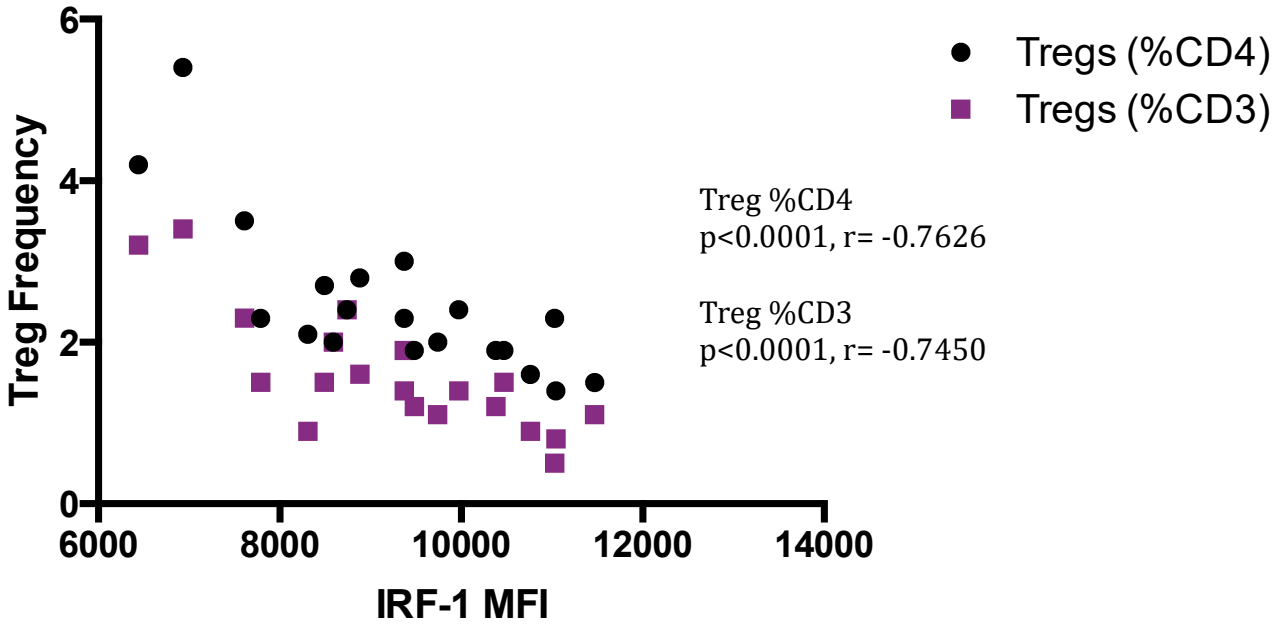

Figure 9: Graphical representation of the association between IRF-1 expression of whole PBMC and Treg frequency (%CD3 and %CD4) in HESN women. An inverse correlation between IRF-1 Expression and Treg Frequency was observed in HESN.

Analysis of IRF-1 expression was performed in PBMC samples from healthy donors. IRF-1 expression was evaluated in bulk CD4+ cells, CD4+CD25- cells and CD4+CD25+FOXP3+ Tregs. The data showed that CD4+CD25+FOXP3+ Tregs have significantly lower expression of IRF-1 compared to bulk CD4+ and CD4+CD25- cells ($p<0.0001$; Friedman Test, Dunns-multiple comparison test) (Figure 10). An analysis of IRF-1 expression in naïve and effector Treg subsets was also performed. The data was analyzed using the Friedman statistical test, followed by the Dunns post-hoc test. IRF-1 expression was significantly decreased in naïve Tregs compared to effector Tregs and CD4+CD25+FOXP3+ Tregs ($p=0.0027$) (Figure 11).

4.4.3. Kinetics of IRF-1 Expression during Treg Induction

To further understand the relationship between IRF-1 and Tregs in human primary cells, we utilized an *in vitro* Treg induction assay to assess IRF-1 expression kinetics in Tregs. We hypothesized that due to the negative relationship between IRF-1 and FOXP3, increasing FOXP3 expression would be associated with reduced IRF-1 expression in Tregs throughout Treg induction.

CD4+CD25- cells from two healthy donors were cultured in the presence of TGF-β (which directly induces FOXP3 expression) recombinant IL-2, anti-CD3 and anti-CD28. After 3 days in culture, CD4+CD25+FOXP3+ Tregs develop, along with activated CD4+CD25+FOXP3- cells.

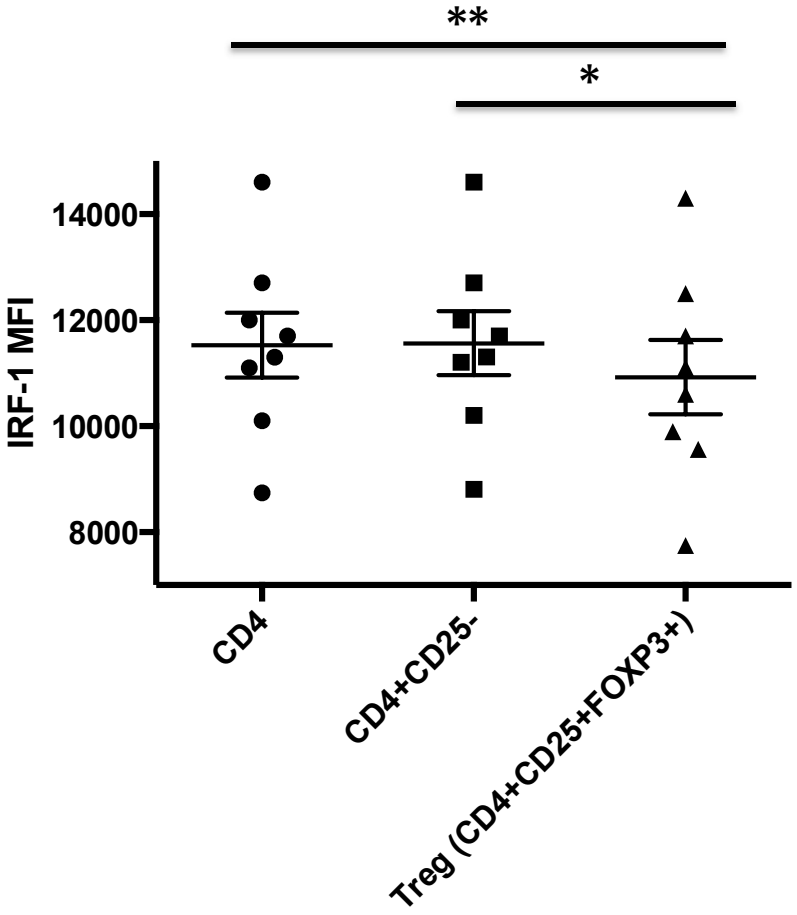

Figure 10: Graphical representation of IRF-1 expression in CD4+ T cell subsets of healthy controls. IRF-1 expression (MFI) was measured by surface and intracellular staining of bulk CD4+, CD4+CD25- cells and Tregs (CD4+CD25+FOXP3+), followed by quantification by flow cytometry. CD4+CD25+FOXP3+ Tregs have significantly reduced expression of IRF-1 compared to bulk CD4+cells and CD4+CD25- cells.

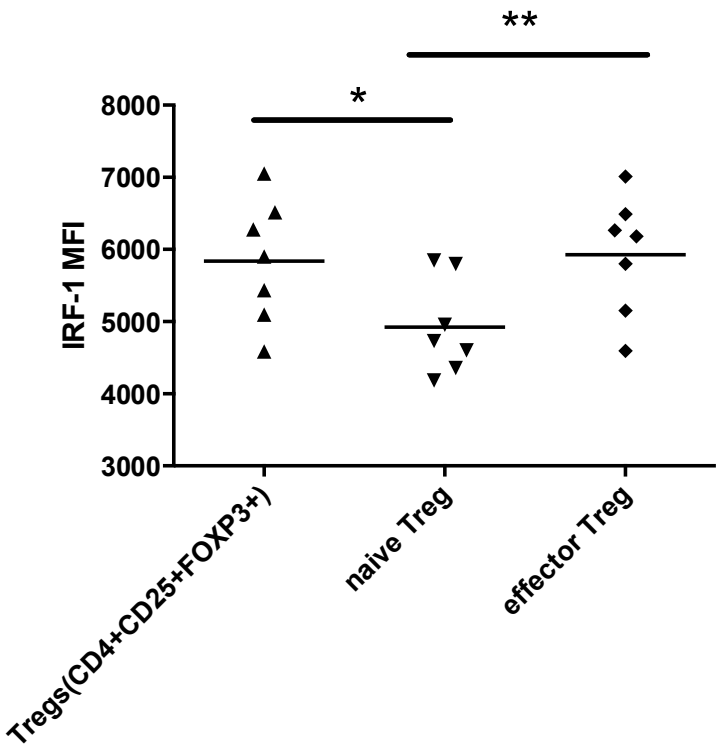

Figure 11: Graphical representation of IRF-1 expression in Treg subsets of healthy controls. IRF-1 expression (MFI) was measured by surface and intracellular staining of conventional Tregs, naïve Tregs and effector Tregs, followed by quantification by flow cytometry. Naïve Tregs have significantly decreased expression of IRF-1 compared to effector Tregs and conventional CD4+CD25+FOXP3+ Tregs.

Treg frequency and FOXP3 expression peaked at DAY3 and by DAY6, dramatically decline (Figure 12). IRF-1 expression was assessed in CD4+CD25- cells, CD4+CD25+ cells and CD4+CD25+FOXP3+ cells at DAY1, DAY3 and DAY6. We expected that low IRF-1 expression would be maintained in Tregs compared to CD4+CD25+ and CD4+CD25- cells. In fact, it was determined that IRF-1 was up-regulated in Tregs throughout Treg differentiation (at DAY1, DAY3 and DAY6), particularly at DAY3, when FOXP3 expression was highest (Figure 13B, 13C). Moreover, Tregs upregulated IRF-1 more than activated CD4+CD25+ T cells and resting CD4+CD25- cells. This result was unexpected since IRF-1 is a negative regulator of FOXP3.

4.4.4. Summary

Analysis of the relationship between IRF-1 and Tregs in HESN revealed no association between IRF-1 genotype and Treg frequency. However, when IRF-1 expression of whole PBMC was analyzed, an inverse relationship between IRF-1 and Tregs was determined. This finding is in line with our hypothesis of reduced IRF-1 expression driving Treg proportions. To address the link between IRF-1 and Tregs in human cells, IRF-1 expression in CD4+ cells was analyzed in primary cells from healthy controls. Compared to other CD4+ T cells, IRF-1 expression was lowest in Tregs, particularly in naïve Tregs – supporting our initial hypothesis. Lastly, analysis of the kinetics of IRF-1 and FOXP3 expression in Tregs during Treg differentiation revealed that IRF-1 is up-regulated with increasing FOXP3 expression in Tregs. Additionally, Tregs maintained higher expression of IRF-1 compared to activated CD4+CD25+FOXP3- cells. This directly proportional relationship between IRF-1 and FOXP3 was not expected and did not support the hypothesis.

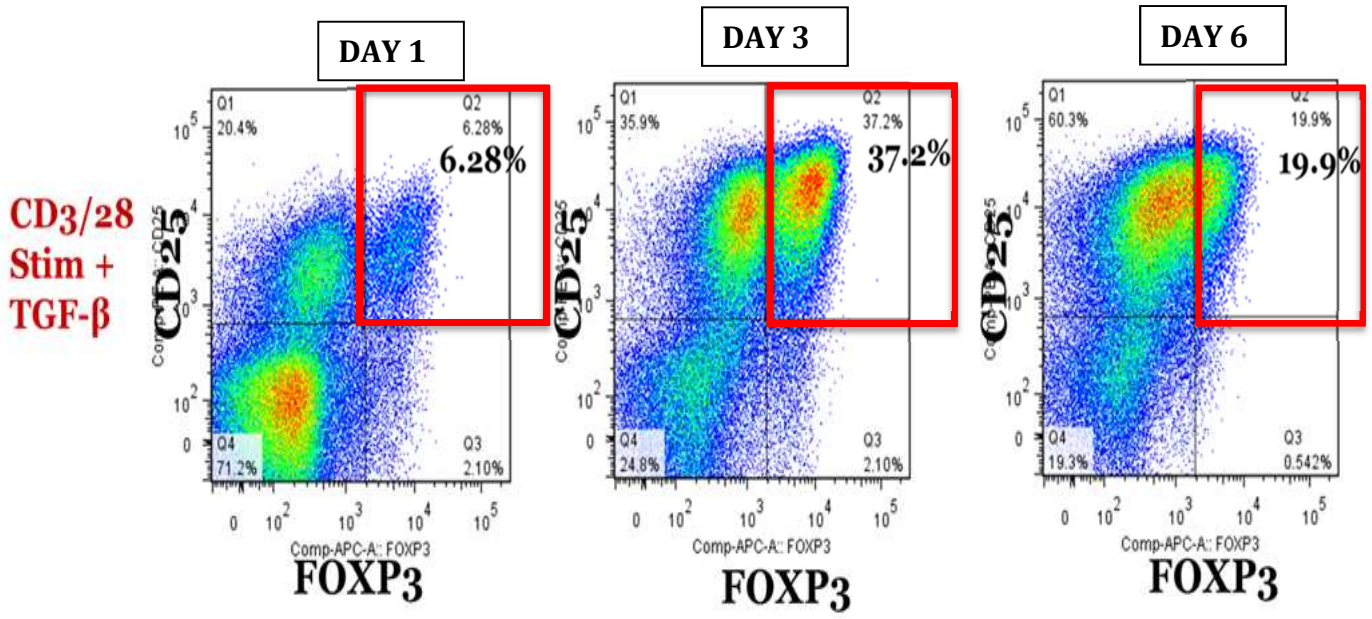

Figure 12: Flow Cytometry Plots of induced CD4+CD25+FOXP3+ Tregs at DAY 1, 3 and 6 of *in vitro* Treg induction. CD4+CD25- cells were purified from PBMC from Donor 154 and incubated in the presence of anti-CD3, anti-CD28 and TGF-β at 37°C for 6 days. Aliquots were removed at DAY 1, 3, and 6 and stained for Tregs. Treg frequency was quantified by flow cytometry. Treg frequency was limited at DAY1, peaked at DAY3 and decreased significantly by DAY 6.

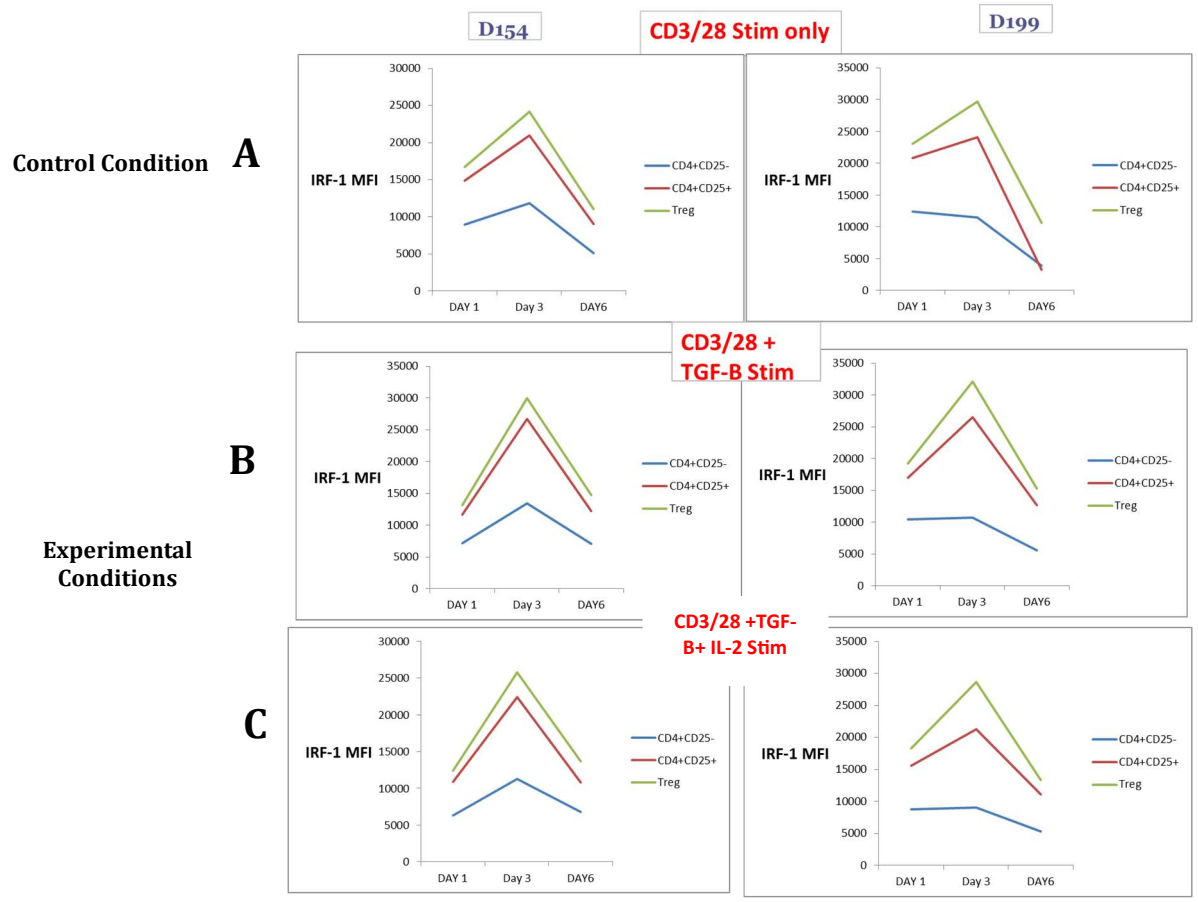

Figure 13: Graphical representation of IRF-1 expression kinetics during *in vitro* Treg induction in CD4+CD25-, CD4+CD25+ and Tregs of Donors 154, 199. IRF-1 expression (MFI) was measured after DAY1, DAY3 and DAY6 following stimulation with anti-CD3, anti-CD28, TGF-β andIL-2. **A.** Stimulation with anti-CD3 and anti-CD28 (control). **B.** Stimulation with anti-CD3, anti-CD28 and TGF-β only. **C.** Stimulation with anti-CD3, anti-CD28, TGF-β and IL-2. In experimental conditions (**B,C**), IRF-1 expression was upregulated in Tregs compared to CD4+CD25+ and CD4+CD25- cells. IRF-1 expression in Tregs peaked at DAY3, followed by downregulation of expression at DAY6.

Chapter 5 **Regulatory T cells in HIV Seroconversion**

5.1 Rational

A preliminary study of pre-HIV-seroconversion samples revealed that increased CD4 and CD8 activated CD38+CD69+HLA-DR+T cells were associated with HIV seroconversion. Since Tregs suppress CD4 and CD8 T cells and have been shown to be elevated in protected HESN individuals, we became interested in the proportion of Tregs in these pre-HIV-seroconversion samples. We hypothesized that increased CD4 and CD8 T cell activation in pre-seroconversion samples may be a consequence of decreased suppression by Tregs –- suggesting that decreased Tregs may be driving increased CD4 and CD8 T cell activation in HIV seroconversion.

5.2 Hypothesis

1. **Decreased Treg frequency is associated with HIV seroconversion**

5.3 Objectives

1. Characterize Treg subsets and frequency in pre-seroconversion samples by flow cytometry
2. Characterize CD4+ memory subsets in pre-seroconversion samples by flow cytometry

5.4 Results

5.4.1. Study Samples

Pre-seroconversion PBMC samples (the last sample prior to HIV seroconversion) were obtained from high-risk female CSW from the Pumwani sex worker cohort in Nairobi, Kenya. Individuals who seroconverted and appropriate age-matched controls were retrospectively selected for the study. The control samples were obtained from age-matched HIV-negative high-risk CSW from the same cohort. In total, 21 frozen PBMC samples were used for this study (12 control, 9 case). Flow Cytometry data was measured for statistical significance using the Mann-Whitney U test.

5.4.2. Regulatory and CD4+ Memory T cell Phenotyping

Phenotypic analysis of pre-seroconversion samples revealed no significant difference in CD4+CD25+FOXP3+CD127low Treg proportions compared to control subjects ($p=0.69$) (Figure 14A). Additionally, there was no difference in naïve or effector Treg proportions between the groups. We observed a significant increase in CTLA-4+expression on naïve Tregs in HIV sero-converters compared to controls ($p=0.05$) (Figure 14B). Similarly, we analyzed proportions of CD4+memory subsets; CD4+naïve (T_{naive}), effector memory (T_{em}), central memory (T_{cm}) and terminally differentiated (T_{eff}) memory subsets, and found no difference in HIV sero-converters compared to controls. Interestingly, we found significantly elevated expression of CTLA-4 on effector memory and central memory CD4+

T cells in HIV-seroconverters (p=0.02 and p=0.02, respectively) (Figure 14 C-D). Furthermore, HIV-seroconverters had significantly increased intracellular CTLA-4 expression in CD4+naïve, effector memory and terminally-differentiated memory subsets (p=0.01, p=0.02, p=0.01, respectively, Figure 14 E-G) compared to controls.

5.4.3. Summary

Contrary to our hypothesis, we did not observe the association of decreased Tregs with HIV seroconversion. This would suggest that decreased Tregs may not be driving increased CD4 and CD8 T cell activation in HIV-seroconverters. Interestingly, we observed increased surface expression of CTLA-4 on naïve Tregs. Phenotypic analysis of CD4+ memory subsets revealed significant up-regulation of surface and intracellular CTLA-4 in HIV-seroconverters compared to controls.

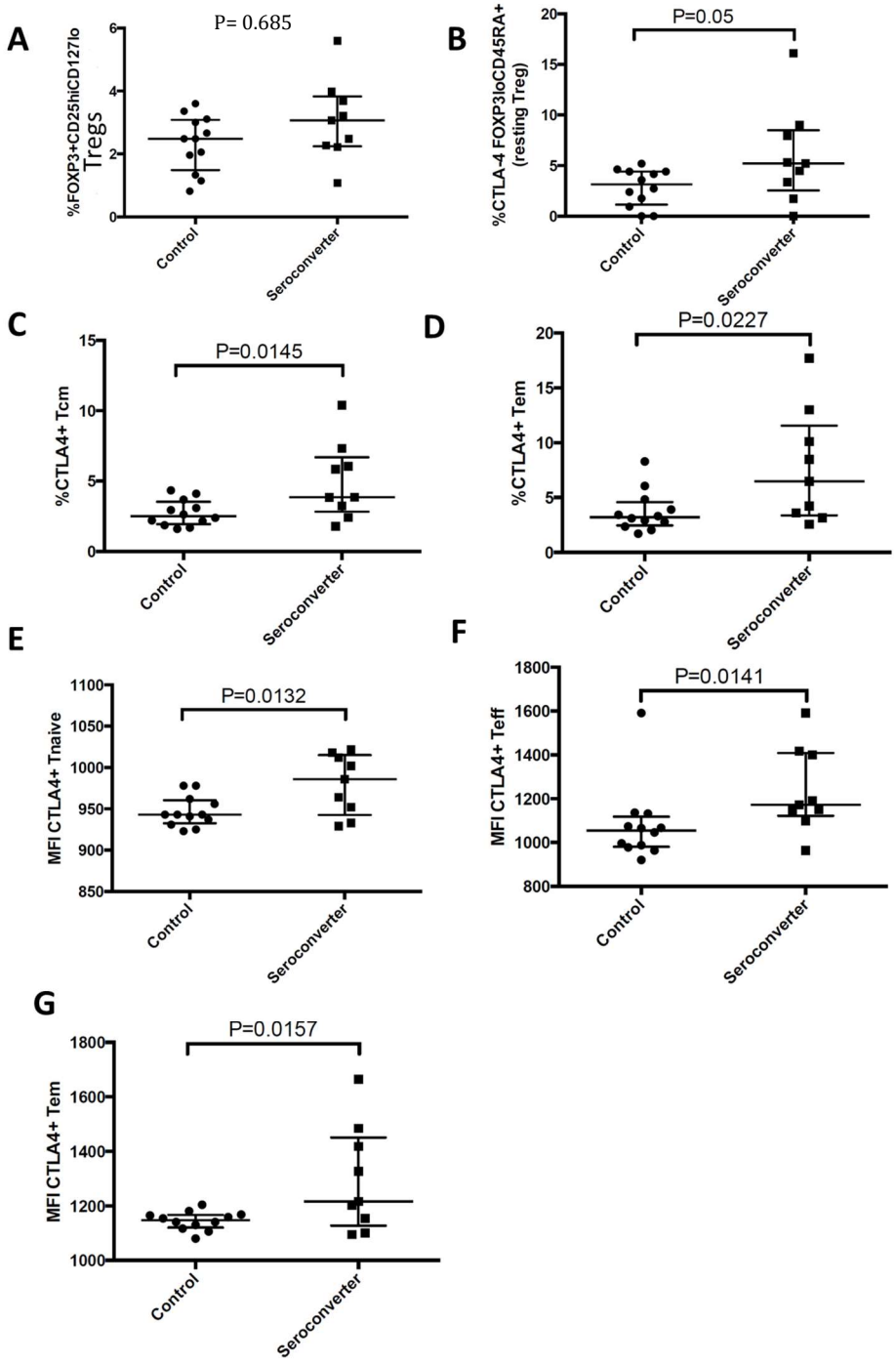

Figure 14: Comparison of Treg frequency, CTLA-4 surface and intracellular expression in CD4+ memory cells between HIV–sero-converters and controls.**(A)**No difference in Treg frequency was observed between groups.**(B)** CTLA-4 expression was elevated in naïve (resting)Tregs in HIV seroconverters compared to controls.**(C-D)**Increased surface CTLA-4 expression was observed on central memory (Tcm) and effector memory (Tem) T cells in HIV sero-converters compared to controls.**(E-G)**Elevated intracellular CTLA-4 expression was observed in naïve (Tnaive), effector (Teff) and effector memory (Tem) T cells in HIV sero-converters compared to controls.

Chapter 6 **Discussion**

6.1 Regulatory T cell Function

The overall goal of the Treg function study was to confirm CD4+CD25+FOXP3+ Treg functionality through *in vitro* experiments, providing evidence for the suppressive function of Tregs in HESN and thus supporting the IQ model. We had initially hoped to use samples from HESN and HIV-S individuals to elucidate possible differences in Treg function between these two groups. However, due to the difficulty in obtaining these samples (Treg function studies are cell-intensive, requiring a large amount of blood from patients), we performed the Treg functional studies in primary cells from healthy controls.

We utilized a unique flow sorting method to deplete CD4+CD25+FOXP3+Tregs from PBMC. Other studies have depleted Tregs in PBMC based on CD25+ expression, however, this also removes the activated CD4+CD25+ population that may be contributing to immune activation. We depleted Tregs by removing the CD25+CD127low cells by flow sorting and achieved an efficient depletion and a pure population of Tregs (Figure 5). To determine the suppressive effects of depleted Tregs, the depleted population was stimulated with different stimulation agents and effector proliferation responses were quantified by flow cytometry.

We hypothesized that CD4+CD25+FOXP3+ Tregs are suppressive in function and *in vitro* depletion of these cells would result in increased CD4 and CD8 T cell responses. Analysis of a healthy donor revealed increased CD4 and CD8 T cell proliferation as a consequence of

Treg depletion, suggesting that CD4+CD25+FOXP3+ Tregs have suppressive effects. Interestingly, this was only observed by stimulation with the allo-pool of cells and not with CD3/CD28 co-stimulation or CEF peptide stimulation. This is likely due to the weak CEF response of the donor or the strong stimulation effect of CD3/28, which may mask the subtle suppressive effect of Tregs. Thus, allo-stimulation may be the best stimulation condition to reveal Treg suppression in *in vitro* Treg depletion experiments.

6.1.1. Limitations and Opportunities

Many studies have previously demonstrated that *in vitro* depletion of Tregs results in increased effector T cell responses [109,138,139]. In this proof of concept experiment, we established the preliminary finding that Treg depletion results in increased proliferation of CD4+ and CD8+ T cells. While this finding is interesting, the evidence was limited to the analysis of only one healthy donor. To conclusively confirm the suppressive effect of CD4+CD25+FOXP3+ Tregs, this study must be replicated with several samples. Other effector functions such as pro-inflammatory cytokine production and expression of activation markers should be analyzed to fully characterize the effect of Tregs. Also, determining the mechanism of suppression (contact-dependent or contact-independent) is important in characterizing Treg function. Furthermore, performing these studies in HESN and HIV-S individuals may be informative in elucidating the role of Tregs in IQ.

6.2 The Relationship between IRF-1, FOXP3 and Tregs in HESN and human primary cells

We first became interested in the IRF-1-Treg relationship when analysis of HESN revealed increased Treg frequency in peripheral blood and independently, reduced expression of IRF-1 by polymorphisms in the IRF-1 locus and differential epigenetic regulation of IRF-1. Several murine studies had already established IRF-1 as a negative regulator of FOXP3 and Tregs, by direct repression of its promoter and indirectly by modulation of DCs. Hence, IRF-1 has a clear negative effect on FOXP3 and Tregs in mice. The goal of this study was to clarify if reduced IRF-1 expression was driving the increased Treg frequency observed in HESN. We examined the association of the IRF-1 protective genotype and Treg proportions in HESN and found no association. However, when we analyzed IRF-1 expression of whole PBMC and Treg proportions, we observed an inverse correlation. This evidence supported our hypothesis of reduced IRF-1 expression driving Tregs in HESN. However, the combined data (Treg proportion/IRF-1 expression) used in this analysis was obtained from the same patients, however, the samples were acquired on different dates (samples were not date-matched). This introduces variables that may impact the observed results. Thus, the inverse correlation between IRF-1 expression and Tregs observed in HESN, while striking, serves as indirect evidence for the hypothesis that reduced IRF-1 expression drives Tregs proportions in HESN.

To further clarify the link between IRF-1 and Tregs, we examined the IRF-1-Treg relationship at the cellular level in healthy controls. We analyzed IRF-1 expression in Tregs compared to different CD4+ T cell subsets and found that CD4+CD25+FOXP3+ Tregs have

lower IRF-1 expression compared to bulk CD4+ cells and CD4+CD25- cells, supporting our hypothesis. Downregulation of IRF-1 expression in Tregs is likely due to the fact that IRF-1 acts as a repressor of FOXP3; since FOXP3 is up-regulated in Tregs, it should have limited IRF-1 expression. When IRF-1 expression was evaluated in Treg subsets, we found that naive Tregs (CD45RA+FOXP3low) had lower expression of IRF-1 compared to effector Tregs (CD45RA-FOXP3high) and conventional CD4+CD25+FOXP3+ Tregs. This was an interesting result since naïve Tregs express less FOXP3 than effector Tregs, yet had lower IRF-1. This can be explained by the fact that naïve Tregs are "resting" Tregs and subsequent activation (and conversion into effector Tregs) may lead to IRF-1 up-regulation. This is supported by *in vitro* studies that show that T cell activation results in IRF-1 up-regulation [115,116,118].

We continued the analysis of the relationship between IRF-1 and Tregs in human cells by examining the kinetics of IRF-1 expression during Treg differentiation. It was expected that increasing FOXP3 expression in Tregs would be associated with reduced IRF-1 expression. The evidence showed the opposite; induced Tregs up-regulated IRF-1 expression throughout differentiation and had higher IRF-1 expression compared to activated CD4+CD25+ cells and resting CD4+CD25- cells, cell populations that do not express FOXP3.There are two possible explanations for why IRF-1 is highly expressed in Tregs throughout Treg differentiation and more than activated/resting cells. First, the *in vitro* Treg induction assay requires co-stimulation of CD3/CD28, this process highly activates T cells and can result in significant up-regulation of IRF-1. Secondly, *in vitro* differentiated Tregs are likely not "real" Tregs but rather unstable Treg-like cells that are over-expressing

FOXP3. We observed at DAY3 of Treg differentiation, CD4+CD25+FOXP3+ "Tregs" are a single distinct population and are maximally expanded (37%). By DAY6, the distinct population of "Tregs" disappears and the proportions decrease (19.9%) (Figure 12). This observation is supported by studies that showed Tregs lose FOXP3 expression and become "exTregs" in inflammatory conditions [140,141]. Thus, the observed up-regulation of IRF-1 expression with FOXP3 is likely a reflection of the *in vitro* Treg induction method. Tregs observed in physiological conditions are likely able to maintain low IRF-1 expression, even when they become activated.

6.2.1. Limitations and Opportunities

The goal of this study was to examine if reduced IRF-1 expression drives Treg proportions in HESN. We established indirect evidence for this with the inverse correlation of IRF-1 expression and Treg proportions in HESN. However, to establish a direct link between IRF-1 expression and Treg proportions, we needed to assess the "cause and effect" relationship between IRF-1 and Tregs. This could be achieved by IRF-1 knockdown in CD4+CD25- cells followed by Treg differentiation; this experiment would provide insight into the effect of IRF-1 on Treg differentiation. We attempted some preliminary studies with siRNA knockdown of IRF-1 in CD4+CD25- primary cells, however our experiments lacked sufficient knockdown of IRF-1 and therefore were discontinued. It may also be prudent to use a different Treg differentiation method than the CD3/28 + TGF-β assay used in this study. This method (due to co-stimulation of CD3+CD28) strongly up-regulates IRF-1, which may obscure subtle effects of IRF-1 knockdown on FOXP3 and Treg development.

One alternative is to use a compound called rapamycin to differentiate Tregs, which has been used in culture to efficiently differentiate Tregs *in vitro* [142]. One area that is lacking in understanding is the effect of IRF-1 on Tregs by DC modulation in humans. This gap in knowledge can be addressed by analyzing the relationship between IRF-1 expression in DCs and Treg proportions in HESN (reduced IRF-1 expression in DCs may be associated with higher Tregs). IRF-1 can also be knockdown in DCs in primary cells or cells lines and the impact on Treg differentiation can be analyzed. These experiments are necessary to fully understand the link between IRF-1 expression and Tregs.

6.3 Regulatory T cells in HIV Seroconversion

Several studies of HESN populations have highlighted the benefits of elevated Tregs in protection from HIV infection. The IQ model predicts that elevated proportions of Tregs decrease overall T cell activation, decreasing the pool of activated target cells that can be efficiently infected by HIV (Figure 1). However, the role of Tregs is HIV seroconversion is unclear. A preliminary study of pre-seroconversion samples obtained from the longitudinal analysis of female CSW from the Pumwani district in Nairobi, Kenya found that increased activated CD4 and CD8CD38+CD69+HLA-DR+T cells in peripheral blood was associated with HIV seroconversion. We hypothesized that since elevated Tregs may limit the number of activated target cells in protection from HIV infection, a decreased proportion may drive up immune activation and lead to HIV seroconversion. We examined the frequency and subset characterization of Tregs in these pre-seroconversion samples and found no difference in Treg frequency or subset proportions compared to the control group. This

suggests that Tregs may not have an impact in driving increased immune activation in HIV seroconverters, thus, other factors may be involved in driving the increased immune activation state of these individuals.

We also analyzed CD4+ memory T cell subsets and found significantly increased CTLA-4 expression on effector memory and central memory CD4+ cells in HIV seroconverters compared to controls. In addition, CD4+naïve, effector memory and terminally-differentiated memory cells in HIV seroconverters had elevated intracellular CTLA-4 expression. Interestingly, when we quantified CTLA-4 expression in Treg subsets, we found significantly increased surface expression on naïve Tregs of HIV seroconverters compared to controls. This was an unexpected finding, as CTLA-4 is associated with suppressive effects of Tregs and therefore is normally up-regulated in effector Tregs, not naive Tregs. We continued this investigation by studying the literature to elucidate the relationship between CTLA-4 expression, Tregs and susceptibility to HIV infection.

The Cytotoxic T Lymphocyte Associated Protein-4 (CTLA-4) molecule is a member of the immunoglobulin superfamily. CTLA-4 is stored intracellularly but is trafficked to the cell surface where it is expressed [143]. It is a negative regulator associated with inhibiting T cell activation[144] and has similar homology to the co-stimulatory molecule CD28 and acts in antagonistic manner. CD28 binds to B7 to deliver the required secondary signal for T cell activation, while CTLA-4 binds to B7 to inhibit T cell activation and proliferation. Studies have shown that CTLA-4 delivers the negative signal by associating with protein phosphatase 2A, which is a negative regulator of TcR activation and early cytokine genes

[145]. Studies have shown that CTLA-4 is a good candidate for targeting immune activation. Blocking of CTLA-4 with monoclonal antibodies has resulted in the stimulation of the immune response against cancer cells[139]. Conversely, CTLA-4 agonists have been implicated in the inhibition of immune activation, which has been shown to be beneficial in targeting autoimmune diseases[146].

Studies examining the expression of CTLA-4 have revealed differential expression in T cell subsets [147]. Studies have shown that resting non-Treg T cells have limited expression of CTLA-4, however, following T cell activation, CTLA-4 is rapidly trafficked from the intracellular stores to the cell surface. Thus, CTLA-4 is preferentially increased (both intracellularly and on the surface) in activated T cells [143]. Analysis of CTLA-4 expression kinetics after PMA/ionomycin stimulation has revealed that CD4+ cells show upregulation of CTLA-4 after 2 hours and have sustained expression for 48 hours post-activation [147]. Also, activated CD4+ cells have been shown to have greater CTLA-4 expression compared to activated CD8+ T cells [143]. Another study examining CTLA-4 expression in T cell subsets revealed that memory CD4+ T cells contain significantly increased intracellular reservoirs of CTLA-4 [147]. Conversely, Tregs have *constitutive* expression of CTLA-4 due to the transcriptional regulation of FOXP3 and following activation, up-regulate surface and intracellular CTLA-4[147]. This is not surprising, as CTLA-4 is necessary for the contact-dependent suppressive effects of Tregs. On the other hand, our finding of elevated surface CTLA-4 in naïve Tregs of HIV seroconverters is unexpected as naïve Tregs tend to have limited CTLA-4 expression and up-regulate CTLA-4 only after activation and conversion to effector Tregs. There are limited studies analyzing CTLA-4 kinetics in human Tregs. One

possible explanation for this unique naïve CTLA-4++Treg subset population in HIV seroconverters may be that elevated immune activation (observed in HIV seroconverters) may be driving increased transition of naïve Tregs to effector Tregs to counteract the increase in immune activation in these individuals. More studies need to be conducted to confirm this hypothesis.

What does that finding of increased CTLA-4 expression in memory CD4+ T cell subsets in HIV seroconverters suggest about HIV susceptibility? The evidence suggests that memory CD4+ cells in HIV-seroconverters are activated, which is consistent with the earlier finding of elevated activated CD4+ and CD8+ T cells in HIV seroconverters. The increased activation of CD4+ T cells may be resulting in more efficient target cells for HIV and thus eventual HIV seroconversion. This evidence also implicates CTLA-4 expression as an indicator of chronic immune activation in CD4+ T cells, which can be combined with other markers such as CD69, HLA-DR and CD38 to analyze *ex vivo* immune activation in CD4+ cells. Overall, this study provides more support for the link between increased immune activation and HIV susceptibility.

6.3.1. Limitations and Opportunities

While the hypothesis that decreased Tregs are driving increased immune activation and HIV seroconversion was disproved, we did observe some significant differences between HIV seroconverters and controls. Currently, genetic and cytokine studies are ongoing on the same samples to further identify the correlates of HIV seroconversion. In this study,

PBMC samples were limited (12 control, only 9 case), therefore, the availability of more samples could have provided stronger statistical results. A major limitation of studying HIV susceptibility in peripheral blood is that HIV is primarily transmitted sexually at the genital tract. The cellular environment in the peripheral blood is not equivalent to that of the genital tract. Thus, examining the cellular environment of the genital tract is essential. In this study, cervical mononuclear cells were not available but could have provided important insight into the mechanism of HIV seroconversion. Yet, this study does provide important supporting evidence for the link between immune activation and increased susceptibility to HIV; the full understanding of this relationship is essential in order to develop effective vaccines and other therapeutic strategies against HIV.

6.4 Significance and Future Directions

Regulatory T cells are crucial regulators of the human immune system; their effects have been implicated in the context autoimmunity, cancer, viral and parasitic infections. In cancer, viral and parasitic infections; Tregs suppress protective anti-tumor, anti-viral and anti-parasite responses. The study of autoimmune diseases and some viral infections have shown that Treg dysfunction or reduced numbers of Tregs are associated with excessive or uncontrolled inflammatory responses. Thus, Tregs can be beneficial or detrimental depending on the specific pathology. Many clinical studies are currently ongoing to target Tregs to provide novel therapeutic methods against these immune-pathologies. In pathologies such as cancer, viral and parasitic infections, in which Tregs are detrimental, strategies such as Treg depletion or neutralization are been utilized to inhibit Treg function [138]. Some of these methods include targeting Treg functional receptors such as CD25, CTLA-4, GITR, and OX40 with monoclonal antibodies. Some strategies include using drugs that inhibit IL-2 secretion, signaling and receptor expression. Other drugs prevent FOXP3 expression and Treg proliferation [138]. In autoimmune diseases, Tregs are beneficial, thus expanding Tregs through adoptive Treg therapy has been shown to be a promising strategy to limit autoimmunity [148]. This method involves isolating Tregs from blood, followed by *ex vivo* expansion using different chemical agents and adoptive transfer back into the patient. Some of these Treg immunotherapies are still being studied, while others are undergoing clinical trials. It is clear that targeting Tregs is a promising approach to developing protective remedies for various pathologies, thus, understanding Treg function and regulation is essential to develop novel therapeutic methods. In this study, we

characterized the effect of IRF-1 on Tregs, a relationship that was not evaluated previously in humans. The evidence suggests that IRF-1 is differentially expressed in Tregs and may have a negative effect on Treg proportions in the periphery as observed in HESN individuals. This preliminary evidence suggests that IRF-1 is an important regulator of Tregs in humans and thus may be targeted to influence Treg development and function. While more studies need to be conducted to fully elucidate this relationship, this study is the first step in understanding the negative regulatory effect of IRF-1 on Tregs in humans.

The central aim of this project was to understand the mechanisms that drive IQ. We investigated the functional role of Tregs and the impact of IRF-1 on Tregs. We observed CD4+CD25+FOXP3+ Tregs are suppressive *in vitro* and determined that IRF-1 may influence Tregs proportions in the periphery. Our evidence supports the role of elevated CD4+CD25+FOXP3+ Tregs in suppressing overall T cell activation, thus, contributing to the Immune Quiescent phenotype. On the other hand, our findings indicate that reduced IRF-1 may be driving the increased proportions of Tregs in HESN, thus, implicating IRF-1 as a driver of IQ. We also observed evidence that CD4+ T cell activation via increased CTLA-4 expression is associated with susceptibility to HIV infection. This is more support for the IQ model of resistance to HIV infection. Overall, this project gives us a better understanding of the mechanisms that drive IQ and can help inform the development of novel therapeutic strategies such as anti-microbial microbicides, which can mimic IQ by reducing immune activation at the genital mucosa and potentially leading to protection from HIV. HIV prevention in the past two decades has focused mainly on the development of HIV vaccines; which have resulted in few successes. The IQ model of HIV resistance supports

the idea that previous failures of HIV vaccines may be due to the indirect activation of the immune system, which may result in the recruitment of more efficient targets for HIV infection. Interestingly, this notion is supported by data from the failed STEP vaccine trial, which delivered HIV gag, pol and nef genes via the recombinant adenovirus sero-type-5 vector (Ad5) [149]. The study was halted midway because the vaccine group showed an increased rate of HIV acquisition compared to the placebo group; especially in vaccinees who had pre-existing immunity to Ad5 [149]. Some researchers have suggested that increased HIV acquisition in the vaccine group maybe the result of increased immune activation from pre-existing adenovirus vector-specific immunity, however, some reports have presented contradictory evidence [150]. Nevertheless, most vaccine studies suggest that HIV vaccines that elicit effective HIV-specific responses provide at least partial protection from HIV. The best HIV prevention strategy may be to combine an IQ-inducing microbicide that is applied vaginally or rectally with a HIV vaccine that elicits an effective HIV-specific response. This combination approach may have a potent effect against HIV. The next steps in clarifying the IQ model of resistance will be to identify microbicides or other drug candidates that can mimic IQ at the genital tract and begin testing in non-human primates, followed by clinical studies in high-risk human populations. If these studies are successful, the IQ model may prove to be paradigm-shifting in terms of HIV prevention and may help lead to the eventual eradication of HIV.

Appendix

Abbreviations

HIV – Human Immunodeficiency Virus

ART – Anti-retroviral Therapy

HESN – HIV-Exposed Sero-negative

CCW – Commercial Sex Workers

HIV-S – HIV-Susceptible

IQ – Immune Quiescence

Th – T helper cell

TcR – T cell Receptor

DC – Dendritic Cell

Tregs – Regulatory T cells

MHC – Major Histocompatibility Complex

FOXP3 – Forkhead box-p3 transcription factor

IRF-1 – Interferon Regulatory Factor-1

PBMC – Peripheral Blood Mononuclear Cells

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