The Functional Role of IRF1 Polymorphisms in Susceptibility to HIV-1 Infection

by

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Abstract

Altered susceptibility to HIV-1 infection has been observed in multiple cohort studies around the world, with a small proportion of HIV-Exposed, Seronegative (HESN) individuals remaining uninfected despite repeated exposure. This is the case with a subset of female sex workers (FSWs) in Nairobi, Kenya who can be epidemiologically defined as resistant to HIV infection. Several correlates of HIV resistance have been proposed; amongst the strongest of these are genetic polymorphisms in the *Interferon Regulatory Factor 1* (*IRF1*). IRF1, the first identified member of the IRF family, is one of the key players in the HIV infection process, important for early HIV replication, HIV disease progression, establishment of latency and initiation of innate antiviral immune responses. Three polymorphisms in *IRF1*, located at 619 (intron 1), the microsatellite region (GT repeat at intron 7) and 6516 (intron 9) of the gene, showed an association with natural protection against HIV-1 infection and reduced likelihood of seroconversion. Peripheral blood mononuclear cells (PBMCs) from patients with protective IRF1 haplotype exhibited significantly lower basal IRF1 expression and reduced responsiveness to IFNγ stimulation. Here I hypothesized that the identified IRF1 polymorphisms directly regulate alternative splicing resulting in altered IRF1 protein regulation and function. Changes in IRF1 regulation will impact the IRF1-mediated host immune responses and directly impact HIV-1 replication thus contributing to the HIV-resistant phenotype. The main objectives of this thesis are to characterize the effect of three IRF1 polymorphisms associated with HIV-resistant phenotype on: (1) IRF1 gene transcription, alternative splicing of IRF1 mRNA and IRF1 mRNA/protein

stability (2) plasma and cervical lavage (CVL) cytokine/chemokine expression and (3) HIV pathogenesis and disease progression. Furthermore since differences in hormone expression are known to regulate IRF1 function we set out to determine if differences in plasma hormone levels contribute to the natural resistance against HIV-1 infection in the Majengo HESN cohort.

These studies revealed that polymorphisms in the *IRF1* gene do not directly affect IRF1 transcription but instead act as intronic splicing regulators. PBMCs from individuals with protective IRF1 haplotype were associated with increased inclusion of exons 7/8 and decreased protein stability when compared to cells from individuals with nonprotective haplotype. Individuals with protective IRF1 haplotype also exhibited significantly higher plasma IL15, IFNγ and IL6 expression and significantly higher CVL IL15, IFNγ, IL2 and sIL2Rα expression (with univariate analysis only) compared to those with nonprotective IRF1 haplotype. Additionally, individuals with protective IRF1 haplotype expressed significantly lower levels of plasma prolactin when compared to individuals with nonprotective haplotype. IRF1 polymorphisms were found to not be associated with HIV disease progression, suggesting that the protective effect of IRF1 polymorphisms is limited to the early stages, prior to establishment of HIV-1 infection. Furthermore, independent of IRF1 polymorphisms, significantly lower plasma prolactin, estrogen, progesterone and cortisol levels were observed in HESN women suggesting that hormonal regulation may be one of the main factors regulating natural resistance to HIV-1 infection in the Majengo HESN cohort.

Dedication

This thesis is dedicated to my grandparents: Hajrudin and Hanumica Sivro and Bešir and Almasa Isaković. The stories of your struggles during the World War II have taught me the true meaning of courage, virtue, honor and integrity. I am proud to be your granddaughter.

Radimlja stećak necropolis, Stolac, Bosnia and Herzegovina

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1. Introduction

1.1 The HIV/AIDS Epidemic

Since the first case of acquired immunodeficiency syndrome (AIDS) was reported in 1981[1], almost 70 million people have been infected with the Human Immunodeficiency Virus (HIV) and an estimated 36 million people have died of AIDS during this period according to the 2013 WHO report. About 6,300 people were newly infected with HIV each day in 2012 (WHO). The burden of the epidemic varies considerably between countries and regions with 95% of new infections and deaths occurring in developing countries[2]. Sub-Saharan Africa accounts for 69% of the people living with HIV worldwide and had the highest adult prevalence (15-49 years) of 4.6% in 2011(UNAIDS). HIV prevalence in Kenya ranges from 4-7% in all provinces except Nyanza province where the prevalence is 15.1% (Cherutich P, Annual Review Meeting 2014, University of Nairobi STD/AIDS Collaborative Group). Of the estimated 100, 000 new infections per year in Kenya, 30% occur in young women (<25 years of age) with majority of these infections occurring in girls under 18 years of age (Kilonzo N, Annual Review Meeting 2014, University of Nairobi STD/AIDS Collaborative Group).

Currently, women comprise more then half of individuals living with HIV worldwide (UNAIDS, 2013). Heterosexual transmission accounts for over 80% of new HIV infections in women, and HIV prevalence in sub-Saharan Africa is 3.4-7 fold higher among adolescent girls compared to adolescent men[3]. HIV acquisition and

prevention in women is highly complex and depends on a combination of biological[4,5], behavioral and social factors[3]. Girls and young women are more vulnerable to contracting HIV due to social factors that include gender norms, violence against women, lack of education and economic stability[6,7]. Female sex workers (FSW) are a key population for HIV transmission and modes of transmission studies suggest that paid sex contributes significantly to the global HIV burden[8]. HIV prevalence in sex workers in Nairobi has been estimated at 29.3%, and condom use is still inconsistent, in particular with sub-groups such as regular clients (Nyaboke D, Annual Review Meeting 2014, University of Nairobi STD/AIDS Collaborative Group). A deeper understanding of protective immune responses against HIV acquisition in women is crucial for the development of successful HIV prevention and therapeutic modalities and the control of the HIV/AIDS epidemic.

1.2 HIV Virology

1.2.1 Classification and structure

HIV belongs to family *Retroviridae*, subfamily *Lentivirinae*, and genus *Lentivirus*. There are two major viral sub-types: HIV-1 and HIV-2. Both HIV strains resulted from a cross-species transmission of simian immunodeficiency virus (SIV), which naturally infects African primates. HIV-1 is thought to be derived from a chimpanzee SIVcpz strain and HIV-2 from a sooty mangabey SIVsmm strain[9]**.** In terms of prevalence**,** HIV-1 has a worldwide distribution while HIV-2 is mainly constricted to Western Africa. Based on their genetic similarities, HIV-1 strains fall into three

divergent lineages: group M, N and O. The majority of the HIV pandemic is compromised of M strains, that have been phylogenetically classified into nine subtypes or clades (A, B, C, D, F, G, H, J and K) and subclades (A1, A2, F1 and F2). The predominant clades in Kenya are A1 and D, while clade B circulates in North America[10,11]. HIV genetic variability is a result of high rates of viral replication combined with high mutation and recombination rates of the viral reverse transcriptase. Recombination between different subtypes has resulted in several circulating recombinant forms (CRFs) and even more unique recombinant forms (URF). The high degree of HIV sequence diversity represents a major challenge for host immune responses, as well as the development of treatment and vaccine strategies.

Structurally, HIV has a single stranded, positive sense ribonucleic acid (RNA) genome of ~9.7 kilobases. HIV gene products can be broadly classified into structural (Gag and Env), catalytic (Pol), regulatory (Tat and Rev) and accessory (Vpu, Vif, Vpr and Nef) categories. Gag and Pol are synthesized as a polyprotein and cleaved by the viral protease into four structural proteins [MA (matrix), CA (capsid), NC (nucleocapsid) and p6] and three enzymes (protease, reverse transcriptase and intagrase). The envelope protein (Env) is cleaved into gp 120 (the outer envelope protein that binds CD4 and co-receptors on T cells) and gp41 (the transmembrane protein that anchors the glycoprotein complex to the surface of the virion). The regulatory proteins Tat and Rev are expressed early in the virus life cycle and play a crucial role in enhancing viral replication. Additionally Tat acts as a robust transactivating protein affecting host gene expression in both infected and non-

infected cells[12]. The accessory HIV proteins (vpu, vif, vpr and nef) distinguish HIV from other retroviruses and play an important role in regulating various host antiviral responses and increasing viral replication.

1.2.2 HIV-1 Life cycle

HIV enters the target cell by an interaction between viral envelope proteins (gp120 and gp41) with the host cell surface receptors (predominantly CD4, CXCR4 and/or CCR5). Activated CD4+ T cells are the major targets for HIV infection, since they express high levels of HIV receptors on their surface and are highly permissive for viral production. To a much smaller degree, HIV can also actively infect innate immune cells including macrophages, myeloid dendritic cells (mDCs), plasmocytoid DCs (pDCs) and Langerhans cells[13,14]. Following binding, the HIV envelope and host cell membrane fuse allowing HIV to enter the cell. Once inside the cell, the viral reverse transcriptase is released leading to the synthesis of a double stranded DNA proviral genome. The reverse transcription is error prone and introduces a mutation approximately $3x10^{-5}$ per replication cycle[15,16], contributing to the extensive viral diversity observed in HIV. The HIV integrase allows the HIV DNA to enter the nucleus and integrate into the host chromosome. Once integrated into the host genome HIV can remain latent for the lifetime of the cell[17]. The virus utilizes several host cell factors to initiate transcription of the viral genome at the 5' long terminal repeat (LTR) region[18]. Translation of HIV transcripts produces polyproteins that are cleaved by HIV protease into individual proteins. Viral assembly occurs at the plasma membrane and is followed by budding of viral

progeny. HIV infected cells can undergo rapid cell death, enter latency or become persistent virus-producing factories[19].

1.3 HIV Treatment and Prevention

1.3.1 HIV treatment

The major achievement in the fight against HIV/AIDS since the identification of HIV as the causative agent of AIDS in 1983, has been the development of antiretroviral therapy (ART). Drugs that target viral enzymes (reverse transcriptase, integrase and protease) and cell binding steps necessary for viral replication and entry are all now FDA-approved and in use globally. Successful ART results in viral suppression, partially restored immune function, improved quality of life, increased life span and reduced viral transmission. ART effectively reduces the transmission of HIV from mother to child[20] and was recently shown to be effective as pre-exposure prophylaxis (PrEP) for HIV uninfected people at high risk for infection[21]. While these benefits are impressive, ART does not eliminate viral reservoirs and there are potential short-term and long-term toxic effects associated with the lifelong adherence including higher than normal risk of cardiovascular diseases, cancer, osteoporosis and other end-stage diseases[22]. Additionally, despite recent advances in treatment delivery only about 34% of people needing antiretroviral therapy worldwide under the 2013 WHO guidelines are actually receiving it, and this number is expected to increase as the number of people living with HIV increases (UNAIDS, 2013). While ART can successfully control viral replication in

HIV infected people, it does not lead to viral eradication due to the early establishment of latent viral reservoirs[23]. With the increasing global HIV burden, and the high costs and adverse effects associated with lifelong antiretroviral therapy recent years have seen an increase in research aimed at finding a cure for HIV.

1.3.2 HIV cure

The inability to cure HIV in part lies in the complexity of the latent HIV reservoir, persistent viremia and an aberrant and dysfunctional host immune response. The establishment of latency within the genome of HIV target cells, despite being a rare event, occurs in the early stages of HIV infection and is a major barrier to viral eradication in infected individuals. HIV latency represents a cellular reservoir of HIV-1 that is unaffected by current ART regimens and undetected by the host immune response. Latency is thought to occur when a newly HIV-1 infected cell exits the cell cycle and returns to the resting state. This is thought to occur most frequently in resting memory CD4+ T cells, however the exact mechanism of proviral latency is not fully understood[24]. Based on prospective clinical studies, it is estimated that the half-life of these stable infected resting cells is 44 months in patients on highly active ART (HAART). Based on this, complete viral eradication would take more then 60 years on ART, making current treatment strategies unable to have an impact on a true HIV cure [24-27]. Complete elimination of the virus has only been achieved in one person, the so-called Berlin patient. In February 2007 a 40 year old HIV infected man with acute myelogenous leukemia, following myeloablative chemotherapy (whole- body irradiation), received a transplantation

of hematopoetic stem cells from a donor with a homozygous mutation (Δ32) in the gene encoding C-C chemokine receptor 5 (CCR5)[28]. A Δ32 mutation in the CCR5 gene has been shown previously to render cells highly resistant to HIV-1 infection[29]. Antiretroviral therapy was terminated and the patient remains without any sign of HIV infection suggesting that a sterilizing HIV cure had been achieved. More recently, two patients with Hodgkin lymphoma received a bone marrow transplant from donors with wild-type CCR5 in order to asses whether chemotherapy treatment could reduce or eliminate the HIV reservoirs by replacing infected cells with those of HIV uninfected donors[30]. Unfortunately when ART was terminated HIV replication eventually resumed in both patients[31].

One of the major obstacles to successful therapy is the inability to clear quiescent, replication –competent provirus in resting CD4+ T cells. Therefore the majority of HIV cure research has been focused on identifying treatments that can activate viral replication in quiescent cells such that these cells become susceptible to cytopathic effects of HIV replication and viral clearance by the host immune response and/or ART. Histone deacetylase (HDAC) inhibitors (such as valproic acid and trichostatin A) were shown to activate the replication of latent virus *in vitro,* however recent evidence suggests that HDAC inhibitors might be less effective in activating latent virus in primary T lymphocytes[32]. As with ART, HIV eradication will most likely require a combination approach: an anti-latency treatment to successfully induce expression of proviral genomes; and strategies to enhance host antiviral immune response and speed up viral clearance[33]. However, ethical issues need to be considered in the approaches that activate viral replication in

individuals who are successfully treated with ART. Recent evidence suggests that early ART treatment immediately post infection has the potential to reduce the size of the latent reservoir and lead to post-treatment viral control in a small proportion of early-treated individuals [34]. This is further supported by a recent case of the "Mississippi baby", an infant born to a HIV-1 infected woman that began receiving aggressive treatment 30 hours after birth[35]. After therapy was discontinued HIV-1 viremia remained undetectable through 30 months of age, suggesting that early ART initiation could interfere with quantities and/or qualities of persistent reservoirs of replication-competent virus. However a mathematical modeling analysis showed that implementation of combined strategies such as early ART and PrEP will not stop HIV-1 transmission completely despite leading to dramatic declines in HIV-1 incidence[36]. Until further progress is realized, a preventative HIV-1 vaccine as a part of the comprehensive prevention and treatment strategies remains the best option in controlling the HIV/AIDS pandemic[37,38].

1.3.3 HIV Vaccine development

While the search for an HIV vaccine remains one of the highest public health priorities, attempts to develop an effective vaccine against HIV-1 have been largely unsuccessful[25,39]. Vaccine efforts have been marked by difficulty in eliciting high–titre broadly neutralizing antibodies and an inability to achieve broad T –cell-mediated protection[40]. The recent Thai RV144 vaccine trial provided the best results so far, with a 31.2% efficacy in the modified intent-to-treat analysis, but showed no protective effect against disease progression in vaccinated study

participants who subsequently became infected[41]. Many of the difficulties in developing a preventive and/or curative strategy lie with the large deversity of HIV and its complex relationship with the host[25,42]. As with all viruses HIV must utilize a number of cellular factors for successful viral replication[42,43]. HIV's replication cycle is therefore dependent on direct and indirect interaction between numerous viral and host proteins[43] and a full understanding of this complex interplay is likely to be key in developing future treatment and prevention modalities.

1.4 HIV pathogenesis and host immune response

1.4.1 Brief overview of host immune system

Skin and mucosal membranes provide the first line of defense against invading pathogens. Beyond their role as a physical barrier, mucous membranes produce mucus and various antimicrobial proteins that trap and inhibit invading microbes. Pathogens that penetrate the physical barrier face the host immune response, which can be broadly divided into innate and adaptive arms.

The innate immune system acts rapidly after an invading pathogen is encountered and involves various cell types including neutrophils, monocytes, macrophages, dendritic cells (DCs), natural killer (NK) and several other cell types that are specific to various tissues. Phagocytic cells, such as neutrophils, monocytes and macrophages, act by engulfing and digesting invading microbes, infected or dying cells or other foreign particles. Dendritic cells act as antigen-presenting cells

(APCs) by capturing antigens, processing them and presenting them on the cell surface along with appropriate costimulatory molecules (e.g. CD86 and CD80). They play a key role in regulation of adaptive immune response and establishment of immunological memory. Dendritic cells can be further divided into myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). Plasmacytoid DCs are known as interferon producing cells and play a major role in antiviral immunity. Interferons (IFNs), commonly know as antiviral cytokines, represent important regulators of both innate and adaptive immunity and provide protective effects against a range of viral pathogens, including influenza and HIV. Activated by monocyte and macrophage-derived cytokines (such as IL15 and IFNγ), NK cells play an important role in clearing virus-infected and cancerous cells. The cytotoxic activity of NK cells is mediated by activating and inhibiting receptors on the cell surface, including Killer Immunoglobulin-like receptors (KIRs), that recognize classical human leukocyte antigen (HLA) class I molecules and modulate immune response to infected or tumor cells. In addition to their cytotoxic function, cells of the innate immune response secrete a range of cytokines and chemokines that act to attract and activate specific arms of the innate and adaptive immune response.

The key defining features of the adaptive immune response are specificity and immunological memory, resulting in a rapid and a robust antigen-specific response upon secondary exposure to a pathogen. The adaptive immune system is primarily mediated by T and B lymphocytes, the latter of which participate in humoral immunity by producing antibodies in response to antigen stimulation. Adaptive immune responses are typically induced at the organized peripheral

tissues called secondary lymphoid organs through which naïve T cells are continually migrating. Naïve T lymphocytes recognize antigens presented by APCs and this interaction initiates a signal transduction cascade resulting in cytokine production, proliferation and T cell differentiation. T lymphocytes can be separated into two major categories: CD4+ T helper (Th) cells and CD8+ T cells. Th cells produce cytokines and coordinate B and T cell immune responses. When activated by APCs, the Th cells can differentiate into Th1, Th2, Th17 and regulatory T cell (Treg) subsets based on production of signature cytokines. Th1 cells secrete IFNγ and tumor necrosis factor α (TNFα) and are primarily involved in defending the host against intracellular pathogens; while Th2 cells secrete interleukins (IL) 4, 5, 10 and 13 and mainly defend the host against extracellular pathogens. Th17 cells produce IL17, IL22 and IL21 and are important in protection against extracellular pathogens, however they can also contribute to severe inflammation and autoimmunity. Tregs, in contrast, play an important role in maintaining immunological unresponsiveness to self-antigens and in suppressing and controlling host immune responses that might be damaging to the host. Cytotoxic CD8+ T lymphocytes (CTL), also known as killer T cells, recognize and destroy cells bearing "foreign" antigens and provide potent defenses against virus infection and intracellular pathogens. Although they differ with respect to their mechanisms of action, synergy between innate and adaptive immunity is essential for an intact and fully functional immune response.

1.4.2 The infected cell and host restriction factors

To enable its replication, HIV must counter host cell factors that restrict viral replication and production. It is generally thought that HIV infection arises from a

single, or relatively few founder viruses, establishing a single focus of infected mucosal CD4+T cells[13,44-47]. The failure of most infected foci to develop into an established infection site may in part be explained by the effects of the host antiviral responses that act to prevent early HIV replication and viral dissemination. IFNs, known for their wide ranging antiviral effects have been shown to inhibit HIV replication in number of *in vitro* studies. IFN induction leads to activation of numerous interferon-stimulated genes (ISGs) some of which function as antiviral restriction factors. IFNα regulated, apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC3G) acts to restrict HIV replication in newly infected cells by editing C to U in HIV DNA negative strand introducing premature stop codons, and inhibiting reverse transcription and chromosomal integration[48-50]. Other IFN-induced HIV restriction factors include Tripartite motif-containing Motif 5α (TRIM5α), tetherin (BST-2) and SAM domain and HD domain-containing protein 1 (SAMHD1), which act to block the uncoating of the incoming virion, block release of enveloped viruses, and inhibit HIV replication in myeloid cells, respectively[51,52]. Treatment of cells with IFNα, β and γ prior to infection induces an antiviral state and prevents productive HIV infection[53-55]. Treatment of cells with IFNα inhibited Vpu-deficient HIV replication through activation of tetherin, which exerts its antiviral function by preventing new virion release. IFNλ treatment was shown to inhibit HIV-1 infection of macrophages through upregulation of extracellular CC chemokines and activation of intracellular innate immune responses (the induction of other IFNs and APOBEC3G/3F)[56]. Together, *in vitro* evidence suggests that IFN-mediated antiviral innate immune responses against

HIV-infection play a crucial role in containing and preventing the dissemination of HIV in the early stages of infection. Unfortunately, numerous HIV evasion mechanisms dampen the therapeutic potential of these anti-HIV host factors. HIV-1, via the action of viral accessory proteins (including Vif and Vpu), blocks IFN driven intrinsic restriction factors (such as APOBEC3G and tetherin) and counteracts protective IFN responses by inhibiting the up-regulation of other antiviral proteins encoded by ISGs in target cells[51,57].

1.4.3 HIV transmission

HIV transmission occurs through contact with infected bodily fluids, via several routes including sexual transmission (homosexual and heterosexual), parenteral (injection drug use, contaminated blood transfusions) and perinatal transmission (mother to child transmission during pregnancy, labor, delivery or breastfeeding). Most HIV infected individuals acquire the virus through sexual contact via the genital and rectal mucosa. The transmission probability per exposure event varies greatly between different tissues, ranging from 1/200 to 1/2000 with vaginal intercourse compared to 1/20 to 1/300 with anal intercourse[58]. Despite lower HIV transmission probability per exposure event, heterosexual transmission accounts for >80% of all new HIV infections in women. The rate of HIV-1 infection varies between different sites of the female reproductive tract and it is influenced by multiple factors including inflammation levels, availability of HIV-1 target cells and hormonal changes. Progesterone-containing contraceptives have been shown to result in thinning of the epithelium and subsequent increase in incidence of SIV infection in macaques[59]. Pregnant women have significantly increased risk of HIV

acquisition, and this was independent of sociodemographic and behavioral factors[60]. The high levels of estrogen and progesterone during pregnancy potentially contribute to increased incidence by inducing structural and immunological changes in the female genital tract (FGT). A deeper understanding of the complex viral-host interaction at the FGT is key to guiding the development of products most suitable for combating HIV infection in women.

1.4.4 Acute infection and the early host immune responses

Following mucosal transmission of HIV, viral RNA is undetectable in the circulation for a period of about 10 days. This is referred to as the eclipse period, or the period from viral entry into a cell to the production of new virions[61]. Virus levels increase exponentially following the eclipse period to reach a peak at around 21-28 days post infection. One of the first signs of an immune response to HIV infection is the surge in the expression of inflammatory cytokines, including IL15, TNFα, IFNα and IFNγ[62], this coincides with a steep rise in HIV-1 viral load and contributes to symptoms of the acute retroviral syndrome[61]. The observed increase in viremia is associated with rapid and transient increase in IFNα and a slow and sustained increase in IFNγ as well as other proinflammatory cytokines[62]. Dendritic cells, specifically pDCs which are recruited to the infection site by virus-mediated induction of MIP-3α (CCL20) secretion by the endocervical epithelium, are thought to be the major producers of IFNα [61], while increased NK and NKT cell activation during acute infection could explain the observed increase in IFNγ[63,64]. There is considerable controversy whether or not this early innate immune response is

beneficial or detrimental to the host. DCs and NK cells are vital mediators of innate immune responses and crucial for the development of adaptive immunity. Concurrently, increased immune activation leads to influx of other immune cells, including macrophages and T cells to the site of the infection, transforming the submucosal environment into an ideal place for viral replication and expansion of the HIV-1 founder population. However, the early induction of innate immunity, including stimulation of IFNs and IFN-mediated immune responses, may prevent or partially control HIV spread through the activation of intrinsic antiviral factors, as well as induction of apoptosis of HIV-1 infected cells[65].

It is generally accepted that the early innate immune responses against HIV-1 are likely a crucial factor in determining the clinical course of the disease. Type I IFN-mediated antiviral activity exerts selective pressure on the transmitted virus pool, with virus isolates from patients during acute HIV infection more resistant to *in vitro* control by IFNα then viruses isolated from the same patients during chronic infection[66]. The establishment of systemic HIV-1 infection by relatively IFNα-resistant founder viruses indicates that IFNα and IFNα-mediated host responses play a crucial role in restricting early HIV replication. It is evident that early innate immune responses limit HIV replication and spread to some degree. However paradoxically these same protective responses contribute to the overall immune activation and enable local expansion and lymphatic dissemination of the virus. The virus replicates rapidly and spreads into other lymphoid tissues, including gut–associated lymphoid tissue (GALT) where a major loss of CD4+ T cells (80%) occurs within the first 3 weeks of HIV-1 infection[67-69]. Following this acute infection

period, virus levels decline with the appearance of innate and adaptive immune responses including increased NK cell activity and HIV specific CD8+ cytotoxic T lymphocyte responses. In addition to host antiviral immune responses, the declining viral load is also a consequence of massive CD4+ T cell depletion and the ever decreasing pool of target cells available for infection (called a 'substrate exhaustion mechanism')[70].

1.4.5 HIV disease progression

HIV disease progression is marked by a progressive loss of CD4+ T cells leading to AIDS and ultimately death. Several mechanisms have been proposed to contribute toward this gradual loss of CD4+T cells including chronic immune activation and cytopathic effects of HIV on CD4+ T cells. Massive destruction of CD4+ T cells in the gastrointestinal tract leads to translocation of the intestinal bacteria and endotoxins, such as lipopolysaccharide (LPS) and this results in induction of proinflammatory cytokines and type I IFNs all of which are thought to contribute to chronic immune activation[71]. Immune activation is considered to be a major determinant of HIV pathogenesis and contributes to the dysfunction of various host immune cells and tissues.

In addition to progressive loss of CD4+ T cells, HIV infection also leads to extensive defects in the humoral arm of the immune response. Although all HIV infected individuals produce anti-HIV antibodies, HIV evolution leads to the accumulation of escape mutants early in the infection process rendering antibody responses ineffective. Additionally, HIV infection causes multiple B cell defects including an increased number of activated and exhausted B cells, increased levels

of immature, translational B cells and a progressive loss of memory B cells[72]. If left untreated, HIV mediated destruction and premature ageing of the host immune system leads to impaired host defense, the development of opportunistic infections and eventually AIDS and death.

While a greater understanding of HIV pathogenesis post initial infection provides valuable information for novel treatment approaches, studies identifying host mechanisms that prevent initial HIV transmission and early infection are crucial for the development of successful prevention strategies. One way to achieve this is through studies on natural protection against HIV infection in HIV-exposed seronegative individuals (HESN).

1.5 Natural protection against HIV infection – HIV-exposed seronegative (HESN) individuals

Exposure to HIV-1 in the absence of infection has been observed in multiple cohort studies around the world[73-78]. Individuals highly exposed to HIV, but who remain uninfected are defined as HIV-exposed seronegative (HESN) and span several different key populations at risk of acquiring HIV, including commercial sex workers (CSWs), men who have sex with men (MSM), infants born to HIV positive mothers, injection drug users, hemophiliacs and discordant couples[73].

Specific adaptive, innate and genetic factors have been correlated with reduced susceptibility to HIV-1 infection[79,80], with innate immune responses

acting as a front line defense against the establishment of productive infection and HIV-1 dissemination.

1.5.1 Role of Immune quiescence in protection against HIV infection

Data from several HESN cohorts suggest that the immune quiescence (IQ) model may be an overreaching hypothesis to explain protection against HIV infection. IQ is a state of low baseline immune activation resulting in decreased susceptibility to HIV infection by limiting the number of available target cells and a reduction in pathways involved in HIV replication. Analysis of T cell phenotypes in the Majengo HESN female sex worker (FSW) cohort demonstrated decreased levels of T cell activation in HESN women when compared to HIV negative controls[81]. These HESN individuals were shown to have reduced frequencies of CD4+ and CD8+ T cells expressing the early activation marker CD69. Additionally, an elevated frequency of regulatory T (Treg) cells, known to suppress T cell activation, was also observed in HESN women[81]. Two different studies employing microarray approaches to analyze gene expression in unstimulated CD4+ T cells[82] and whole blood[83] from HESN women in the Majengo FSW cohort found lower levels of expression in genes involved in T cell receptor signaling and genes required for HIV replication compared to HIV-negative controls. Evidence for T cell- immune quiescence is not limited to the Majengo HESN cohort. Similar observations were made in HESN men who have sex with men (MSM) showing low frequency of CD4+ and CD8+ T cells expressing activation markers HLA-DR, CD38, CD70 and Ki67[84]. Studies of serodiscordant couples found decreased expression of the activation markers CD38[85], HLA-DR and CCR5[86] on systemic CD4+ T cells from the

uninfected partner. In addition to a decrease in systemic immune activation, reduced mucosal inflammation was observed at the FGT of HESN women. Reduced expression of proinflammatory cytokines and chemokines, including MIG and IP-10, was observed in cervical lavage (CVL) samples from Majengo HESN women[87]. These chemokines play a crucial role in recruitment of activated T cells to the site of the infection, and a decrease in their expression may result in fewer HIV target cells at the point of initial viral exposure.

1.5.2 Innate immunity in resistance to HIV infection

Studies in HESN individuals suggest that IFNs and IFN mediated responses play an important role in natural protection against HIV infection. IFNα induced host restriction factor APOBEC3G was found to be significantly higher in PBMCs and in cervical tissues of HESN individuals exposed to HIV through a sexual intercourse[88]. The observed increase in APOBEC3G expression correlated with reduced susceptibility of PBMCs from these individuals to *in vitro* infection with a HIV-$1_{Ba\text{-}L}$ R5 strain. Together, these data provide evidence that a potent IFN-induced antiviral immune response including factors such as APOBEC3G could offer a strong barrier against HIV infection, both during the initial exposure at mucosal surfaces as well as systemically.

NK cells, major producers of IFNγ, are thought to play a pivotal role in natural protection against HIV-1 infection in numerous HESN cohorts around the globe. HESN individuals exposed to HIV through sexual intercourse with a known HIV infected partner demonstrate significantly elevated expression of IFNγ by

PMA/ionomycin-activated NK cells [89]. Studies done in Vietnamese HESN intravascular drug users, show an increased percentage of NK cells producing IFNγ, TNFα and β chemokines (CCL3, CCL4 and CCL5) either after *in vitro* activation or without stimulation compared to healthy controls and HIV infected individuals [75]. Recent results from the Majengo HESN cohort also demonstrate that activated NK cells from HESN women are capable of more robust degranulation and produce higher levels of IFNγ when compared to cells from susceptible uninfected women[90]. Additionally, NK cells expressing the protective killer cell immunoglobulin-like receptor (KIR) 3DS1, associated with reduced risk of HIV-1 infection[91] and delayed progression to AIDS[92] have been shown to produce more IFNγ in unstimulated state [93] and mediate strong inhibition of HIV-1 replication *in vitro*[94]. Taken together, studies from a number of different HESN cohorts indicate that IFNγ production by innate immune cells, particularly NK cells, may play a major role in natural protection against HIV infection.

Increased innate antiviral responses have also been observed in the FGT of HESN women. Proteomics analyses of cervical mucosal secretions identified several anti-proteases, including Serpin A1, Serpin A3, Cystatin B and A2ML1, to be significantly over-expressed in HESN women[95,96]. These innate proteins are known to have anti-inflammatory and anti-HIV activity that may limit immune activation and target cell availability at the site of infection. Innate immune responses represent the first line of defense against HIV and could prevent the spreading of the virus to the systemic compartment, therefore allowing time for HIV-specific responses to develop.

Overall, natural resistance in the context of HIV infection seems to be associated with the host's capacity to induce strong innate antiviral immune responses while at the same time controlling the inflammation conditions and limiting the number of target cells at the initial exposure site – a process that likely involves host genetic factors.

1.5.3 Host genetics and natural resistance to HIV infection

Host genetics plays an important role in determining susceptibility to HIV infection and the rate of disease progression. Many of the protective genetic variations associated with protection from infection and reduced disease progression are found in genes that control viral entry (e.g. *CCR5*, *CCR2* and *SDF1*)[44], innate immune regulation (e.g. *MBL2*)[97] and adaptive immune recognition by T cells (*HLA*)[98,99].

One of the best described genetic mutations shown to provide resistance to HIV acquisition is the *CCR5*Δ32 mutation. Sequencing of the *CCR5* gene in HESN cohorts identified a 32 –base pair deletion that was common in Caucasians, with an allele frequency range from 5 to 15% but is almost entirely absent in non-European populations[79]. A 32 –base pair deletion in the open reading frame of the *CCR5* leads to a generation of a truncated, non-functional protein and therefore an absence of the surface CCR5 expression. Given its role as a major HIV co-receptor, it is unsurprising that the lack of surface CCR5 would provide strong protection from HIV-1 infection. Cross-sectional and longitudinal analysis indicated that homozygous and heterozygous *CCR5*Δ32 genotypes are associated with reduced

susceptibility to HIV infection among high-risk HIV seronegative MSM. However, while the *CCR5*Δ32 mutation may partly explain HESN phenotype in Caucasian population it does explain altered susceptibility to HIV infection in African HESN cohorts, who reside in regions with the highest HIV/AIDS burden in the world. Several HESN cohorts have described association between specific single nucleotide polymorphisms (SNPs) and decreased susceptibility to HIV-1 infection[44-46,100], with polymorphisms identified in the *interferon regulatory factor 1* (*IRF1*) gene being one of the strongest and best described correlates of protection in the Kenyan Majengo HESN cohort.

1.5.4 IRF1 polymorphisms as a correlate of protection in HESN individuals

Microsatellite genotyping and genomic sequencing of the *IRF1* gene led to the identification of three polymorphisms that showed an association with the HIV–resistant phenotype: 1) at nucleotide 619, 2) the microsatellite region and 3) at nucleotide position 6516 of the *IRF1* gene (Figure 1.1A). The 619A, 12 GT microsatellite repeat (179) and 6516G alleles were all associated with resistance to HIV-1 infection and a reduced likelihood of seroconversion[100,101]. All three polymorphisms were shown to be in high linkage disequilibrium (LD), and haplotype analysis suggested that the protective effect of these polymorphisms was additive[46]. Western blot analysis of PBMCs showed significantly lower basal IRF1 expression and reduced responsiveness to IFNγ stimulation in patients with protective IRF1 genotypes [101]. As IRF1 was identified to be an essential initiator of HIV-1 LTR transcription in PBMCs from subjects with different IRF1 polymorphisms was also investigated. Unstimulated PBMCs from individuals with

protective IRF1 haplotype showed a significantly reduced ability to transactivate the HIV-1 LTR during initial stages of infection when infected with a single-cycle VSV-G pseudotyped HIV-1 virus (HIV-1_{VSV-G})[102], suggesting a reduced ability to support HIV replication. While individuals with both protective and non-protective IRF1 haplotype had a robust response to infection by pseudotyped HIV-1, this response was transient and controlled in individuals with protective IRF1 haplotype[102]. Because all three polymorphisms are located within introns (619 in intron 1, MS in intron 7, and 6516 in intron 9), how these non-coding regions affected the IRF1 protein expression is unclear. However, polymorphisms in intronic regions of genes have been shown to act as regulatory elements and affect transcription, mRNA splicing, mRNA stability or promoter activity[103-106].

However the protective IRF1 haplotype does not account for the entirety of the HIV-resistant phenotype in HESN individuals from the Majengo cohort. It was recently shown that HESN individuals that lack the protective IRF1 haplotype also exhibit reduced IRF1 protein expression in PBMCs[107] indicating that additional modes of IRF1 regulation such as epigenetic regulation might be at play. Furthermore, PBMCs from HESN individuals showed a robust but transient IRF1 response to exogenous IFNγ stimulation while PBMCS from HIV-susceptible individuals showed a prolonged and sustained increase in IRF1 expression[107].

This timely silencing of IRF1 response in HESN individuals with and without protective IRF1 haplotype may be critical during the early stages of HIV infection and may play an important role in altered susceptibility to HIV-1 infection. IRF1 is

an important immunoregulatory host cell factor and differences in IRF1 expression and regulation could have a significant impact on the rate of HIV acquisition and the generation of host immune responses.

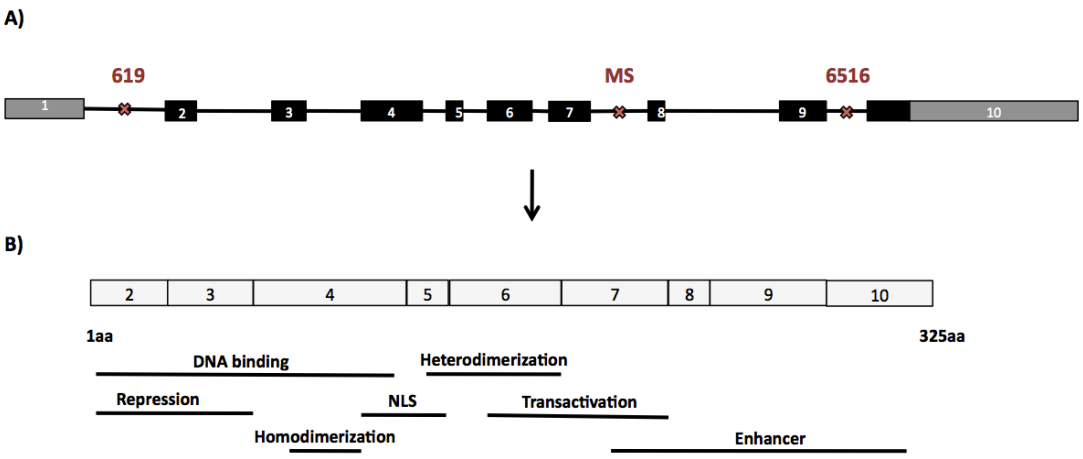


Figure 1.1 Schematic representation of IRF1 structure.

A) *IRF1* gene structure. Coding sequence goes from 235bp (exon2) until 1212bp (first 123bp of exon 10). Polymorphisms associated with HIV-resistant phenotype 619, MS, 6516 are located in introns 1, 7 and 9 respectively. **B)** IRF1 exon and protein domain organization.

1.6 Interferon Regulatory Factor 1 (IRF1)

1.6.1 The IRF Family of transcription factors

Members of the IRF family of transcriptional factors play a crucial role in multiple biological processes including regulation of host immune responses, cell growth and carcinogenesis. Phylogenetic analysis suggest that the genetic origin of IRFs coincided with the appearance of multicellularity in animals and it is thought to have coevolved in parallel with the Rel/NFκB family, with which it shares an important role in regulating host immune responses[108]. The evolution of these transcriptional factors was likely driven by increased demand for more complex regulation of both embryogenesis and immunity in multicellular organisms. *IRF* genes are not found outside the taxon *Metozoa*, and are present in all five principle metazoan groups including simple organisms such as sea sponges, which branched from vertebrates more then 550 million years ago[39,109]. Ten members of the IRF family have been identified in vertebrates, with IRF10 absent in humans and mice[25,110]. IRFs, depending on their mechanism of action, can be grouped into transcriptional-activators (IRF1, IRF3, IRF7 and IRF9), and transcriptional-repressors (IRF2 and IRF8). Some IRFs (IRF1, IRF2, IRF5, IRF4 and IRF8), depending on the target genes, can act both as activators and repressors [42,111]. Structurally, all IRFs share a high degree of homology in the NH2-terminal DNA binding domain characterized by a series of five well-conserved tryptophan-rich repeats. The DNA binding domain recognizes a DNA sequence known as interferon stimulated response element (ISRE), and similar regulatory elements in IFN and ISGs that are crucial in regulating host antiviral immune responses. The COOH-

terminal domain acts as a regulatory domain and is responsible for the functional properties of different IRF members by regulating IRF interaction with other host and viral proteins. Two types of association domains have been identified within the COOH-terminal, IRF-associated domain 1(IAD1) which is conserved for all IRFs except IRF1 and IRF2, and IAD2 that is shared by IRF1 and IRF2. The nature of the protein-protein interactions directed by IADs likely determines whether the resulting complex acts as a transcriptional activator or repressor.

Some viruses, such as Human Herpes Virus-8 (HHV8) encode for IRF homologs, named vIRFs, that inhibit the host interferon responses and specifically, IRF1, thereby counteracting the activity of the host immune system[43,112]. Many viruses have evolved different mechanisms to inhibit and/or exploit IRF responses either by encoding for their own vIRFs or through complex manipulation and control of host IRFs. HIV replication is controlled by a complex interplay between viral and host factors, including members of the IRF family. In early phases of HIV-1 infection, when the viral transactivator Tat is absent or present at low levels, IRF1 is an essential factor for successful HIV replication [113].

1.6.2 IRF1 Structure and Expression

The human *IRF1* gene has been mapped to chromosome 5q31.1 and contains 10 different exons, 9 of which are translated. The *IRF1* gene is 3567 bp long with a coding sequence going from 235 bp (exon 2) to 1212 bp (exon 10) and encoding 325 amino acids (aa). Exon 10 is quite large (988 bp) but codes for only 40 aa in addition to the stop codon and 3' untranslated sequence. The IRF1 protein has distinct domains that mediate transcriptional activity, DNA binding, nuclear

translocation and heterodimerization with IRF8 (Figure 1.1B)[114]. As discussed previously, all IRFs contain a well-conserved NH2-terminal DNA binding domain, characterized by five conserved tryptophan repeats[115]. The DNA-binding domain forms a helix-turn-helix structure that recognizes a specific DNA sequence corresponding to the ISRE (G(A)AAAG/CT/CGAAAG/CT/C)[116]. IRF1 can bind to the target DNA as a monomer or a dimer. The DNA binding domain (1-120 aa) spans exons 2-4 and it includes a repression domain (N-terminal 60 aa), a homodimerization domain (90-115 aa), and a nuclear localization sequence (116-139 aa)(Figure 1.1B). The IRF COOH-terminal region includes heterodimerization, transactivation and enhancer domains (Figure 1.1B). The transactivation domain spans exons 6 and 7 and includes IAD2 which is important for protein-protein interactions and therefore IRF1 specificity [108]. The enhancer domain spans exons 7, 8 and 9 and plays a role in protein stability and localization through ubiquitination and SUMOylation of the IRF1 protein.

IRF1 activity is regulated through a variety of post-translational modifications, including phosphorylation, ubiquitination and SUMOylation. The IRF1 COOH-terminal domain includes several tyrosine residues, and phosphorylation of these residues by casein kinase II was shown to increase IRF1 activity[117]. The IRF1 protein is highly unstable, with an approximate half-life of 30 minutes in cultured cells, compared to other IRFs (eg. IRF2 has half-life of approximately 8 hours)[118]. Protein degradation is regulated via the ubiquitin-proteosome pathway, which is a major route of cellular degradation for short-lived regulatory proteins[119]. Amino acids 255-300 contain lysine residues that are

subject to ubiquitin modification while amino acids 311-325 are thought to be involved in delivery of the IRF1 protein to the proteosome[120]. Interestingly specific lysine residues within DNA-binding domain have been recently shown to act as ubiquitin-acceptor sites and contribute to IRF1 protein degradation[121]. Furthermore, the DNA-binding domain was ubiquitinated only in its non-DNA binding state, showing that IRF1 protein is "protected" from degradation when part of an active transcription pre-initiation complex, but can be rapidly degraded if non-functional or when it has completed its function. In addition to ubiquitination, C-terminal IRF1 lysine residues are substrates for SUMOylation, a posttranslational modification by which a SUMO moiety is attached to the target protein affecting subcellular localization, transcriptional activity and protein stability[122]. IRF1 is modified by SUMO-1 and this leads to a decrease in protein degradation and altered transcriptional activity[123].

Interactions between IRF1, other IRFs and other host transcriptional factors represent important control mechanisms of IRF1's function and specificity. The heterodimerization domain is important for interaction with IRF8, and this complex formation was shown to repress IFN and IRF1 mediated activation of ISRE-driven genes[124,125]. IRF2 is another IRF1 antagonist that represses IRF1 function by competing for the same DNA binding sites[126]. Protein-protein complexes play an important role in IRF1's ability to affect gene expression and IRF1 is known to interact with other transcriptional factors including NFκB. The IRF1/NFκB complex formation is important for the induction of several promoters including the inducible nitric oxide synthase (iNOS)[127] and HIV-1 LTR Enhancer [128].

IRF1 is regulated at multiple levels from transcription to posttranslational modifications and protein-protein interactions. It seems likely that intronic IRF1 polymorphisms, which are associated with altered susceptibility to HIV infection in the Majengo HESN cohort, could through alternative splicing of the IRF1 mRNA impact the expression, regulation and the overall function of IRF1.

1.6.3 IRF1 splice variants

Alternative splicing is a process by which multiple mRNAs are generated from a single pre-mRNA molecule resulting in functionally distinct proteins. Greater then 90% of multiexon genes are alternatively spliced in mammals, producing on average at least 7 mRNA variants per gene[129]. Alternative splicing of mRNA allows for generation of many gene products with different functions from a single coding sequence. Ten IRF1 splice variants labeled as splice patterns 1-10, have been identified, and are differentially represented in different human tissues. Alternative splicing of IRF1 was shown to occur more frequently in cancerous than normal tissue[130,131]. Several studies have reported exon 2 or both exon 2 and 3 skipping in IRF1 gene transcripts in bone marrow and in PBMCs from healthy donors and patients with chronic myeloid leukemia and myelodisplastic syndrome[130,132]. The identified splice variants lack the DNA-binding domain and are non-functional as transcription factors and do not seem to affect wild-type IRF1 mediated gene regulation. More recently, Lee *et al.* identified five IRF1 splice variants with skipping of exons 7, 8 or 9, alone or in some combination in human normal and cancerous uterine cervical tissues[131]. While these variants also lacked transcriptional activity, they also appear to compete with wild-type IRF1 and therefore lead to

overall decrease in IRF1 function. Furthermore, alternative splicing of exons 7, 8 and 9 disrupts the ubiquitin target sites and results in increased protein stability and a longer half-life. Therefore, IRF1 variants lacking exons 7, 8 and 9 or some combination of thereof have longer half-lives and are able to block wild-type IRF1 activity adding to the complexity of IRF1 regulation. Alternative splicing of the IRF1 mRNA produces functionally distinct IRF1 proteins impacting the overall IRF1 mediated host responses.

1.6.4 IRF1 Function

IRF1 is constitutively expressed at low levels in almost all cell types including immune cells such as T cells, macrophages and NK cells[133]. IRF1 can be strongly induced by various cytokines (such as IL12[134] and IFNγ[135]), hormones (prolactin)[136] and certain viral infections[137]. IRF1 affects the transcription of specific genes and exerts immunomodulatory, antiviral, antiproliferative and antitumor effects. [114]. The discovery of IRFs dates back to 1988 when IRF1 was identified as the transcriptional regulator of IFNα/β[138]. IRF1 plays a central role in host defense, including activation and attenuation of the host immune response, regulation of immune cell differentiation and the regulation of cell growth and homeostasis. Once activated IRF1 is known to stimulate expression of IFN-inducible genes including iNOS (important for clearance of intracellular pathogens), 2'5'-oligoadenylate synthetase (2'5'-OAS, antiviral response), and transporter associated with antigen processing 1 (TAP1) and low molecular weight protein 2 (LMP2), which are important for antigen processing and presentation[114]. IRF1 was shown to directly regulate the expression of several cytokines, including IL6[139],

IL15[140], IL12 and IL4[141]. Analysis of IRF1 knock-out mice indicates that IRF1 is a key regulator of macrophage function, DC differentiation and maturation, NK responses, Th1/Th2 differentiation and MHC class I and II expression[115,142,143]. Several studies have demonstrated that the NK cell number and function (such as cytotoxicity and IL12-dependent IFNγ production) are absent in IRF1 knockout mice[140,144,145]. This is largely attributed to the lack of IL15 production, as IL15 is transcriptionally regulated by IRF1 and essential for NK cell development [140]. In other cells of the innate immune system, IRF1 is required for the full maturation of neutrophils and macrophages[146] and it is known to negatively regulate pDC differentiation[143].

In terms of adaptive immunity, IRF1 has a profound effect on T cell differentiation and subset distribution. IRF1 knockout mice display a profound reduction in mature CD8+ T cell numbers in the thymus and peripheral lymphoid organs as well as reduced CTL activation[147]. IRF1 promotes Th1 differentiation through several mechanisms[148,149]. Firstly IRF1 regulates the expression of IL12 by macrophages, a cytokine that is essential for the Th1 differentiation. Secondly, IRF1 positively affects the number and function of NK cells that in turn produce IFNγ to stimulate macrophage secretion of IL12. Finally, IRF1 is known to suppress Th2 differentiation via repression of IL4 expression[141].

In addition to its immunoregulatory role, IRF1 functions as a tumor suppressor by controlling apoptosis through transcriptional regulation of various tumor suppressive genes including caspases and tumor-necrossis factor-related apoptosis inducing ligand (TRIAL)[150]. A loss of IRF1 expression and function has

been linked to human malignancies in several clinical studies[150]. The relatively frequent allelic loss of IRF1 in women with breast cancer was associated with an increased risk of reoccurrence and death highlighting the tumor suppressive role of IRF1 in the clinical setting[151]. However, the exact mechanism of IRF1-mediated tumor suppression remains to be determined.

IRF1 has been implicated in various human diseases, including viral infections such as Hepatitis B and C[152], West Nile virus[153], respiratory viruses such as Influenza and Rhinovirus[154] and HIV[46,155,156]. As an important transcriptional regulator critical for immunity against pathogens and tumor suppression, IRF1 could represent an attractive therapeutic reagent for treatment of infectious diseases, autoimmune disorders and various malignancies. A better understanding of the protective effects of IRF1 polymorphisms in HESN individuals, and the complex relationship between HIV and IRF1 may represent an important factor in the fight against HIV/AIDS pandemic.

1.7 HIV and IRF1 – a story of manipulation and control

Many viruses have evolved mechanisms to evade and manipulate various aspects of the IRF1 pathway in order to promote viral pathogenesis. During the HIV infection process, IRF1 acts as a double-edged sword, essential for driving early viral replication as well as enabling protective antiviral responses. The complex interplay between HIV-1 and IRF1 likely has a strong impact on HIV replication and generation of host immune responses.

1.7.1 IRF1 and its role in early HIV replication

Like most viruses, HIV depends on host cellular factors to synthesize and assemble new virions. One of the most important aspects of the HIV-1 life cycle is the modulation of its replication by the complex interplay between viral and host proteins acting on the viral promoter, the 5′ LTR region. The HIV-1 5′LTR binds viral regulatory proteins and cellular transcription factors and can be subdivided into core promoter elements, enhancer, modulator, negative regulatory elements and the Tat responsive element (TAR)[157]. The LTR enhancer region contains two NFκB binding sites (-109 to -79) that play central roles in Tat-dependent and Tat-independent HIV transcription in blood CD4+ T lymphocytes[158]. Region +200 to +217 downstream of the LTR was shown to be homologous to the ISRE present in the promoter of ISGs[159]. This region represents a binding site for members of the IRF family, and is essential for efficient HIV-1 transcription and replication[157,160]. The HIV-1 accessory protein Tat acts as a transcriptional activator of HIV-1 gene expression and is essential for HIV-1 replication. However, after viral entry HIV-1 promoter activation is required to achieve Tat expression, and this is initially mediated by host transcriptional machinery. IRF1 was shown to increase HIV-1 LTR-directed gene expression in a dose dependant fashion in Jurkat cells, in the early stages of HIV replication when Tat is absent or present at low levels[137]. IRF1 further cooperates with Tat to amplify Tat-mediated HIV-1 transcription through direct physical interaction between Tat and the IRF1 COOH-terminal. In addition, Sgarbanti *et al*. showed that HIV-1 upregulated IRF1 early in the infection, prior to the induction of Tat, in both Jurkat T cells and primary CD4+ T

cells, stimulated with anti-CD3 mAb[137,161]. IRF1 was also shown to form a functional complex with NFκB at the LTR κB sites, and this was shown to be required for full activation of the HIV-1 LTR enhancer[128]. Overall these data clearly demonstrate that in early stages of HIV infection, when viral transactivator Tat is absent or present at low levels, the IRF1/NFκB complex represents an essential factor in HIV-1 LTR transcription and thus HIV replication. Interestingly, different tissues of the FGT have been shown to exhibit varying degrees of HIV-1 transcription[162]. It seems possible that tissue specific differences in IRF1 and/or NFκB expression could drive the observed differences in HIV-1 transcription.

1.7.2 IRF1 and HIV pathogenesis

IRF1's role in HIV pathogenesis is largely determined by its physical interaction with the viral transactivator Tat. In addition to acting as an intracellular transcription factor, Tat can be secreted by infected cells and act extracellularly [163]. Healthy cells can absorb secreted Tat, allowing it to affect both infected and uninfected cells. Tat is able to activate and/or suppresses expression of numerous cellular genes, contributing to the HIV-mediated deregulation of the host immune responses. Extracellular Tat was shown to upregulate IRF1 expression in Jurkat cells[164]. This initial Tat-mediated upregulation in IRF1 expression likely represents an important mechanism in preparing new cells for viral infection. Furthermore, once inside the cell Tat can directly interact with IRF1 and manipulate host antiviral responses allowing HIV replication and spread. Internalized Tat forms

a heterocomplex with IRF1 thereby impairing IRF1 transcriptional activity[137,164]. For example, binding of the Tat-IRF1 complex results in the repression of the LMP2 proteosome subunit expression, and consequently, changes in antigen processing and presentation[164].

Tat mediated induction and manipulation of IRF1 also plays a role in Tat-induced apoptosis of CD4 and CD8 T cells, which is a hallmark of HIV disease progression. Tat has been implicated as the inducer of apoptosis in both infected and uninfected T cells, potentially by Fas-dependent mechanisms[165]. It seems likely that these effects are mediated in part by IRF1 which is shown to be an essential regulator of apoptosis in several cell types driven by IRF1 mediated induction of caspase 1 and 7[115] and FasL[111,166]. HIV-1 mediated control of IRF1 expression and the close relationship between Tat and IRF1 suggest that IRF1 could emerge as one of the key players in HIV-1 mediated T cell depletion and disease progression. However, the exact role of IRF1 in HIV-1 induced apoptosis remains to be defined.

IRF1, and its antagonist IRF2 and IRF8 have been implicated in HIV pathogenesis in monocytes. HIV-1_{BAL} infection of mDCs led to a time-dependent induction of specific IRFs [155] where the early and persistent induction of IRF1 was coupled with up-regulation of its two inhibitors IRF2 and IRF8. IRF8 was induced early in the infection process but then steadily decreased while IRF2 was upregulated later in infection. By modifying IRF-1 response via time-dependent increase of IRF1 inhibitors (IRF2 and IRF8), HIV induced a distinct subset of ISGs without detectable induction of antiviral Type I and II IFN responses. [155]

Together this indicates that in addition to IRF1's role in driving HIV replication, the regulation of IRF1 and its antagonists (IRF1 and IRF8) is an important strategy utilized by HIV to counteract IFN- mediated host defenses and drive disease progression.

1.7.3 IRF1 and its role in HIV latency

Multiple mechanisms have been implicated in the establishment and maintenance of low level of transcription during HIV-1 latency, including the absence of viral transactivator Tat and its associated cofactors[24,42,167]. Varying expression of Tat and host transcription factors, such as NFκB and IRF1, in activated versus resting cells is likely to contribute to the establishment of productive versus latent infection and influence viral reactivation[113]. Latent proviruses can be reactivated by stimulating the host T cells with mitogens and anti-CD3-CD28 antibodies[43,168,169]. Both IRF1 and NFκB are present at higher levels in activated T cells compared to resting cells and could contribute to viral reactivation[138,170,171]. Furthermore, HIV induced proinflammatory cytokines such as IL6, TNFα and IL1β lead to cell activation and are strong activators of IRF1 and NFκB expression[111,115,171]. Thus, when Tat is absent or present at low levels, increased intracellular levels of IRF1 and NFκB in activated cells would create an environment favorable for viral replication, resulting in viral reactivation.

Other members of the IRF family, IRF2 and IRF8 in particular, play an important role in governing the HIV-1 LTR/IRF1 interaction and may, in part, regulate HIV latency. IRF2 and IRF8 are known IRF1 antagonists; IRF2 directly

competes with IRF1 for ISRE binding spots at the target promoter, while IRF8 forms a heterocomplex with IRF1 preventing the protein-protein interaction and hindering its transcriptional activity. IRF8, but not IRF2, by interfering with the IRF1-Tat protein complex formation, inhibits the IRF1-Tat mediated HIV-LTR transactivation [137,157,172]. Additionally, IRF8 was shown to play an inhibitory effect on HIV-1 replication in human CD4+ T lymphocytic and monocytic cell lines, [137,158,160]. Further evidence suggests that IRF8 plays an essential role in maintaining viral latency by transcriptional repression of the HIV-1 ISRE element[159,172]. These intricate interactions between HIV-1 and host IRF1, IRF2 and IRF8 are likely to have an important role in regulating HIV-1 latency and reactivation. Strategies to modulate proviral latency and host antiviral immune response through manipulation of the IRF proteins could allow for reactivation of latent viral reservoirs and allow for successful HIV-1 clearance and perhaps HIV cure.

1.7.4 IRF1 and altered susceptibility to HIV-1 infection

IRF1 is an essential component of the host innate and adaptive immune response against a diverse array of pathogens, including HIV-1. IRF1 is a key regulator of macrophage function, DC differentiation and maturation, NK responses, Th1/Th2 differentiation and MHC class I and II expression[115,142,143]. Interestingly, all of these have been linked with the HIV-resistant phenotype in HESN individuals [80,173], highlighting the potential role of IRF1-mediated anti-HIV immune responses in decreased susceptibility to infection. Several HESN studies have reported an increase in NK cell number and function in protected

individuals[75,90], and IRF1 through transcriptional regulation of IL15 is an essential factor for NK cell development and function. The epithelial microenvironment and early innate immune responses are generally thought to represent the main barrier that the HIV-1 has to overcome during transmission. IFNs, transcriptionally regulated by IRFs, are secreted by infected cells and induce an antiviral state, by stimulating the expression of ISGs [174]; many of which have direct intrinsic antiviral effects or act to recruit and enhance adaptive immune responses. Multiple studies have demonstrated that IFNs inhibit HIV replication both in primary cells such as macrophages and PBMCs and in monocyte and T cell lines[54,56,175], indicating that it is possible to elicit anti-viral immune responses capable of inhibiting HIV replication and preventing infection. IFNλ, the primary interferon that regulates mucosal responses to viral infections, is transcriptionaly regulated by IRF1[154] and was shown to inhibit HIV infection in macrophages[176]. These early antiviral immune responses may provide protection immediately after exposure and potentially contribute to the low probability of vaginal transmission, restricted cellular tropism observed in HIV infection and the failure of most exposures to result in infection [47,177]. However, these early immune responses can also facilitate viral dissemination by contributing to the generation of more HIV-target cells via immune activation and recruitment of systemic T cells into the tissue. Thus a controlled, early robust antiviral IFN/ISG response, such as the IRF1 response observed in HESN individuals[107] may be sufficient in controlling viral replication at the exposure site while at the same time preventing detrimental immune activation and systemic infection.

Study Rationale, Hypotheses and Objectives

Host genetic polymorphisms and variation in gene expression have been demonstrated to have an impact on HIV acquisition and disease progression in numerous studies. Previous studies from the Majengo HESN cohort have identified three polymorphisms (SNPs at nucleotide location 619 and 6516 and a MS GT repeat) located in the intronic regions of the *IRF1* gene that showed an association with the HIV-resistant phenotype and a reduced likelihood of seroconversion. Further it was demonstrated that:

1. PBMCs from individuals with protective IRF1 haplotype (619AA, 179+179+, 6516GG) exhibit significantly lower IRF1 basal expression
2. PBMCs from individuals with protective IRF1 haplotype demonstrate significantly lower HIV-1 LTR transcription and decreased ability to support HIV replication *in vitro*.

What remains unclear however is the connection between polymorphisms in the intronic regions of the IRF1 gene and how this could impact IRF1 protein expression. Given IRF1's role as an important mediator of innate and adaptive immunity we were interested in assessing the impact of IRF1 polymorphisms on host immune responses. Furthermore, while the protective IRF1 haplotype was demonstrated to restrict HIV replication during the early stages of infection, its impact on disease progression in individuals that seroconvert remained unexplored. We further used this opportunity to characterize IRF1 tissue specific expression, as differential IRF1 tissue expression could be a contributing factor to the differences between HIV transmission probability in different human tissues. Here we set out

to further characterize the mechanism and the impact that IRF1 polymorphisms have on host immunity and HIV disease dynamics.

Furthermore, since IRF1 polymorphisms do not account for all cases of resistance to HIV-1 infection in the Majengo cohort, other mechanisms of IRF1 regulation could be at play in HESN individuals without protective IRF1 haplotype. There is an increasing amount of evidence suggesting that hormones play a crucial role in development and maintenance of host immune responses as well as the rate of HIV acquisition in women. Prolactin is a known transcriptional regulator of IRF1 expression and differences in prolactin expression could contribute to IRF1 regulation. Here we also investigated the differences in systemic hormone expression with respect to IRF1 polymorphisms and HESN status.

This thesis included 3 major hypotheses:

1. IRF1 polymorphisms, that were associated with decreased susceptibility to HIV-1 infection, directly regulate alternative splicing of the IRF1 mRNA resulting in altered IRF1 protein regulation and function. Changes in IRF1 regulation will impact the IRF1-mediated host immune responses contributing to the HIV-resistant phenotype.
2. Higher IRF1 expression is expected in tissues associated with increased susceptibility to HIV-1 infection and higher rate of HIV-1 replication.
3. The HIV resistant phenotype overall, and IRF1 protective haplotype specifically will be associated with decrease in systemic hormone expression (prolactin, cortisol, estrogen and progesterone).

These hypotheses will be tested in the following specific aims:

1. To determine IRF1 transcript and protein levels in various human tissues including different anatomical sections of the FGT (Section 3).
2. To determine the effect of IRF1 polymorphisms, associated with HIV-resistant phenotype, on alternative splicing of the IRF1 mRNA and IRF1 mRNA/protein stability (Section 4).
3. To characterize the association between IRF1 polymorphisms associated with HIV-resistant phenotype and systemic (plasma) and mucosal (cervical lavage, CVL) cytokine/chemokine expression (Section 5).
4. To determine if IRF1 polymorphisms associated with HIV-resistant phenotype play a role in HIV pathogenesis and disease progression in HIV-infected ART-naïve individuals (Section 6).
5. To quantiate systemic hormone levels with respect to IRF1 polymorphisms and the HESN status (Section 7).

2. GENERAL MATERIALS AND METHODS

2.1 Ethics

Informed written consent was obtained from all study participants and the studies were approved by University of Manitoba and Kenya National Hospital Institutional Review Boards.

2.2 Study Participants

Samples used in the studies were obtained from local donors in Winnipeg, Manitoba or from a well described female sex-worker (Majengo, ML) cohort established in the Pumwani district of Nairobi, Kenya in 1985. Samples obtained from the local Winnipeg donors were used to optimize experimental conditions or conduct basic methodological studies. All subjects were presumed to be HIV negative.

All enrollees of the ML cohort in Nairobi were tested for HIV-1 at a biannual resurvey visit by serological test and PCR. Since 1985 more then 4,000 participants have been enrolled in the ML cohort, approximately 600 of whom are in active follow-up. Cohort participants fall in one of four epidemiological categories: (1) HESN: individuals who were HIV-1 negative at enrolment and remained negative for over 7 years while continuing active sex work; (2) HIV-1 infected: individuals who tested HIV-1 positive at enrolment; (3) Seroconverters: individuals who tested HIV-1 negative at enrolment and seroconverted during follow-up; (4) HIV-Susceptible (HIV-S): individuals who tested negative at enrolment but do not fulfill the time requirement to be classified as HESN. Based on epidemiological evidence majority of

these women will succumb to infection pressure and can be defined as relatively susceptible to HIV infection.

At biannual resurvey visits women complete a behavioral interview, are screened for sexually transmitted diseases and donate samples for immunological assays. A total of 21ml of heparinised blood is collected for immunological assays and 7ml of blood in EDTA is collected for CD4 counts and vial loads. Women have regular access to medical treatment (including ART for eligible HIV infected individuals), counseling and free condoms.

2.3 Sample Preparation

2.3.1 Peripheral Blood Mononuclear Cells (PBMCs) Collection and Processing.

PBMCs were isolated from the whole blood by density gradient centrifugation using the Ficoll-Hypaque isolation method. Once plasma was removed (Section 2.3.2), the remaining whole blood samples were diluted with PBS + 2% FBS, layered over Ficoll and centrifuged at 1400rpm for 25minutes. The PBMC layer was collected, washed with PBS + 2% FBS, centrifuged at 1600rpm for 10 minutes, resuspended and washed with R10 culture media.

For prolonged storage, samples were stored in freezing media (10%DMSO in FBS) in liquid nitrogen at – 193 °C. When using frozen PBMC samples, cells were fast-thawed in a 37°C water bath for 1-2 minutes, resuspended in 8ml of R10 culture media and centrifuged at 1200 rpm for 5 minutes to remove any residual DMSO. Cells were washed with R10 culture media and left to rest at 37°C until further

treatment. Cell count and cell viability was determined using a Trypan blue exclusion method[178].

2.3.2 Plasma Collection and Processing.

Peripheral blood was collected in EDTA tubes and spun down at 1,500rpm for 7 minutes. Plasma was collected and stored at -70°C. Samples were shipped to Winnipeg, Manitoba for further analysis.

2.3.3 Cervicovaginal Lavage (CVL) Collection and Processing.

The cervix was washed with 2ml of sterile 1x PBS and the lavage was collected from the posterior fornix into a 15 ml conical tube. The samples were placed on ice and transported from the Majengo clinic to the laboratory. Obtained samples were centrifuged at 1400rpm for 7 minutes to remove cell debris and supernatant was stored at -70°C. Samples were shipped to Winnipeg, Manitoba, Canada for analysis.

Commercial reagents used:

Lymphoprep, Axis-Shield, (Product No. 11 14547)

Trypan Blue, Sigma-Aldrich (Cat. No. T8154)

RPMI 1640 media, Hypoclone (Cat. No. SH30027.01)

DMSO – Dymethyl Sulphoxide, Hybri-max® (100ml), Sigma (Cat. No. D2650)

FBS, Sigma-Aldrich (Cat. No. F1051-100ml)

Penicillin- Streptomycin Solution 100X, Corning-Cellgro (Cat. No. 30-002-CI)

Dulbecco's Phosphate-Buffered Saline (PBS), Gibco™ Invitrogen Corporation (Cat. No. 21600-044)

Self prepared solutions:

Freezing media: 10% DMSO in FBS

R10 cell culture media: RPMI-1640 supplemented with 10% heat inactivated FBS and 1% Penicillin-Streptomycin solution.

2.4 HIV Testing.

Plasma samples from the Majengo cohort were tested for HIV biannually using the Recombigen (Trinity Biotech, Ireland) enzyme-linked immunosorbent assay (ELISA). Detect HIV1/2 Immunoassay (Adaltis Inc.) was used to confirm the positive results. Samples that tested positive in both assays were considered HIV-1 positive.

2.5 CD4 Count Analysis.

CD4 counts were measured for all HIV infected participants of the Majengo cohort. Whole blood collected in the EDTA tubes was analyzed using the BD Tritest reagents (CD3/CD4/CD8). CD4+ T cell count was determined by multiplying the lymphocyte counts by the percentage of CD4+ T cells.

2.6 Viral Load Analysis.

Plasma viral loads were determined for 263 HIV positive samples that were sequenced for the identified IRF1 polymorphisms. HIV RNA in EDTA plasma was extracted and quantified using the automated Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 Test (Roche Diagnostics). This assay has a lower limit of detection of 40 HIV RNA copies/ml with a dynamic range of: 40-10^7 HIV RNA copies/ml.

2.7 IRF1 Sequencing and Microsatellite Typing

Genotyping for three polymorphisms in the IRF1 gene, located at 619 (intron 1), microsatellite region (intron 7) and 6516 (intron 9) was conducted.

2.7.1 IRF1 619 & 6516 Sequencing.

Two DNA segments that contained the two single nucleotide polymorphisms in the IRF1 gene (intron1-619 and intron 9- 6516) were amplified using following primers: 619FW: 5'GCTCGCCACTCCTTAGTCG3' and 619RV: 5'CAGTAAGCAGCCCTTGCC3' (Tm=55°C); 6516FW: 5'AGGGTGAGTCTGCACTGGAA3' and 6516RV: 5'CTTGGCAGTGGGGTCACA3' (Tm=53°C). Initial PCR consisted of the following reaction: 19.75µl 2 x PCR Master Mix, 0.5µM of each primer, 0.25µl Taq, 23µl H20 (18µl H20 + 5µl Betaine solution for 619 fragment) and 2µl DNA sample. PCR was carried out on an ABI thermocycler (Veriti 96 Well Thermal Cycler, ABI) as follows: 94°C for 3min, (94°C 30s, 58°C 30s, 72°C 1min) x36, 72°C 10min. The rest of the sequencing procedure was carried out at the DNA core of the National Microbiology Laboratory, Public Health Agency of Canada. Prior to sequencing, PCR products were purified using Millipore's Multiscreen filter plates. Sequencing was conducted using the ABI PRISM BigDye Terminator Version 3.0 Cycle Sequencing system (Applied Biosystems) using following primers: 619FW: 5'CTCCTTAGTCGAGGCAAGACG3' (Tm: 53°C) and 619RV: 5'AGCCCTTGCCACCAGCACA3' (Tm: 55°C); 6516FW: 5'AGGGTGAGTCTGCACTGG3' and 6156RV: 5'CTTGGCTGTTGAGGGGC3' (Tm: 53°C). Sequencing PCR was set up as follows: 2µl Big Dye, 2.7 µM primer (forward or reverse) and 2µl purified initial PCR

products. PCR conditions were as follows: 96°C for 3min, (96°C 30s, 53°C 30s, 60°C 4min) x80. All PCR products were ethanol precipitated and resolved using ABI Prism 3100 Genetic Analyzer. Results were subsequently analyzed using Sequencher 4.0.5 software (Sequencher, Gene Codes Ann Arbor, MI, USA) by comparison to the published GenBank IRF1 sequence (L05072).

2.7.2 IRF1 Microsatellite Typing.

The IRF1 microsatellite marker located in the intron 7 of the IRF1 gene contains a variable number of GT dinucleotide repeats. Microsatellite typing was adapted from previously described methods[46]. Custom primers targeting the intron 7 of the IRF1 gene were obtained from Invitrogen: FW: 6-FAM-5'ATGGCAGATAGGTCCACCGG3' and RV: 5'TCATCCTCATCTGTTGTAGC3' (Tm=55°C). PCR amplification was conducted using the Type-it Microsatellite PCR Kit from Invitrogen with the following reaction mixture: 12.5µl of 2X Type-it Multiplex PCR Master Mix, 2.5µl of 10x primer mix (2µM each primer), 3µl RNase-free water and 2µl DNA. PCR was performed on the ABI thermocycler as follows: 95°C 5min, (95°C 30s, 57°C 1:30min, 72°C 30s)x28, 60°C 30min. Using the amplified PCR product, the following 20µl reaction was resolved on a 3130xl Genetic Analyzer with Dye Set D and FragmentAnalysis36_POP7 template: 3µl PCR product, 2µl of 1/5 MapMarker® CST ROX 100-225 standard and 15µl HiDi formamide. Full run module settings are outlined in Table 2.1. Data analysis was performed using Gene Marker 1.59 Software (SoftGenetics LLC, USA).

Table 2.1. Microsatellite Run Module Settings

Oven_Temp.	60
Poly_Fill_Vol	6500
Current_Stability	5.0
Pre_Run_Voltage	15.0
Pre_Run_Time	180
Injection_Voltage	1.2
Injection_Time	23
Voltage_Number_Of_Steps	20
Voltage_Step_Interval	15
Data_Delay_Time	60
Run_Voltage	15.0
Run_Time	1000

Commercial reagents used:

Type-it Microsatellite PCR Kit, Qiagen (Cat. No. 206243)

Commercial reagents used:

Taq DNA Polymerase, Recombinant, Invitrogen (Cat. No. 10342-020)

Betaine solution, 5M, PCR Reagent, Sigma (Cat. No. B0300)

HiDi Formamide, Invitrogen (Cat. No. 4311320)

MapMarker® CST ROX 100-225, BioVentures, Inc.

Self prepared solutions:

2x Master Mix for PCR		
Concentration of stock buffer	*Quantity(µl)*	*Final Concentration in 2x Master Mix*
1M Tris-HCl pH 9.0	144	120mM Tris-HCl pH9.0
50mM MgCl	60	3 mM $MgCl_2$
1M $(NH_4)_2SO_4$	30	30mM $(NH_4)_2SO_4$
1mM dNTP Mix	200	200uM dNTP Mix
DMSO	100	10% DMSO
1% Gelatin	10	0.01% Gelatin
S.D.D. H20	366	
Total volume	910	

2.8 RNA Isolation

Several RNA isolation methods were used depending on the type of analysis to be conducted.

2.8.1 RNA Preparation for Microarray Analysis

Total mRNA was prepared from PBMCs according to the manufacturers' instruction using TRIzol Reagent (Invitrogen). In brief, cells were spun down (1,600rpm, 10min), culture medium was removed and the cells were washed with 1ml of sterile 1 X PBS buffer. Following the wash, cells were lysed with one milliliter of TRIzol Reagent and incubated at room temperature for 5 minutes to permit complete dissociation of nucleoprotein complexes. Following homogenization, the sample was mixed with 0.2 ml of chloroform and spun at 11,000g for 15 minutes at 4°C. Following centrifugation the upper aqueous phase containing RNA was transferred to a clean microcentrifuge tube. RNA was precipitated with 0.5 ml of isopropyl alcohol. Samples were incubated at RT for 10 minutes and then centrifuged at 11,000g for 10 minutes at 4°C. The RNA pallet was washed with 75% ethanol and resuspended in sterile double-distilled water. The Qiagen RNase-free DNase Set was used to eliminate contaminating genomic DNA. RNA samples were concentrated and purified using the Qiagen RNeasy MinElute Kit following manufacturer's instruction. RNA sample was eluted with 14µl of RNase-Free water and stored at -80°C until further use.

2.8.2 RNA Preparation for qRT-PCR Analysis

For qRT-PCR analysis RNA was extracted using a combination of TRizol Reagent and the Qiagen RNeasy Plus Mini Kit. The same TRizol Reagent procedure as outlined above was followed to obtain the upper aqueous phase following addition of chloroform. One volume of 70% ethanol was added to the aqueous phase. After mixing, sample was transferred to RNeasy spin column and centrifuged for 15s at more then 8,000g. Rest of the procedure was carried out using Qiagen RNeasy Plus Mini Kit as per manufacturers instruction. RNA sample was eluted with 30µl of RNase-free water and stored at -80°C until further use.

Commercial kits used:

RNase-free DNase Set, Qiagen (Cat No. 79254)

RNeasy Plus Mini Kit, Qiagen (Cat. No. 74134)

RNeasy MinElute Kit, Qiagen (Cat. No. 74204)

Commercial reagents used:

TRIzol Reagent, Invitrogen (Cat. No. 15596-018)

Chloroform Biotech grade, ≥ 99.8% contains 0.5-1 % ethanol as stabilizer, Sigma-Aldrich (Cat. No. 498189-1L)

Isopropanol/2-Propanol, Fisher Scientific (Cat. No. A464-4)

2.9 RNA Quantification

The amount and quality of the RNA samples used in microarray experiments was determined using the Agilent Bioanalyzer 2100 system according to the RNA Nano

6000 Series II Kit protocol. RNA samples used for the qRT-PCR were quantified using the Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, USA).

Commercial reagents used:

RNA Nano 6000 Series II Chips: Agilent Cat. No. 5067-1511

Reagents: Cat. No. 5067-1512

Ladder: Cat. No. 5067-1529

Equipment used:

Agilent Bioanalyzer 2100 (Agilent Technologies Inc., USA)

NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, USA)

2.10 cDNA Synthesis

One hundred nanograms of extracted RNA were used to make cDNA using the Qiagen QuantiTec Reverse Transcription Kit following manufacturers instructions. Briefly, RNA was treated with gDNA Wipeout buffer for 5 min at 42°C and cDNA synthesis was performed using random primers and reverse transcriptase for 20 min at 42°C. The reverse transcriptase reaction was inactivated at 95°C for 3 min and chilled to 4°C. Final volume of 20µl was aliquoted into 4x5 µl and stored at -80°C until further use.

Commercial kits used:

QuantiTec Reverse Transcription Kit, Qiagen (Cat. No. 205311)

2.11 qRT-PCR

Quantitative Real Time PCR was performed using the LightCycler 480 System (Roche Applied Sciences) and Qiagen QuantiTec SYBR Green PCR Kit. The resulting PCR products were analyzed using the LightCycler 480 Software 1.5.0 SP4 (Roche Applied Sciences). The qRT-PCR reaction was set up according to the manufacturer's instructions. In brief, a 20 µl reaction was set up as follows: 10 µl 2x QuantiTec SYBR Green PCR Master Mix, 1µl Primer mix (0.2µM final concentration each primer), 1µl template cDNA (4µl for exon splicing assay) and 8µl sdH2O (5µl for exon splicing assay). RT-PCR was ran on Lightcycler 480 (Roche, USA) at following conditions: Pre-incubation: 95°C for 15 min, Amplification: 45 x(94°C 15s, Tm 30s, 72°C 30s), Melting Curve: 95°C 5s, 65°C 1min, 95°C continuous and Cooling: 40°C 10min.

Standard curve was generated for each primer set in order to quantify the relative abundance of target genes using control cDNA. IRF1 transcription levels were determined using primers specific to exon 1 (Table 2.2). The human 18S gene was used as an internal control, and was amplified on an aliquot of the identical amount of cDNA samples used to characterize specific exon levels. The full list of primers used for RT-PCR is listed in Table 2. GraphPad PRISM Version 5.0a was used to graph and analyze the qRT-PCR data.

Commercial kits used:

Qiagen QuantiTec SYBR Green PCR Kit (Cat. No. 204143)

Equipment used:

LightCycler 2.0 Real-Time PCR System (Roche Applied Sciences)

LightCycler Software 3.5.3 (Roche Applied Sciences)

2.12 Exon Microarray

In order to analyze alternative splicing events in the IRF1 mRNA we used GeneChip® Human Exon ST Arrays (Affymetrix). The GeneChip® exon arrays are the first experimental tool that allows examination of alternative splicing events on the whole genome scale on a single array. The array consists of 1.4 million probe sets, with ~40 probes per gene and ~4 probes specific for each exon. Multiple probes per exon allow exon-level analysis and the ability to distinguish between different isoforms of a gene.

2.12.1 Sample Preparation

Total RNA was prepared as outlined in the RNA preparation section (Section 2.8.1). A total of 3.2µl of 350ng/ml RNA was used as a starting material. Sample amplification and preparation for microarray hybridisation was performed as outlined in the GeneChip® Whole Transcript (WT) Sense Target Labeling Assay Manual. Briefly, the RNA sample was depleted of rRNA minimizing the background and increasing the array detection sensitivity and specificity. Following rRNA reduction, double-stranded cDNA was synthesized with random hexamers tagged with a T7 promoter sequence. The template double-stranded cDNA was then amplified by T7 polymerase producing many copies of antisense cRNA. In the second cycle, cDNA synthesis random hexamers were used to produce >5.5µg of single-stranded DNA in sense orientation with incorporated dUTPs in order to enhance fragmentation and robustness of the assay. This single-stranded DNA sample was then treated with a combination of enzymes that specifically recognize

the unnatural dUTP residues and break the DNA strand into 40-70bp fragments. DNA fragments are labeled by deoxynucleotidyl transferase (TdT) with Affymetrix DNA Labeling Reagent covalently linked to biotin. Terminally labeled fragments, were hybridized onto the Affymetrix Human Exon 1.0 ST array, stained and washed, and finally scanned using the Affymetrix GeneChip® Scanner 3000 7G (Affymetrix Inc., USA).

2.12.2 Pre-processing

Obtained CEL files were imported into Partek® Genomic Suite Software (Partek Inc., USA) for pre-processing and analysis. Probe intensities were quintile normalized and summarized using the RMA algorithm. IRF1 haplotype was added as a categorical attribute (factor variable) and samples were labeled as either protective (619AA, 179+179+, 6516GG) or non-protective (619CC, 179-179-, 6516TT) IRF1 haplotypes. ML Number was added as a subject ID variable (random effect). Exploratory analysis (PCA and QA/QC) was performed in order assess the quality of the microarray data and to test for outliers.

2.12.3 Analysis and Visualization

Alternative splicing was analyzed using the Partek® Genomic Suite Software (Partek Inc., USA) following the *Exon Workflow* protocol. Alternative Splice ANOVA with IRF1 haplotype as Alternative Splice Factor was run to detect alternatively spliced genes. The gene view module was utilized in order to visualize the IRF1 exon expression profile.

2.12.4 Conformation

Exon microarray results were confirmed by qRT-PCR as outlined in Section 2.11. Primers specific to exon 2, exon 3, exon 7 and exon 8 were designed and used to quantify the expression of these particular exons in mRNA samples from selected HIV-1 resistant subjects with distinct IRF1 haplotypes. The positions and sequences of primers are described in Table 2.2. The human 18S gene was used as an internal control, and was amplified on an aliquot of the identical amount of cDNA samples used to characterize specific exon levels. GraphPad PRISM Version 5.0a (GraphPad Software Inc., USA) was used to graph and analyze the qRT-PCR data.

Table 2.2. IRF1 exon specific primers

IRF1 target exon	Custom Oligonucleotide Primers	Tm
Exon 1	FW: 5' CCTCTGCCTTCTTCCCTC 3'	58
	RV: 5' CTGCTGCAGGAGCGATTC 3'	58
Exon 2/3	FW:5' CCAACATGCCCATCACTCG 3'	60
	RV: 5' AACAGGCATCCTTGTTGATGTC 3'	60
Exon 7/8	FW: 5' AGCAGCACTCTCCCCGACTG 3'	60
	RV: 5' TTCCCTTCCTCATCCTCATCTGTTG 3'	60

Commercial kits used:

GeneChip® Human Exon 1.0 ST Array, Affymetrix (Cat. No. 900651)

RiboMinus™ Transcriptome Isolation Kit, Invitrogen (Cat. No. K1550-02)

Magna-Sep™ Magnetic Particle Seperator, Invitrogen (Cat. No. K1585-01)

GeneChip® WT Sense Target Labeling and Control Reagents, Affymetrix (Cat. No. 900652)

GeneChip® Eukaryotic Poly-A RNA Control Kit, Affymetrix, (Cat. No. 900433)

GeneChip® WT cDNA Synthesis and Amplification Kit, Affymetrix (Cat. No. 900673)

GeneChip® WT terminal Labelling Kit, Affymetrix (Cat. No. 900671)

GeneChip® IVT cRNA Cleanup Kit, Affymetrix (Cat. No. 900547)

GeneChip® Sample Cleanup Module, Affymetrix (Cat. No. 900371)

GeneChip® Hybridization Control Kit, Affymetrix (Cat. No. 900454)

GeneChip® Hybridization, Wash, and Stain Kit, Affymetrix (Cat. No. 900720)

Equipment used:

GeneChip® Hybridization Oven 640, Affymetrix (Cat. No. 800138)

GeneChip® Fluidics Station 450, Affymetrix (Cat. No. 00-0079)

GeneChip® Scanner 3000 7G, Affymetrix (Cat. No. 00-0212)

2.13 Exon Splicing Assay

In order to test if specific IRF1 polymorphisms located at the intronic regions of the IRF1 gene can act as splicing regulators we performed an exon splicing assay. Dinucleotide repeats located in intronic regions of genes are known modifiers of RNA splicing. To examine the effect of microsatellite GT repeat on splicing of the surrounding IRF1 exons (exon 7 and 8) the IRF1 exon7-intron7-exon8 segment with partial flanking intron sequences from two individuals with protective and non–protective IRF1 MS genotype respectively. The fragment was cloned into the splicing reporter vector pDUP175. A schematic representation of the exon splicing assay is shown in Figure 2. 1.

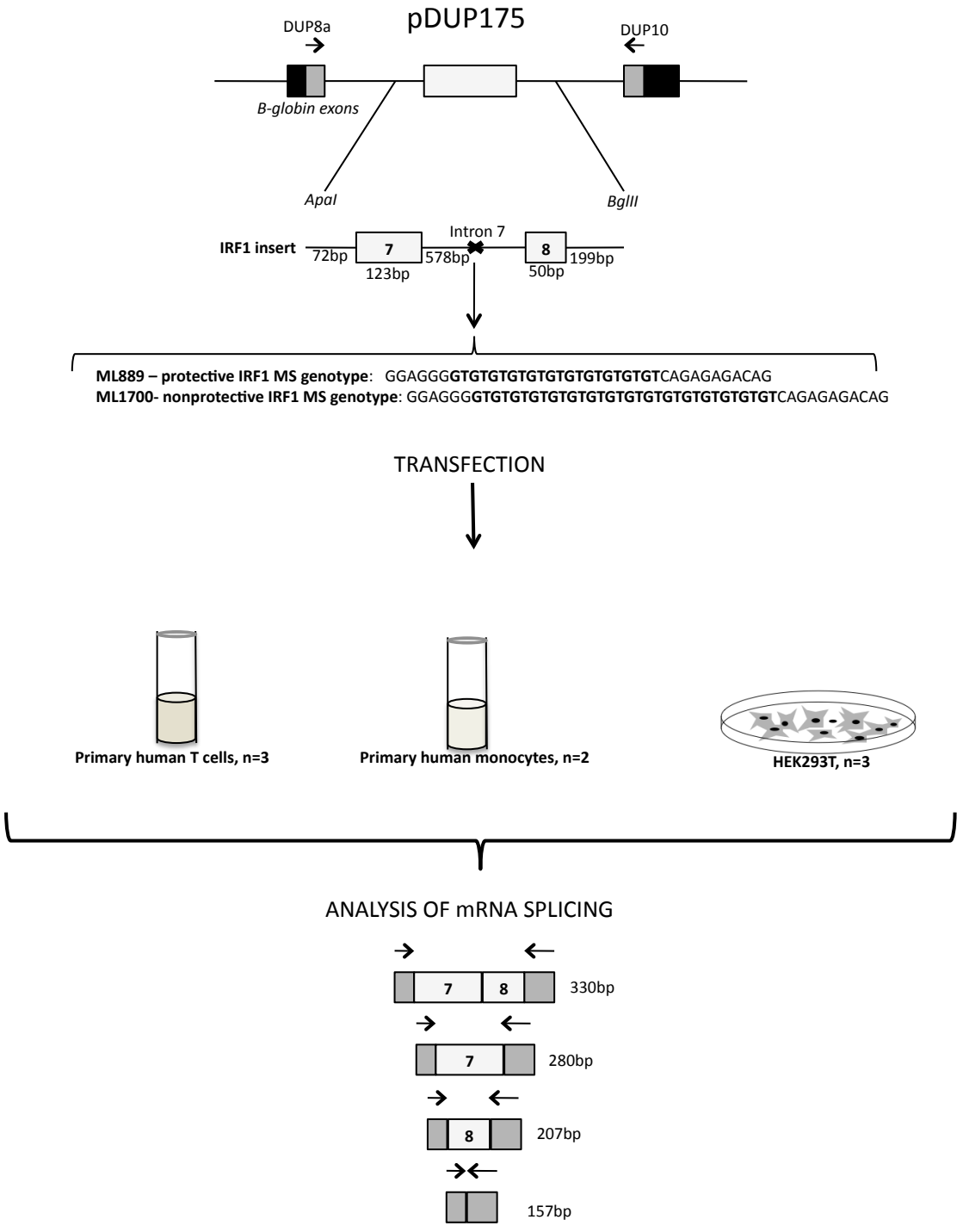


Figure 2.1 Schematic representation of the exon splicing assay.

2.13.1 Plasmid Construction

The pDUP175 splicing reporter[179] vector was a generous gift from Dr. Jiuyong Xie, Department of Physiology, University of Manitoba, and was used as a plasmid backbone. Cloning sites for the splicing assay were: *Apa*I (GGGCCC) and *Bgl*II (AGATCT). A 1000bp fragment starting from 72bp upstream of exon 7 to 199bp downstream of exon 8 was amplified and inserted between the *Apa*I and *Bgl*II sites of the pDUP175 vector.

2.13.2 Cloning

Amplification: The following primers were used to amplify the 1022bp intron6 to intron8 region of the IRF1 gene from individuals with protective and nonprotective IRF1 MS marker: Intron6FW: 5'-TTACGGGCCCATCGGAGGGCGCTCGATGTCT-3' and Intron8RV: 5'-ATCGAGATCTAGCCAGGCCTCCCACCTGCCT-3'. The PCR consisted of the following reaction: 2µl 10x Pfx PCR buffer, 2µl 10X Pfx Enhancer buffer, 0.4µl 50mM $MgSO_4$, 1µl 10mM dNTP mix, 1.5µl of 10mM both primer Intron6FW and Intron8RV, 0.25µl Pfx, 200ng DNA toped up to 20µl with sdH20. PCR reaction was run on the thermocylcer (Ventri 96 Well Thermal cycler, ABI) at following conditions: 94°C 2min, (94°C 15s, 60°C 30s, 68°C 1 min)x45, 68°C 7min. Following amplification, PCR products were resolved on a 1% agarose gel, the desired bands were cut out and gel purified using a QiAquick Gel Extraction Kit from Qiagen. Purified products were stored at -20°C until further use. The plasmid diagram and primer location are illustrated in Figure 2. 1.

Restriction digest (RD). RD was set up using *Apa*I and *Bgl*II restriction enzymes for both the DUP175 plasmid and amplified inserts (exon 7-intron7-exon 8). First an *Apa*I RD reaction for the insert was set up as follows: 25µl insert, 3µl Buffer 4 and 2µl *Apa*I incubated for 2 hours at 30°C. RD for DUP175 vector reaction was set up as follows: 5µl vector, 1µl React 4 buffer, 0.5µl *Apa*I and 3.5µl H20 incubated for 1h at 30°C and stored at -20°C. The RD products were gel purified to remove any buffers and enzymes and eluted with 25µl H2O. The *Bgl*II RD was set up as follows: Insert: ~23µl eluted insert, 3µl React 3 buffer, 2µl *Bgl*II and 2µl H2O for 2h at 37°C; Vector: ~23µl purified vector, 3µl React 3 buffer, *Bgl*II 1µl, 3µl H2O for 1h at 37°C. Following two rounds of digestion the total product was resolved on a 1% agarose gel and digested bands were cut out and gel purified using a QiAquick Gel Extraction Kit (Qiagen).

Ligation. Several ligation reactions were set up with following insert to vector ratio: 1:1, 2:1 and 3:1. The reaction consisted of 0.25µl of ligase, 2µl of ligase buffer, vector and insert and sdH2O to bring the volume up to 10 or 20 µl depending on the amount of vector and insert used. Ligation was carried out at 4°C overnight.

Transformation. 70 µl of DH5α bacterial suspension was added to the final ligation product. Cells were heat shocked by incubation on ice for 30 minutes and then at 42°C for 1 minute. Transformed cells were plated out on a LB+AMP plate (ampicillin 100mg/ml) and plates were incubated overnight at 37°C. Colonies were re-plated and screened for plasmid insert by colony PCR. The colony PCR Mix was set up as follows: 15µl of 2x PCR buffer, 0.7µl DUP8a, 0.7µl DUP10, 0.2µl Taq and 13µl

s.d.d.H2O. To each cold PCR mix small amount of colony was added using a pipette tip. The following PCR protocol was then conducted: 95°C 5min, (95°C 40s, 58°C, 40s, 72°C 1:20 min)x 35 cycles, followed by 72°C 7min. Colonies confirmed to carry the plasmid inserts were grown in 5ml LB+Amp overnight. The plasmid was then extracted using a QIAprep Spin Miniprep Kit (Qiagen) following the manufacturers' instructions.

Conformation. The identity of the resulting constructs was confirmed by sequencing using the DUP8a and DUP10 primers designed to amplify the 150bp plasmid region flanking the insert (DUP8A: 5'- CTCAAACAGACACCATGCATGG -3', DUP10: 5'- CAAAGGACTCAAAGAACCTCTG -3'). Sequencing was performed at the DNA core, Public Health Agency of Canada as described previously (Section 2.7.1) and data was analyzed using a Sequencher 4.0.5 software (Gene Codes Corporation, USA).

The following plasmids were generated: pDUP889 (plasmid containing the 1000bp insert from ML889 who has the protective IRF1 MS genotype) and pDUP1700 (plasmid containing the 1000bp insert from ML1700 who has the non-protective IRF1 MS genotype).

2.13.3 Cell Culture

The human embryonic kidney 293T cell line (HEK293T) and local donor PBMCs were transfected with the plasmid constructs. The 283T cells were grown in DMEM medium (Fisher) with 10% heat-inactivated FBS, and 1% Penicillin-Streptomycin. The local donor PBMCs were maintained in R10 cell culture media. All cells were maintained at 37°C in 5% CO_2 atmosphere.

Primary Cell preparation: 6 tubes of blood were obtained from 3 different local Winnipeg donors. PBMCs were isolated as described in Section 2.3.1. The human T Cell Enrichment Kit (StemCell Technologies, CA) was used to isolate primary T cells via negative selection following manufactures' instructions. Human monocytes were isolated by depletion of non-monocytes (negative selection) using the Monocyte Isolation Kit II (StemCell Technologies, CA) and MACS Separator (Myltenyi Biotech Inc., USA) following manufacturers' instruction.

2.13.4 HEK293T cell Transfection

HEK293T cells were grown to 80-90% confluence in T75 flasks and subcultured into a 12-well plates with $0.25x10^6$ cells/well 24hours before transfection. Cells were grown in 1ml of DMEM + 10% FBS and were 50-80% confluent on the day of the transfection. Immediately prior to transfection, 0.5ml of media was removed from the cells. The transfection procedure was performed with Lipofectamine LTX Plus reagent kit (Invitrogen). For each well to be transfected, 1µg of DNA was diluted in 200µl of DMEM without serum. Plus Reagent was added (1µl/1µg of plasmid DNA) and incubated at RT for 5 minutes. For each well of cells 3µl of the LTX reagent was added to the above mixture and incubated at RT for 30 minutes. The DNA-Lipofectamine complex was added to each well containing cells. Four hours after transfection 0.5ml of DMEM + 10% FBS was added to each well (total of 1ml/well). Transfection was carried out for 24 hours prior to analysis.

2.13.5 Primary Cell Transfection

Primary T cells were transfected using the Human T Cell Nucleofector® Kit (Amaxa) following manufacturers' instructions. A total of 5µg of DNA plasmid was used for

transfection with a Nucleofector® Program U-014 setting on a Nucleofactor™ 2b Device (Lonza) for high viability. The human Monocyte Nucleofector® Kit (Amaxa) was used for transfection of primary human monocytes. A total of 5µg of DNA plasmid was used for transfection with a Nucleofecor® program Y-001 setting.

2.13.6 Total mRNA and cDNA Preparation.

Total mRNA from transfected cells was prepared using a combination of TRizol Reagent and the Qiagen RNeasy Plus Mini Kit as described in Section 2.8.2. RNA was quantified using the Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, USA). One hundred nanograms of extracted RNA was used to make cDNA using the Qiagen QuantiTec Reverse Transcription Kit as described in Section 2.10.

2.13.7 Analysis of mRNA Splicing.

Following cDNA synthesis, a PCR reaction was performed using the following conditions: 95°C 5min, (95°C 40s, 58°C 40s, 72°C 1:20) x35, 72°C 7 min; using the DUP8a and DUP10 primers. The PCR reaction mix (25µl) was set up as follows: 12.5µl 2x PCR buffer, 4µl cDNA, 0.7µl 10mM-DUP8a, 0.7µl 10mM-Dup10, 0.25µl Taq and 6.85µl sdH20. Final products were resolved on a 3% agarose gel and Quantity One Software 4.6.3 (Bio-Rad) was used to quantify the band intensity.

Commercial kits used:

QuantiTect Rev. Transcription Kit, Qiagen (Cat. No. 205311)

QuantiTect SYBR Green PCR kit, Qiagen (Cat. No. 204145)

QIAquick Gel Extraction Kit, Qiagen (Cat. No. 28704)

QIAprep Spin Miniprep Kit, Qiagen (Cat. No. 27104)

EasySep® Human T Cell Enrichment Kit, StemCell Technologies (Cat. No. 19051)

Monocyte Isolation Kit II human, Miltenyl Biotec (Cat. No. 130-091-153)

Human T Cell Nucleofector® Kit, Amaxa (Cat. No. VPA-1002)

Human Monocyte Nucleofector® Kit, Amaxa (Cat. No. VPA-1007)

Commercial reagents used:

DMEM/HIGH WITH HEPES 1000, Fisher (SH3024902)

HEK293T Cell Line, Thermo Scientific (Cat. No. HCL4517)

FBS, Sigma-Aldrich (Cat. No. F1051-100ml)

Penicillin- Streptomycin Solution 100X, Corning-Cellgro (Cat. No. 30-002-CI)

LB broth, Miller (Cat. No. 7279)

LB agar, Miller (Cat. No. 71752-5)

Ampicilin, Sigma-Aldrich (Cat. No. A0166)

Taq DNA polymerase, Invitrogen (Cat. No. 18038-018)

Platinum Pfx DNA polymerase, Invitrogen (Cat. No. 11708-013)

T4 DNA Ligase, Invitrogen (Cat. No. 15224-017)

BglII, Invitrogen (Cat. No. 15213-010)

ApaI, Invitrogen (Cat. No. 15440-019)

Subcloning Efficiency DH5α Competent Cells, Invitrogen (Cat. No. 18265-017)

Lipofectamine LTX & Plus Reagent, Invitrogen (Cat. No. 15338-100)

Actinomycin D, Sigma-Aldrich (Cat. No. 1410)

Equipment:

Nucleofector® Device (Lonza)

MACS Separator (Myltenyi Biotech Inc., USA)

2.14 IRF1 Protein Expression Determination via Western Blot

PBMCs from individuals with different IRF1 haplotypes were isolated from whole blood by the Ficoll-Hypaque method. One million cells from each subject were washed with PBS + 2% FCS twice and then lysed with 10 µl per half million cells of M-PER Lysis buffer (Thermo Scientific, USA). If not immediately used protein samples were stored at -70°C. Protein samples were quantified using the Pierce™ BCA Protein Assay Kit following manufacturers instructions.

For each subject 15µg of protein sample was brought to a final volume of 20µl with sdH2O and 2µl of 10X Western Blot sample buffer. Samples were then boiled for 3 minutes and loaded into 10% SDS –polyacrylamide gel. Precision plus Protein™ Standard (Bio-Rad) was run on each gel. Gel electrophoresis was performed at 160 volts for 55 minutes. Gel separated proteins were transferred onto a nitrocellulose membrane using a semi-dry transfer apparatus (BioRad) for 30 minutes at 15 volts. The membrane was blocked with 5% nonfat milk in PBS with 0.05% Tween -20 (PBS-T) for 60 minutes at RT. Following a 30 minute wash with PBS-T, the membrane was incubated overnight with shaking at 4°C with anti-human IRF-1 polyclonal antibody (1/500 in 2% nonfat milk in PBS-T). After overnight incubation, the membrane was washed 6 times for 5 minutes in PBS-T to remove unbound antibodies. The membrane was then incubated for 1 hour with shaking at room temperature with peroxidise-conjugated AffinityPure goat anti-rabbit IgG (H+L) (1/16000 in 2% nonfat milk in PBS-T). The membrane was washed 6 times for 5 minutes and then an additional 2 hours in PBS-T. Immunoreactive bands were detected by ECL Advance™ Western Blotting Detection Kit. Following IRF1

detection, the membrane was stripped (175µl beta-mercaptoethanol, 5ml of 10% SDS, 1.56ml of 1M Tris pH6.8 and 18.2ml H2O) for 30 minutes at 55°C with shaking. As an internal control, the membrane was then re-probed for house-keeping protein actin with goat anti-actin polyclonal antibody (1/1000 in 2% nonfat milk in PBS-T for 2 hours) and peroxidase-conjugated AffiniPure rabbit anti-goat IgG (H+L) (1/20000 in 2% nonfat milk in PBS-T for 1 hour). Semi-quantitative analysis was conducted via band densitometry using a Fluorchem™8800 Imaging System (Altha Innotech Corporation US). The IRF1/actin ration was used for intra group comparisons.

Commercial kits used:

Pierce™ BCA Protein Assay Kit, Thermo Scientific (Cat. No. 23227)

Amercham ECL Advance Western Blotting Detection Kit, GE Healthcare Life Sciences (Cat. No. RPN2135)

Commercial reagents used:

M-PER Mammalian Protein Extraction Reagent, Thermo Scientific (Cat. No. 78503)

Precision Plus Protein ™ Standards All Blue, Bio-Rad (Cat. No. 161-0373)

Rabbit anti-human IRF-1, Santa Cruz (Cat. No. sc-497)

Goat anti-human actin, Santa Cruz (Cat. No. sc-1616)

Peroxidase–conjugated AffiniPure Goat anti-Rabbit IgG (H+L), Jackson ImmunoResearch (Cat. No. 111-035-003)

Peroxidase-conjugated AffiniPure Rabbit anti-Goat IgG (H+L), Jackson Immunoresearch (Cat. No. 305-035-003)

Ammonium Persulfate (APS), Thermo Scientific (Cat. No. 17874)

Temed, Bio-Rad (Cat. No. 161-0801)

Self prepared solutions:

10x Western Blot sample buffer: 11.7 ml 0.5M Tris (pH 6.8), 6.3g Glycerol, 1.7g SDS, 1.55 g DDT, 1.7% Brown phenolblue. Dissolve well and store at -20°C.

10% Seperation gel: 3.6ml of ddH2O, 2.5ml of 1.5M Tris (pH 8.8), 0.1ml 10%SDS, 3.33ml of Acrylamide, 0.005ml of TEMED, 0.5ml of Ammonium Persulfate

Stacking gel (5ml): 3.05 ml ddH2O, 1.25ml 0.5M Tris (pH 6.8), 0.05ml 10% SDS, 0.65ml Acrylamide, 0.005ml TEMED, 0.25ml 1% Ammonium Persufate

12.5X Running buffer (1L): 15g of Tris base, 72g of Glycine, 5g of SDS. Dissolve in ddH2O and bring to 1L final volume.

Stripping buffer: 175µl Beta-mercaptoethanol, 5ml of 10% SDS, 1.56ml of 1M Tris pH 6.8 and 18.2ml H2O

Equipment used:

Trans-Blot® SD Semi-dray Transfer Cell (Bio-Rad, Model: Trans-Blot® SD cell)

Fluorchem ™ 8800 Imaging System (Alpha Innotech Corporation, US)

Microplate Spectrophotometer (Molecular Devices, Sunnyvale, USA)

2.15 mRNA stability assay

For analysis of mRNA stability $6x10^6$ PBMCs were cultured in RPMI+10%FBS and exposed to actinomycin D[180,181] (final concentration 10µg/ml) for up to 20 hours. One million cells were collected at 0, 0.25, 0.5, 1, 3 and 20 hours and cells were lysed with TRizol Reagent (Invitrogen). RNA was isolated and prepared for qRT-PCR analysis as described in Sections 2.8.2, 2.9 and 2.10. IRF1 exon 1 expression was measured by qRT-PCR as outlined in Section 2.11.

Commercial reagents used:

Actinomycin D, Sigma-Aldrich (Cat. No. A1410-2MG)

2.16 Protein Stability Assay

For analysis of protein stability $4x10^6$ PBMCs were cultured in RPMI+10%FBS and exposed to cycloheximide[182] (final concentration 50µg/ml) for up to 12 hours. Samples were collected at 0, 3, 5 and 12 hours and cell lysates were prepared and protein expression levels were detected by Western blotting using anti-IRF1 antibody as described previously (Section 2.14). Actin was used as a loading control.

Commercial reagents used:

Cycloheximide, Sigma-Aldrich (Cat. No. C4859-1ML)

2.17 Millipore Miliplex Cytokine/Chemokine Assay

To simultaneously analyze multiple cytokines/chemokines from small volumes of plasma and CVL samples the Milipore Milliplex Cytokine/Chemokine Kits were used as per manufacturers' protocol with minor adjustments. In brief, 200µl of Wash Buffer (Human Cytokine/Chemokine Magnetic Bead Panel) or Assay Buffer (Human Cytokine/Chemokine Magnetic Bead Panel III) was added to a 96-well plate. After shaking for 10min at RT and decanting, 25µl of standards and controls were added to the appropriate wells, followed by addition of 25 µl of assay buffer and appropriate matrix to the background, sample and control wells. Twenty five µl of thawed plasma samples at RT were added to the plate, followed by 25 µl of fluorescent beads bound with anti-human cytokine/chemokine antibodies. Plates were incubated at 4°C with shaking either overnight (Human Cytokine/Chemokine

Magnetic Bead Panel) or for 2h at RT with shaking (Human Cytokine/Chemokine Magnetic Bead Panel III). The plates were washed 2x (MAG_2X wash protocol on Bio-Plex Pro™ II Wash Station, Bio-Rad) before adding 25µl of detection antibodies followed by incubation for 1h at RT with shaking. Then, 25 µl of Streptavidin-Phycoerythrin was added to each well and incubated at RT for 30min with shaking. The plates were washed 3x with wash buffer using the MAG_3X wash protocol on Bio-Plex Pro™ II Wash Station. Finally 150µl FACSFlow Sheath Fluid was added to the wells and plate was read on a Bio-Plex™ 200 System (100 beads per region, 60s sample timeout, gates: low: 5000, high: 25000, Bio-Rad). All plasma and CVL samples were thawed once only and assays were performed in duplicate.

Commercial kits used:

Human Cytokine/Chemokine Magnetic Bead Panel (Cat. No. HCYTOMAG-60K, HCP3MAG-63K-PX9 and HCP3MAG-63K-PX11)

Human Cytokine/Chemokine Magnetic Bead Panel III (Cat. No. HCYP3MAG-63K, HCYTMAG-60K-PX29, HCYTMAG-60K-PX30, HCYTMAG-60K-PX39, HCYTMAG-60K-PX42)

Commercial reagents used:

BD FACSFlow™ (Cat. No. 342003)

Equipment used:

Bio-Plex Pro™ II Wash Station, BIO-RAD (Cat. No. 300-34377)

Bio-Plex™ 200 System, BIO-RAD (Cat. No. 171-000205)

2.18 Millipore Miliplex Hormone Assay

Extraction of Plasma Human Samples (for the Steroid/Thyroid Hormone Magnetic Bead Panel): In order to analyze plasma cortisol, estradiol and progesterone levels, plasma samples need to be extracted to eliminate inhibitory and possible interfering substances. The Waters Extraction Method (Waters, USA) was used as per manufacturers' protocol. Briefly, 250µl of plasma was acidified by adding 250µl of 1% TFA. Samples were then loaded onto a Waters 96-well extraction plate, that has been previously equilibrated with 2 x 1ml 0.1% TFA (Solvent A). Wells were washed 3x with 1ml of Solvent A. Samples were eluted with 0.5 ml of acetonitrile/water/TFA (Solvent B). Loading, washing and elution steps were done at vacuum setting of Hg 2-5. The eluted samples were dried using SpeedVac at highest vacuum setting and reconstituted with 250µl of Milipore Miliplex Kit Assay Buffer.

Sample preparation for the Human Pituitary Magnetic Bead Panel 2: Plasma samples were diluted 1:10 with Assay Buffer supplied in the kit prior to assay.

Immunoassay procedure: To analyze hormone levels in small volumes of plasma we used a Milipore Milliplex Hormone Magnetic Bead Panels as per manufacturers protocol with minor adjustments. Briefly, 200µl of Assay Buffer was added to the 96-well plates. After shaking for 10min at RT and decanting, 25µl of Assay Buffer was added to all wells (for the Steroid/Thyroid Hormone Magnetic bead Panel) or 25µl of 1:10 Matrix Solution to Background, Standard and Quality Control wells with 25µl of Assay Buffer to Sample wells (for the Pituitary Magnetic bead Panel 2). Then, 25µl of Assay Buffer was added to the Background wells and 25µl of Standards and

Controls were added to the appropriate wells. Prepared samples (25µl) were added to the plate, followed by 25µl of HRP Conjugate to all wells (only for Steroid/Thyroid Hormone Magnetic bead Panel) and 25 µl of fluorescent beads bound with anti-human cytokine/chemokine antibodies (for both panels). Plates were incubated at 4°C with shaking overnight. The plates were washed 2x (MAG_2X wash protocol on a Bio-Plex Pro™ II Wash Station, Bio-Rad) before adding 25µl of detection antibodies followed by incubation for 1h at RT with shaking. Then, 25 µl of Streptavidin-Phycoerythrin was added to each well with incubation at RT for 30min with shaking. The plates were washed 3x with wash buffer using the MAG_3X wash protocol on Bio-Plex Pro™ II Wash Station. Finally 100µl FACSFlow Sheath Fluid was added to the wells and plates were red on Bio-Plex™ 200 System (100 beads per region, 60s sample timeout, gates: low: 5000, high: 25000). All plasma samples were thawed once only.

Commercial kits used:

Human Pituitary Magnetic Bead Panel 2 (Prolactin), Millipore (Cat. No. HPTP2MAG-66K)

Steroid/Thyroid Hormone Magnetic bead Panel (Cortisol, Estradiol and Progesterone), Millipore (Cat. No. STTHMAG-21K)

Commercial Reagents used:

Acetonitrile, Sigma-Aldrich (Cat. No. 34851-4L)

Trifluoroacetic acid (TFA), Sigma-Aldrich (Cat. No. T6508-100ML)

BD FACSFlow™, BD Biosciences (Cat. No. 342003)

Self prepared solutions:

Solvent A: 0.1% TFA in water

1% TFA in water

Solvent B: Acetonitrile/water/TFA (60%/40%/0.1%, v/v/v)

Equipment used:

Extraction Plate Manifold for Oasis 96-Well Plates (Product No. 186001831)

Oasis® HLB LP 96-Well Plate 60µl (60mg), Waters, (Part No. 186000679)

SAVANT DNA 120 SpeedVac Concentrator, Thermo SCIENTIFIC (Cat. No. 20-548-130)

Bio-Plex Pro™ II Wash Station, BIO-RAD (Cat. No. 300-34377)

Bio-Plex™ 200 System, BIO-RAD (Cat. No. 171-000205)

3. Tissue specific IRF1 expression

3.1 Rationale

Human tissues show considerable variation in terms of their level of susceptibility to HIV infection[58,183]. HIV gains access into the body during sexual intercourse by crossing the epithelial barrier that covers mucosal surfaces of the gastrointestinal and male and female genital tracts. The transmission probability per exposure event varies greatly between different tissues, estimated at between 1/200 to 1/2000 in the FGT and 1/20 to 1/300 in the intestinal tract[58]. The rate of HIV transmission across different tissues likely depends on multiple factors including structural differences, the density of HIV-1 target cells, inflammation levels, host antiviral immune responses and the presence of host factors required for successful HIV replication. IRF1 protein expression has been demonstrated in number of human tissues[184,185] and in almost all cell types including cells of the innate and adaptive immune systems[143,144,148]. Being necessary for early HIV replication prior to Tat production, differential IRF1 tissue expression could be a limiting factor for productive HIV replication in various tissues.

3.2 Hypothesis

Higher IRF1 expression is expected in tissues associated with increased susceptibility to HIV-1 infection and higher rate of HIV-1 replication, such as tissues of the intestinal and reproductive tracts.

3.3 Objective

To determine relative IRF1 transcript and protein levels within various human tissues including different anatomical sections of the FGT.

3.4 Section specific methods

3.4.1 Tissue specific IRF1 mRNA expression analysis

To identify tissue specific patterns of IRF1 expression, qRT-PCR was performed on the FirstChoice® Human Total RNA Survey Panel (Ambion) as outlined in Section 2.11. RNA samples from each tissue were a pool of 3 tissue donors of various ages, genders, ethnicities and causes of death. All donors were negative for HIV and HCV. All reactions were performed in duplicate and expression levels were normalized to 18S RNA.

3.4.2 Tissue specific IRF1 protein expression analysis

To identify tissue specific patterns of IRF1 protein expression Western blot analysis was performed on Customized Human Normal Tissue Blot (containing cervix, colon, liver, rectum, small intestine, spleen, testis and thymus tissue samples, ProSci Incorporated) and whole cell lysates obtained from normal human ectocervix, endocervix and endometrial tissue as outlined in the Section 2.14. Actin was used as a loading control.

Study population:

The customized human tissue blot consists of tissue lysates with 15µg of total cellular protein in each lane. Proteins were separated on 4-20% SDS-PAGE gel and

transferred onto nitrocellulose membrane. No information was provided on the origin of the samples or demographics of the study population.

FGT tissue samples were provided by Dr. Kristina Broliden (Karolinska Institute, Sweden) and included tissues from 7 healthy women (mean age 48, range 42-57 years). All study participants were HIV uninfected with no clinical symptoms of sexually transmitted infections during three months prior to undergoing hysterectomy for non-malignant and non-inflammatory conditions (heavy menstrual bleeding and/or benign myoma) at St. Goran Hospital in Stockholm, Sweden. Informed consent was obtained from all study participants and ethical approval was obtained from Regional Ethical Review Board in Stockholm.

3.4.3 Statistical analysis

Statistical analysis was performed using the GraphPad Prism (version 5, GraphPad software, La Jolla, CA) and p values <0.05 were considered to be statistically significant.

3.5 Results

3.5.1 IRF1 mRNA expression across different human tissues

To understand the expression of IRF1 mRNA in different tissues we carried out a qRT-PCR assay measuring IRF1 exon 1 expression in a panel of human tissues. IRF1 exon 1 was chosen as a target since it is not spliced-out and would allow us to measure total mRNA levels. We observed IRF1 expression in all tissues examined, with the colon, small intestine and thymus showing the highest relative levels of expression (Figure 3.1). Of interest in terms of HIV-1 infection, the cervix, adipose

tissue, esophagus, liver and testes had the lowest levels of IRF1 mRNA transcripts. The observed differences between the colon and cervical IRF1 transcript expression could potentially contribute to the differences in susceptibility and the rate of HIV acquisition between FGT and intestinal tract.

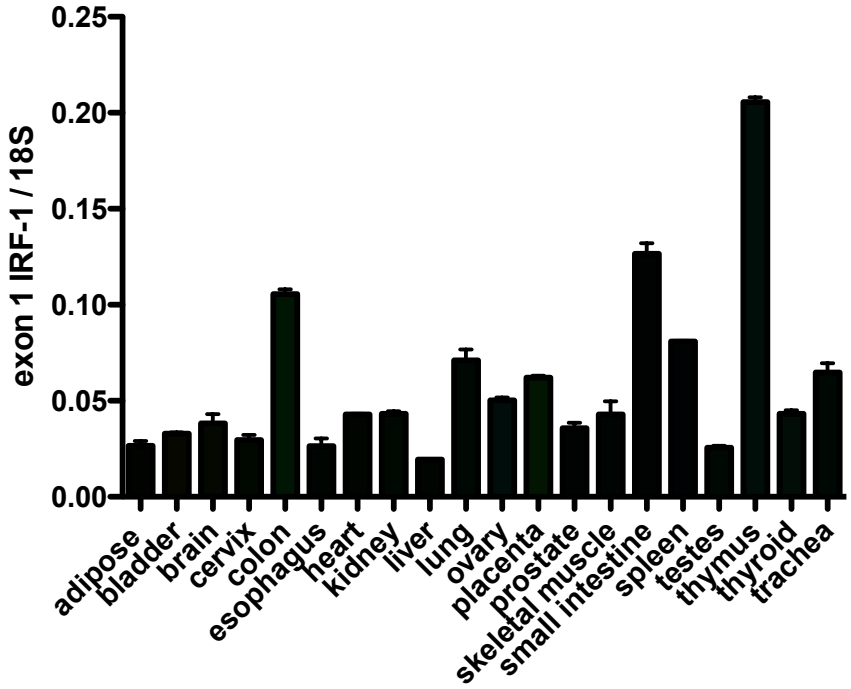


Figure 3.1 IRF1 transcript expression across different human tissues.

qRT-PCR specific for IRF1 exon 1 was conducted on a panel of RNA from various human tissues. Samples were run in duplicate and expression levels were normalized to 18S (Kruskal Wallis test, p=0.0062**).

3.5.2 IRF1 protein expression across different human tissues

To determine IRF1 protein expression in different human tissues we performed a Western blot analysis of Customized Human Normal Tissue Blot (ProSci Incorporated) containing tissue samples from the cervix, colon, liver, rectum, small intestine, spleen, testis and thymus. Unlike with IRF1 transcription, relatively constant expression of IRF1 protein was observed across all tissues (Figure 3.2). Of interest IRF1 protein expression in the cervix appeared to be higher then IRF1 protein expression in colon tissue. Actin levels were almost undetectable in liver and thymus tissue samples; potentially reflecting the decreased proportion of actin with respect to total cellular protein. Reasons for the lack of correlation between mRNA and protein levels potentially include posttranscriptional regulation and differences in mRNA and protein half-lives[186]. The observed differences between IRF1 mRNA and protein expression in various tissues may also in part be attributed to different sampling techniques and differences in study participant demographic and clinical characteristics.

To further characterize IRF1 protein expression in FGT tissue we analyzed biopsies of normal human ectocervix, endocervix and endometrium for IRF1 protein expression by Western blot. IRF1 protein expression was significantly different between the different compartments of the FGT (ANOVA <0.0001, Figure 3.3), with the highest levels observed in ectocervix, followed by the endocervix and the endometrium. This remained true when IRF1 protein levels were normalized to actin (Figure 3.3B) as well as relative to input protein alone (Figure 3.3C).

Differences in IRF1 protein expression potentially reflect the differences in FGT tissues susceptibility to early HIV infection.

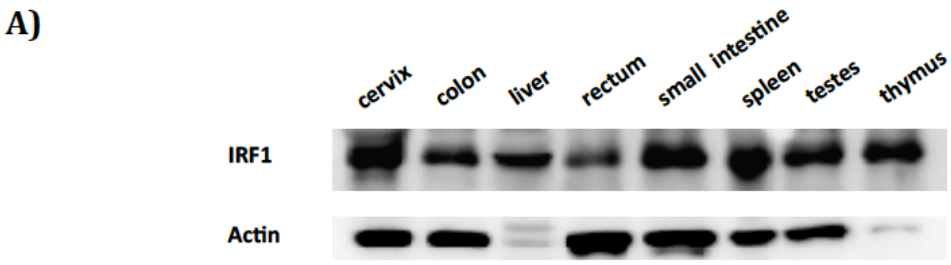
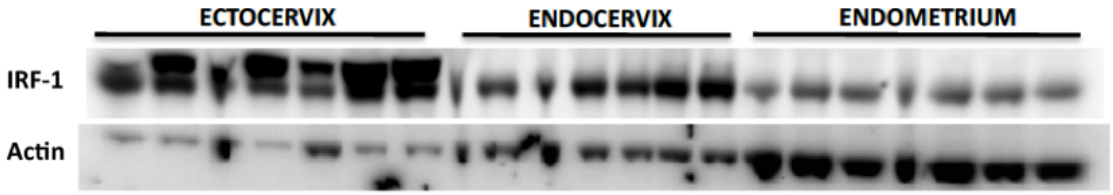


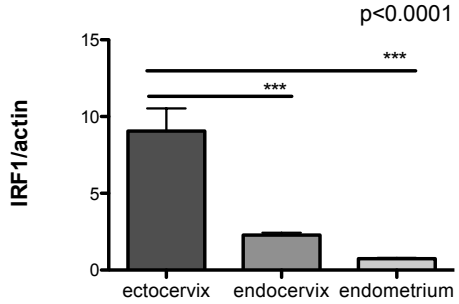
Figure 3.2 IRF1 protein expression across human tissues.

Western blot analysis was performed on a protein blot containing proteins from number of different human tissues. Relatively high level of IRF1 protein expression was observed across all tested tissues. Actin levels were almost undetectable in liver and thymus samples.

A)



B)



C)

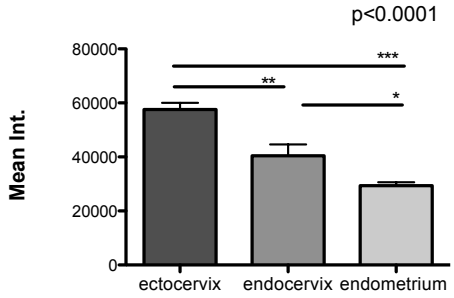


Figure 3.3 IRF1 protein expression in different sections of the FGT.

A) IRF1 western blot analysis of whole-cell lysates prepared from human ectocervix, endocervix and endometrium tissues (n=7). Equal amount of protein was loaded for all samples (15µg). Actin protein was used a loading control. **B)** Bars represent the mean of densitrometric quantization of IRF1 relative to actin protein levels from gels obtained from 7 independent samples. **C)** Bars represent the mean of densitrometric quantization of IRF1 relative to total input protein from gels obtained from 7 independent samples. (One-way ANOVA with Tukey's multiple comparisons test).

3.6 Discussion

Sexual transmission through the lower genital and rectal mucosa accounts for most current and new HIV-1 transmissions[58]. Significant differences exist between different tissues with respect to their level of susceptibility to HIV-1 infection. This can likely be attributed to a number of different factors, including structural differences, level of inflammation, availability of target cells, presence of host restriction factors and the expression of host factors utilized by HIV during early viral replication[61]. Like most viruses, HIV relies on host cell proteins to synthesize and assemble new virions, especially during the early stages of infection. The host IRF1/NFκB complex is essential for HIV transcription in the early stages of HIV infection, when viral transactivator Tat is absent or present at low levels[128]. This study examined IRF1 transcripts and protein expression in various human tissues, including different tissues within the FGT. IRF1 transcript levels varied significantly between different human tissues with the cervix having one of the lowest and colon one of the highest levels of IRF1 mRNA expression. We observed significantly higher IRF1 protein expression in the ectocervix compared to edocervix and endometrium.

Higher IRF1 mRNA expression in the colon and small intestine compared to the cervix could be a contributing factor to the roughly 10 fold higher transmission probability per exposure event in the intestinal tract compared to the FGT[58]. However a similar trend was not observed with IRF1 protein levels measured on the commercially available human tissue blot, where we observed notably higher IRF1 protein levels in cervix tissue lysates. These differences are most likely due to posttranscriptional regulation and differences in rates of mRNA and protein

degradation. Additionally, different sections of the female genital tract exhibit unique structural and physiological characteristics. HIV penetration and infection has been demonstrated in all sections of the FGT, including vaginal, ectocervical, endocervical and endometrial tissues[58]. Furthermore, significant differences in HIV transcription levels have been observed between different sections of the FGT. The ectocervix was shown to be more conductive to HIV-1 replication than the endometrium with 30-fold more HIV-1 transcripts in the ectocervix than in the endometrium on day 3 post infection[162]. Our results show significantly higher IRF1 protein expression in the ectocervix compared to the endocervix and the endometrium. Being a crucial factor in early HIV-1 transcription, increased ectocervical IRF1 protein levels observed in our study could explain increased level of HIV-transcription taking place in the ectocervix compared to endometrial tissues. Furthermore in addition to the most abundant IRF1 protein isoform at ~48kDa we observed an additional protein band (~56kDa) in ectocervix likely to be an alternate IRF1 protein isoform (Figure 3.3A). This suggests differences in IRF1 protein isoform abundance between different sections of the FGT.

Commensial microorganisms populate the lower FGT and human intestine and increased IRF1 protein levels observed in ectocervix and increased IRF1 mRNA levels in intestinal tract tissues could be attributed to the continuous microbial stimulation. The intermediate expression of IRF1 in the endocervix and the lowest expression in endometrium could potentially be attributed to the fact that this portion of the FGT is less exposed to external pathogens and stimuli.

Our results indicate that there is significant amount of heterogeneity in IRF1 protein and mRNA tissue expression. Being crucial for the initial HIV replication, IRF1 could be one of the main drivers of tissue-specific early HIV-1 transcription and should be considered as an important factor in efforts to decrease or prevent HIV-1 transmission. Reducing IRF1 expression at the ectocervix may significantly limit HIV replication and lead to reduction of HIV acquisition in women.

3.7 Limitations and Opportunities

Commercial samples used for the analysis of IRF1 mRNA and protein levels across different human tissues were obtained from a diverse group of study participants with no control for age, gender, and/or cause of death. Furthermore, the companies provided no information on the sampling techniques. While understanding tissue-specific IRF1 expression increases our knowledge of the general IRF1 immunobiology and potential IRF1 roles in tissue specific rates of HIV transmission, cell-specific analysis of IRF1 expression and regulation should also be determined. Depending on sample availability IRF1 protein levels in different sections of the FGT should be further investigated in terms of IRF1 polymorphisms associated with the HIV-resistant phenotype.

4. Effect of IRF1 polymorphisms on IRF1 mRNA splicing and mRNA/protein stability

4.1 Rationale

Three polymorphisms (SNPs at nucleotide location 619 and 6516 and a MS GT repeat) located at the intronic regions of the IRF1 gene (intron 1, 9 and 7 respectively) were shown to be associated with HIV-1 resistant phenotype and changes in IRF1 protein expression. The protective IRF1 alleles (619A, 179 MS and 6516G) were associated with decreased basal IRF1 protein expression and reduced responsiveness to IFNγ stimulation. The exact mechanism of how intronic polymorphisms in the IRF1 gene lead to changes in IRF1 protein regulation is unknown. Polymorphisms located in intronic regions of genes have been shown to act as regulatory elements and affect multiple processes including promoter activity, transcription, alternative splicing, and mRNA and protein stability[106]. Dinucleotide repeats, like the MS GT repeat in IRF1 intron 7, are known modifiers of transcription and splicing[104,106]. For examples, microsatellite GT repeats in the heme oxygenese-1 gene have been associated with cardiovascular mortality in number of different studies[187,188]. Furthermore, a polymorphic GT repeat in the human $Na^{+}Ca^{2+}$ exchanger intron 2 was shown to act as a strong intronic splicing enhancer[189]. This study examined the effect of three IRF1 polymorphisms associated with HIV-resistant phenotype on transcription and alternative splicing of IRF1 mRNA and IRF1 mRNA/protein stability.

4.2 Hypothesis

IRF1 polymorphisms, associated with the HIV-resistant phenotype, directly regulate alternative splicing of the IRF1 mRNA resulting in altered protein regulation.

4.3 Objective

To determine the effect of IRF1 polymorphisms associated with HIV-resistant phenotype on IRF1 transcription and alternative splicing of the IRF1 pre-mRNA and IRF1 mRNA/protein stability.

4.4 Section Specific methods

4.4.1 Study cohort

Samples used in this study were from the well-described Majengo Sex Worker Cohort, Nairobi, Kenya[107,190]. Based on their IRF1 genotypes study participants were separated in two groups: those who had protective haplotype associated with reduced susceptibility to HIV-1 infection (619AA, 179+179+, 6516GG, n=12) and those with nonprotective haplotype (619CC, $179^{-}179^{-}$, 6516TT, n=18). There was no significant difference in age between individuals based on their IRF1 haplotype (mean 36 years for protective vs mean 32 years for nonprotective, p=0.2779).

4.4.2 Statistical analysis

Haploview software (Broad Institute, USA) was used for the analysis of linkage disequilibrium between different IRF1 polymorphisms. Quantity One software (Bio-Rad Laboratories, CA) was used to quantify the intensity of the DNA and protein bands. GraphPad Prism 5.0a software (GraphPad Software, USA) was used to

analyze and graph the PCR, qRT-PCR and Western blot data. Differences between the groups were determined by parametric t-test and results were considered significant if $p<0.05$. Affymetrix CEL files were imported into and analyzed with Partek Genomic Suite (Partek Inc., USA) as described in Sections 2.12.2 and 2.12.3.

4.5 Results

4.5.1 IRF1 sequencing and microsatellite typing

Two single nucleotide polymorphisms, at location 619 (A>C) and 6516 (G>T) and a single microsatellite allele (IRF1 179) were shown to be associated with HIV-resistant phenotype in the Majengo HESN cohort in Nairobi Kenya[46]. Previously 1241 women were sequenced for the identified polymorphisms, 59 of which were HIV uninfected with at least one protective IRF1 genotype. However, due to loss to follow up, at the start of this project only 25 HIV uninfected individuals with at least one protective IRF1 genotype were still regularly sampled during biannual resurveys. To address this, IRF1 sequencing and microsatellite typing was performed for 544 new cohort participants. The overall numbers and genotype frequencies for all three IRF1 polymorphisms agree with the results from the previous studies and are indicated in Table 4.1. To test for non-random association of IRF1 polymorphism, linkage disequilibrium analysis was performed (Table 4.2). High D' values (0.9-0.994) indicate that the three IRF1 polymorphisms are co-inherited 90-99.4% of the time. More importantly high r^2 value (0.736-0.936) indicates that they are present at similar allelic frequencies, suggesting that the

resulting phenotype is likely the result of combined effect of all three polymorphisms.

Table 4.1. IRF1 619, 6516 and MS genotype frequencies in newly sequenced individuals

IRF1 polymorphism	IRF1 genotype	n	Genotype frequency*
619 (N=528)	AA	34	6.4 %
	AC	233	44.1%
	CC	261	49.4 %
6516 (N=432)	GG	27	6.3%
	GT	193	44.7%
	TT	212	49.1%
MS (N=493)	179179	42	8.5%
	179X	187	37.9%
	XX	264	53.5%

*Genotype frequency=(genotype/ N)*100; e.g. AA= (34/528)*100

Table 4.2. Linkage disequilibrium (LD) analysis for the 618, 6516 and MS IRF1 polymorphisms in newly sequenced individuals

IRF1 polymorphisms	D'	LOD*	r^2
619, 6516	0.994	137.4	0.936
619, MS	0.9	110.92	0.742
6516, MS	0.92	88.13	0.736

*Logarithm of the odds

4.5.2 IRF1 transcription levels

Lower IRF1 protein expression was previously observed in PBMCs from individuals with protective IRF1 haplotype, however the exact mechanism of how these intronic polymorphisms regulate IRF1 protein levels remained unknown. One of the possibilities was that IRF1 polymorphisms could affect IRF1 gene transcription. To test this, IRF1 mRNA levels in 12 individuals with protective and 11 with nonprotective IRF1 haplotypes were measured by qRT-PCR (Figure 4.1). IRF1 exon 1 was chosen as a target since it is not translated and would not be affected by alternative splicing allowing us to measure total mRNA. There was no observed significant difference in IRF1 exon 1 expression between individuals with protective and non-protective IRF1 haplotype ($p=0.6039$). These results suggest that IRF1 intronic polymorphisms associated with HIV-resistant phenotype do not affect IRF1 mRNA transcription and that other modes of IRF1 regulation could be at play.

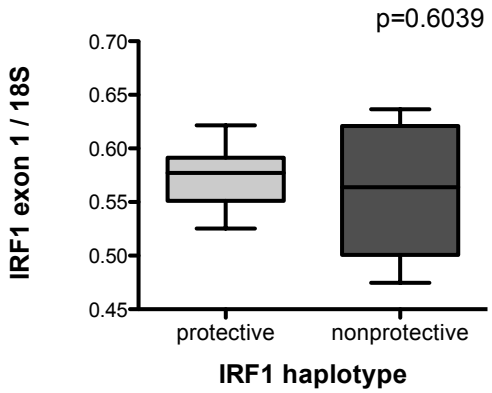


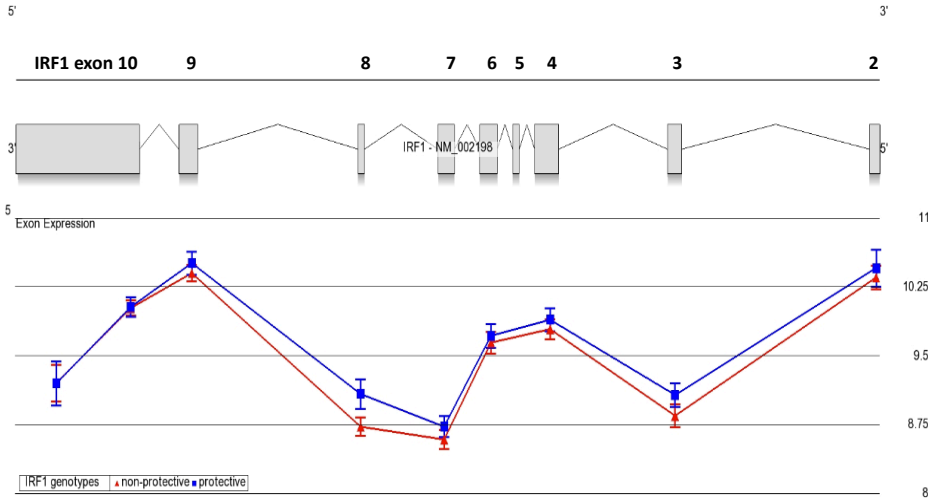
Figure 4.1. The IRF1 total mRNA expression in *ex vivo* unstimulated PBMCs from individuals with protective and nonprotective IRF1 haplotypes.

qRT-PCR analysis of intracellular IRF1 exon 1 mRNA expression in *ex vivo* PBMCs from individuals with protective (n=12) and non-protective (n=11) IRF1 haplotypes. Bars represent mean values. Expression levels were normalized to 18S. Not significant ($p>0.05$).

4.5.3 Alternative splicing of the IRF1 mRNA

To confirm the IRF1 mRNA expression levels and investigate alternative splicing of the IRF1 in PBMCs from individuals with protective (n=12) and nonprotective (n=18) IRF1 haplotypes, the IRF1 exon expression profile was examined via the Affymetrix Exon 1.0 ST array. Overall, the groups demonstrated a similar pattern of exon expression. However, alternative splicing event was observed in probe set 2875356 which corresponded to IRF1 exon 8 (Figure 4.2A). No difference was observed in the expression of other IRF1 exons, including exons of the DNA binding domain (exon 2, 3 and 4). Quantitative RT-PCR analysis of IRF1 exon 7/8 and exon 2/3 was done in order to validate the exon array results. Significantly higher expression of IRF1 exon 7/8 ($p=0.041$) was observed in samples from individuals with the protective IRF1 haplotype compared to nonprotective IRF1 haplotype, while no difference was observed in IRF1 exon 2/3 expression (Figure 4.2B). IRF1 exons 7/8 seem to be preferentially spliced in PBMCs from individuals with nonprotective IRF1 haplotype. Interestingly, alternative splicing of IRF1 exons 7, 8 and 9 was previously described as a cancer-specific splice variants in cervical cancer[131]. IRF1 exon 7/8 are part of the IRF1 transactivation and enhancer domain (Figure 1.1B) and exon 7/8 splice variants are likely to differ in their transcriptional activity on the IRF1 target promoters. Furthermore, splice variants lacking exon 7/8 were previously shown to have increased protein stability due to disruption of ubiqitin target sites[131].

A)



B)

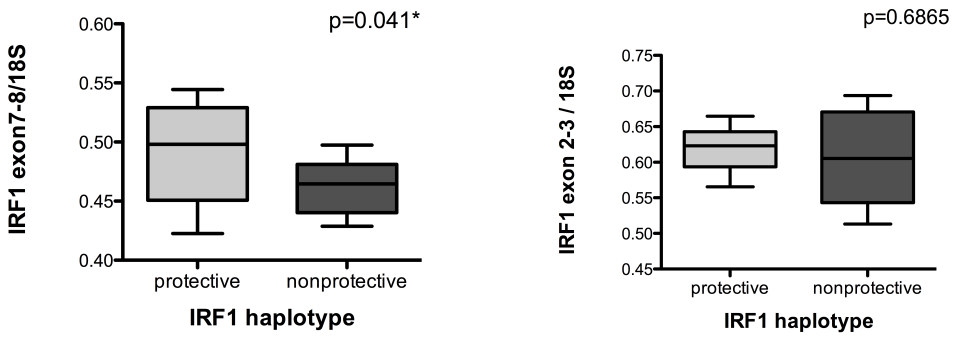


Figure 4.2. Exon splicing profile of the IRF1 mRNA.

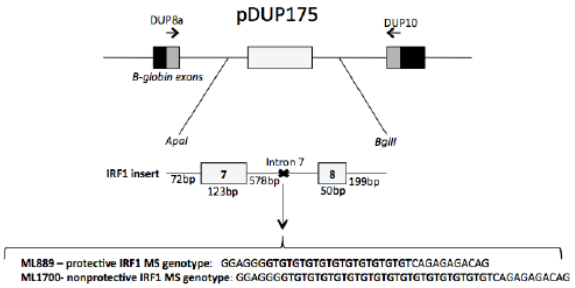
A) Exon structure of the IRF1 gene and IRF1 exon expression profile resulting from the Affymetrix Exon 1.0 ST array. IRF1 exon expression was analyzed on mRNA samples from individuals with protective (n=12, blue line) and nonprotective (n=18, red line) IRF1 haplotypes. Alternative splicing event was observed in probe set 2875356 corresponding to IRF1 exon 8 **B)** qRT-PCR analysis of intracellular IRF1 exon2/3 and exon7/8 mRNA expression in *ex vivo* PBMCs from individuals with protective (n=12) and non-protective (n=11) IRF1 haplotypes. Bars represent mean values. Expression levels were normalized to 18S. Not significant ($p>0.05$).

4.5.4 Exon splicing assay

Intronic microsatellite GT repeats were previously shown to act as regulators of alternative splicing in the human $Na^{+}Ca^{2+}$ exchanger gene[188]. In order to assess whether different lengths of GT repeat in IRF1 intron 7 could affect alternative splicing of the adjacent exons (7 and 8) we performed an exon-splicing assay. IRF1 exon 7 – intron 7- exon 8 segment with partial flanking intron sequences was cloned from individuals with a protective (ML889) and a nonprotective (ML1700) IRF1 MS genotype. The segment was inserted between constitutive β-globin exons of the splicing reporter vector DUP175 (Figure 4.3A and Figure 2.1). The constructed DUP889 and DUP1700 clones were transfected into HEK293 cells, and the expressed mRNAs were analyzed for the inclusion of exon 7 and 8 by PCR and gel electophoresis. Analysis of the total RNA extracted from cells transfected with DUP889 and DUP1700 constructs revealed 3 predominant bands at ~330bp, 208bp and 157bp (Figure 4.3B). Bands were sequenced and shown to correspond to the full length, Δ7 and Δ78 variants, respectively. The DUP889 construct with a $(GT)_{11}$ in intron 7 resulted in higher expression of variants lacking exon 7 and 8 compared to DUP1700 with $(GT)_{16}$. Thus the shorter GT repeat located in IRF1 intron 7 from individuals with the protective IRF1 MS genotype results in increased splicing of the flanking exons. This is opposite to the pattern observed with all three IRF1 polymorphisms in *ex vivo* PBMCs, where individuals with the protective haplotype (619AA, 179+179+, 6516GG) had lower levels of exon 7/8 splicing (Figure 4.2).

In order to confirm that the observed splicing patterns are not cell type specific, we transfected human primary T cells and monocytes with pDUP175 and plasmid constructs DUP889 and DUP1700 (Figure 4.4). As before, the expressed mRNAs were assayed for exon 7 and 8 inclusion by qPCR. Again, as was shown in the HEK293 cell line, in primary T cells and monocytes, the DUP889 construct with a $(GT)_{11}$ in intron 7 resulted in higher expression of variants lacking exons 7 and 8 compared to DUP1700 with $(GT)_{16}$ (Figure 4.4A and B). To the best of our knowledge this is the first study to show that the length of GT repeats effects RNA splicing in primary cells. Results indicate that irrespective of the cell type, shorter GT repeats in intron 7 lead to preferential splicing of the flanking exons. These results indicate that the protective IRF1 MS genotype (shorter GT repeat), independent of the 619 and 6516 polymorphisms, leads to increased splicing of the flanking exons, opposite of what is observed with the protective haplotype which contains all three protective polymorphisms.

A)



B)

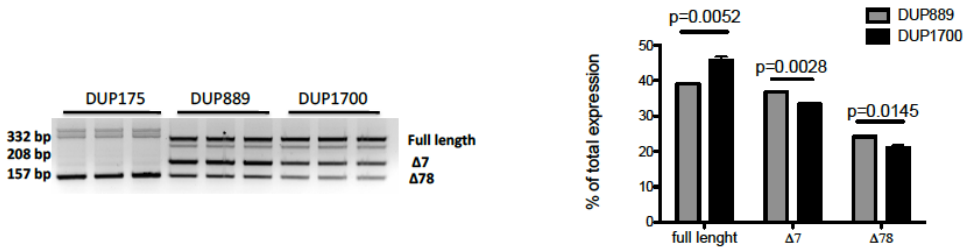


Figure 4.3 Effect of IRF1 MS polymorphisms on alternative splicing of the surrounding exons (7 and 8) in HEK293T cells.

A) Schematic representation of the exon splicing constructs. Fragments containing IRF1 MS polymorphisms (intron 7) and surrounding exons (7 and 8) from individuals with protective (ML889) and nonprotectve (ML1700) genotype were cloned into a splicing reporter vector pDUP175. The IRF1 insert replaces pDUP175 sequence between the *ApaI* and *BglII* sites. HEK293T cell line was transfected with pDUP175 and IRF1 plasmid constructs and exon splicing patterns were analyzed by PCR and gel electrophoresis. The position of the primers used for PCR assays are shown by arrows with primer names labeled. **B)** Exon splicing pattern in HEK293T cell line (n=3). Identity of splice variants was confirmed by sequencing. Each splice variant was quantified and described as a percentage of total expression (full length +Δ78+Δ8).

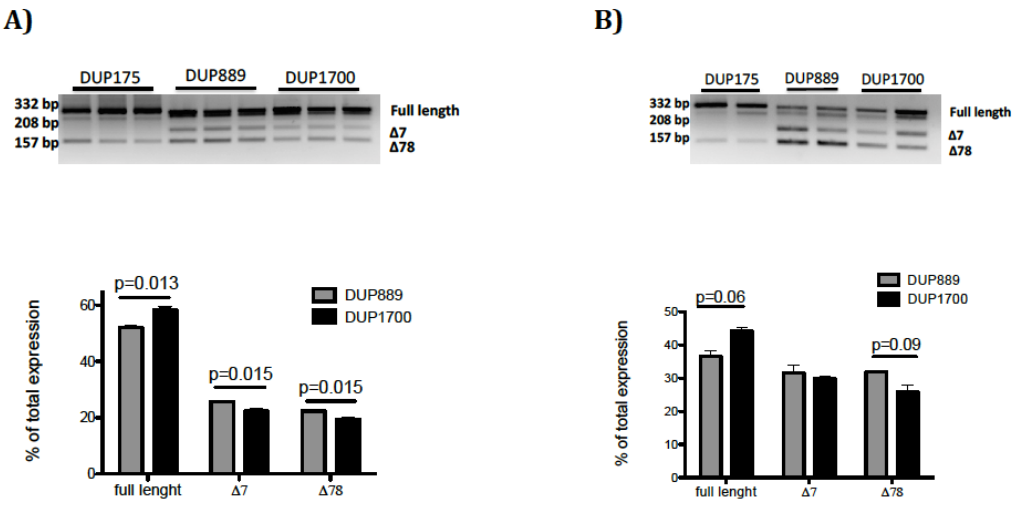


Figure 4.4 Effect of IRF1 MS polymorphisms on alternative splicing of the surrounding exons (7 and 8) in human primary cells.

Primary human T cells and monocytes were transfected with control plasmid pDUP175 and plasmid constructs containing IRF1 MS polymorphisms (intron 7) and surrounding exons (7 and 8) from individuals with protective (ML889) and nonprotectve (ML1700) genotype. Exon splicing patterns were analyzed by PCR and gel electrophoresis. **A)** Splicing pattern in human primary T cells (n=3) **B)** Splicing pattern in human primary monocytes (n=2); Identity of splice variants was confirmed by sequencing. Each splice variant was quantified and described as a percentage of total expression (full length +Δ78+Δ8).

4.5.5 IRF1 mRNA stability

To study whether IRF1 polymorphisms, associated with HIV-resistant phenotype, influence mRNA stability we evaluated the rate of mRNA decay in *ex vivo* PBMCs from individuals with protective and nonprotective IRF1 haplotypes. The mRNA levels were determined by qRT-PCR following inhibition of the RNA transcription by actinomycin D for 0, 0.25, 0.5, 1, 3 and 20 hours. No differences in the rate of mRNA decay were observed between samples from individuals with protective and nonprotective IRF1 haplotypes (Figure 4.5). These observations indicate that IRF1 polymorphisms (619, MS and 6516) do not affect IRF1 mRNA stability.

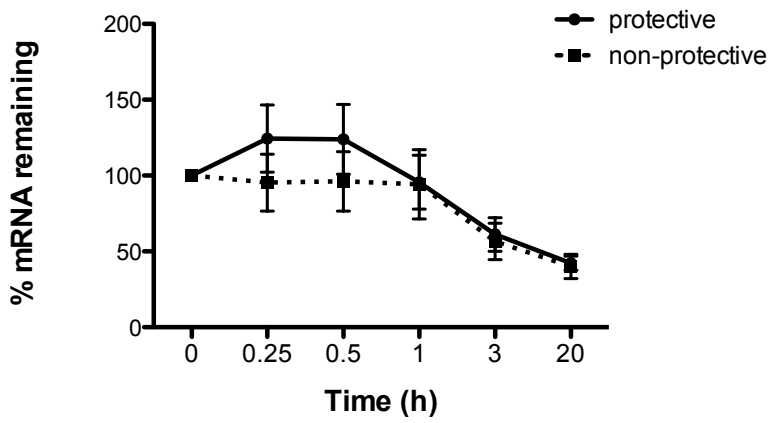


Figure 4.5. IRF1 mRNA stability in *ex vivo* PBMCs from individuals with protective and nonprotective IRF1 haplotypes.

qRT-PCR analysis of IRF1 exon 1 mRNA expression in *ex vivo* PBMCs treated with actinomycin D from individuals with protective (n=5) and non-protective (n=5) IRF1 haplotypes. Expression levels were normalized to 18S and represented as % of mRNA remaining.

4.5.6 IRF1 Protein stability

Protective IRF1 alleles (619A, 179 MS and 6516G) have been associated with decreased basal IRF1 levels as well as a transient IRF1 response to infection by a pseudotyped HIV-1[46,102]. Here we analyzed the stability of the IRF1 protein in *ex vivo* PBMCs from individuals with protective and nonprotective IRF1 haplotypes. Protein synthesis was blocked by the addition of cycloheximide for 0, 3, 5 and 12 hours. Western blot analysis showed that at 12 hours post cyclohexamide addition, significantly higher percentage of the initial IRF1 protein remained in PBMCs from individuals with nonprotective IRF1 haplotype (Figure 4.6). Our results showed that IRF1 protein in *ex vivo* PBMCs from individuals with protective IRF1 haplotype was degraded faster compared to the protein from PBMC samples of individuals with nonprotective haplotype. Thus IRF1 polymorphisms associated with HIV-resistant phenotype associate with the increased rate of IRF1 protein degradation.

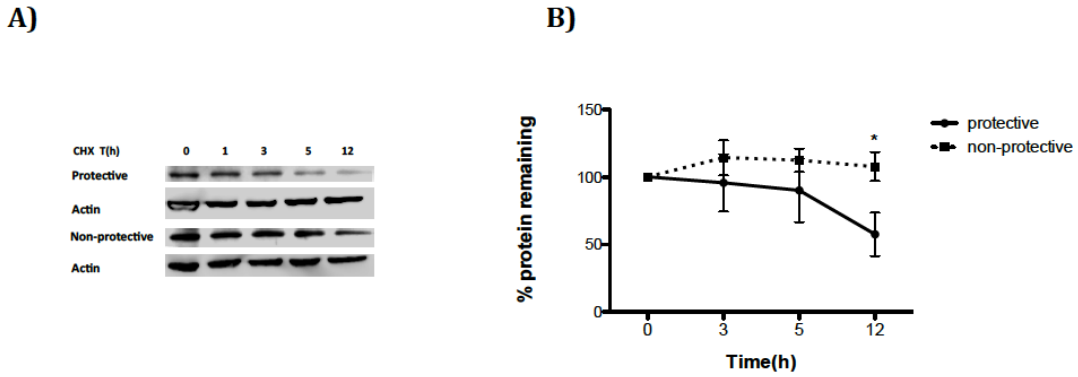


Figure 4.6. IRF1 protein stability in *ex vivo* PBMCs from individuals with protective and nonprotective IRF1 haplotypes.

PBMCs were treated with cycloheximide for indicated periods of time and Western blotting was used to examine protein stability. Actin was used as a loading control. **A)** Western blot from representative samples is shown (protective IRF1 haplotype- ML 887 and nonprotective IRF1 haplotype- ML1803). **B)** Quantification of IRF1 protein relative to actin levels, represented as the % of initial protein expression (n=5).

4.6 Discussion

All three IRF1 polymorphisms associated with HIV-resistant phenotype are located in the intronic regions of the IRF gene (619 in intron 1, 6516 in intron 9 and MS GT repeat in intron 7) and the exact mechanism of how they regulate IRF1 protein expression has not been determined. Here we examined the effect of IRF1 polymorphisms, associated with HIV-resistant phenotype, on IRF1 transcription, alternative splicing of the IRF1 mRNA and IRF1 mRNA/protein stability. Our data shows that identified IRF1 polymorphisms associated with HIV-resistant phenotype do not affect IRF1 transcription or the rate of mRNA decay but act as intronic splicing regulators. The Exon Array results indicate that PBMCs from individuals with protective haplotype (619A>C, 6516 G>T and MS 12 GT repeat) showed higher inclusion of IRF1 exons 7/8 compared to PBMCs from individuals with nonprotective haplotype. However, contrary to these findings when IRF1 MS polymorphism was cloned into the splicing-reporter vector DUP175, the shorter GT repeats associated with the protective IRF1 MS genotype resulted in increased splicing of the flanking exons 7 and 8. Additionally, it was shown that IRF1 protein in *ex vivo* PBMCs from individuals with the protective IRF1 haplotype was degraded faster when compared to the IRF1 protein in samples from nonprotective IRF1 haplotype.

Both the Exon ST. 1.0 Array and qRT-PCR analysis of the IRF1 mRNA, showed no difference in total mRNA expression or the rate of mRNA degradation between samples from individuals with protective and nonprotective IRF1 haplotypes

indicating that other methods of IRF1 regulation might be at play. Increased exon 7/8 inclusion (decreased exon7/8 splicing) was observed in *ex vivo* PBMCs from individuals with protective IRF1 haplotype, compared to samples from individuals with nonprotective IRF1 haplotype. Higher expression levels of IRF1 variants lacking exons 7, 8 and 9 alone or in some combination were previously observed in human cancerous cervical tissues[131]. IRF1 splice variants lacking exons 7,8 and 9 were shown to result in increased IRF1 protein stability and longer half-life due to disruption of the ubiquitin target sites. Interestingly, while these splice variants lacked transcriptional activity, they were shown to compete with wild-type IRF1 and lead to decrease in the overall IRF1 function. We observed higher levels of splice variants lacking exons 7/8 in *ex-vivo* PBMCs from individuals with nonprotective IRF1 haplotype. It seems possible that the increase in these splice variants in individuals with nonprotective IRF1 haplotype and the associated decrease in IRF1 function could contribute to increased susceptibility to HIV-1 acquisition.

The *in vitro* splicing assay results presented here indicate that the intronic GT repeat acts as a direct splicing regulator of the flanking exons. Interestingly, when IRF1 exon7-intron7-exon8 fragment containing GT repeat of varying lengths was cloned into a splicing reporter vector DUP175, the protective MS genotype (shorter GT repeat) resulted in increased splicing of the flanking exons. Intronic GT repeats were previously shown to act as splicing regulators [188]. To the best of our knowledge this is the first study to show that the length of an intronic GT repeat affects mRNA splicing in primary cells. Furthermore, discrepancy between *in vitro*

data looking at IRF1 MS genotype alone and *ex vivo* data looking at IRF1 haplotype indicates that IRF1 polymorphisms associated with HIV-resistant phenotype do not act independently of each other. All three IRF1 polymorphisms are in high linkage disequilibrium, and hyplotype analysis suggested that the protective effect of these polymorphisms was additive[46]. Furthermore, multiple mechanisms regulate alternative splicing; regulation is not restricted to the presence and the sequence of specific splicing sites but also depends on presence and binding of splicing factors as well as complex interactions with the transcription and chromatin machineries[191]. Overall, these results suggest that the observed HIV-resistant phenotype is likely the effect of all three protective IRF1 polymorphism acting together.

Our results show that IRF1 protein in *ex vivo* PBMCs from individuals with protective IRF1 haplotype is degraded faster compared to the protein from PBMC samples of individuals with nonprotective haplotype. This in part explains previous observations showing that protective IRF1 alleles (619A, 179 MS and 6516G) associate with decreased basal IRF1 levels as well as a transient IRF1 response to infection by a pseudotyped HIV-1[46,102]. Overall, our data suggests that individuals with protective IRF1 haplotype seem to have tighter IRF1 regulation. Lower basal IRF1 expression together with the ability for faster IRF1 protein degradation following stimulation could significantly limit HIV-1 transcription and replication, while at the same time allowing for the initiation of IRF1 mediated host immune responses. Persistently higher IRF1 protein levels observed in samples from individuals with nonprotective IRF1 haplotype, were previously shown to

support higher levels of HIV-1 transcription during initial stages of infection[102]. However the increase in splice variants lacking exons 7/8 in samples from individuals with nonprotective IRF1 haplotype could interfere with the overall IRF1 function and lead to a decrease in IRF1 mediated immune responses.

These studies provide further insight into the mechanism of IRF1 polymorphisms associated with HIV-resistant phenotype. While numerous studies have shown association of different polymorphisms with altered susceptibility to HIV-1 infection, this is one of the rare studies that provide a mechanism for such an action. Considering IRF1's role in the early HIV replication, latency and pathogenesis, modulation of IRF1 expression and regulation may potentially be utilized in the development of both preventative and therapeutic strategies.

4.7 Limitations and Opportunities

IRF1 is regulated through a variety of posttranslational modifications (phosphorilation, ubiquitination and SUMOylation) and the extent of these should be examined in samples from individuals with different IRF1 haplotypes. The observed differences in IRF1 protein stability are likely due to changes in the IRF1 protein ubiquitination and/or SUMOylation. Depending on sample availability cell specific IRF1 regulation should be evaluated as well. IRF1 protein variants lacking exon 7/8 should be generated and their effect on HIV acquisition and replication should be examined. Furthermore, the presence of IRF1 protein isoforms lacking exon 7/8 in *ex vivo* samples needs to be confirmed. This could potentially be

achieved by combination of IRF1 immunoprecipitation, 2D gel electrophoresis and mass spectrometry. The effect of the GT repeat length on splicing of the flanking exons should be confirmed by primer extension assay using radioactive labeling, considered to be the gold standard for evaluation of mRNA splicing.

5. Effect of IRF1 polymorphisms on systemic and mucosal cytokine/chemokine expression

5.1 Rationale

IRF1, by binding to the ISRE present in numerous immunological regulatory genes, plays a central role in regulating both innate and adaptive immune responses. Analysis of IRF1 knock-out mice indicates that IRF1 is a key regulator of CD8+ T cell maturation, macrophage function, DC differentiation and maturation, NK responses, Th1 differentiation and antigen presentation[114,115,140,143]. Interestingly, several of these processes have been linked with natural resistance to HIV-1 infection [80,173]. This highlights the potential role of IRF1-mediated immune responses in decreased susceptibility to HIV-1. A state of low baseline immune activation (immune quiescence, IQ) has been suggested to decrease susceptibility to HIV infection by limiting the number of available target cells[192]. An immune quiescent state has been observed at both the mucosal and the systemic compartments of HESN individuals at both the extra- and intra-cellular levels[81]. HESN women from the Majengo cohort were shown to have a significantly lower genital tract expression of IL1α, CXCL9 and CXCL10 as well as increased expression of antiproteases that have both anti-inflammatory and antiviral functions[87,95]. Of relevance, IRF1 has been shown to directly regulate the expression of several cytokines/chemokines (e.g. IFNβ[118] and IL15[193]). While individuals with both protective and nonprotective IRF1 haplotypes had a robust IRF1 response to

infection by a pseudotyped HIV-1, this response was transient and immediately controlled in the individuals with protective IRF1 haplotype[102]. As demonstrated in Section 4.5.6, IRF1 protein from PBMCs in individuals with protective IRF1 haplotype seems to be degraded faster then the protein from nonprotective haplotype. It thus seems likely that controlled and transient IRF1 expression associated with protective IRF1 haplotype may be sufficient to impede the establishment of HIV infection and at the same time allow for generation of IRF1 mediated immune responses. Furthermore, differences in IRF1 splice variants described in Section 4.5.3 could also contribute to changes in downstream gene regulation and affect the plasma and CVL cytokine/chemokine balance.

5.2 Hypothesis

IRF1 haplotypes regulate plasma and CVL cytokine/chemokine expression.

5.3 Objective

To characterize the association between IRF1 polymorphisms associated with HIV-resistant phenotype and systemic (plasma) and mucosal (CVL) cytokine/chemokine expression.

5.4 Section specific methods

Detailed description of methods used to quantify plasma cytokine/chemokine expression can be found in Section 2. 17.

5.4.1 Study cohort

Samples used for this study were obtained from the Majengo FSW Cohort in Nairobi, Kenya [190]. Overall, 138 HIV-negative individuals were enrolled in this study. DNA samples of individuals involved in this study were sequenced for previously identified IRF-1 polymorphisms [46]. Based upon IRF1 genotypes, samples were separated into two groups: those who had protective haplotype associated with reduced susceptibility to HIV-1 infection (619AA, 179+179+, 6516GG); and non-protective haplotype (619CC, 179-179-, 6516TT). As shown previously[46] and demonstrated in Section 4, all three polymorphisms are in strong linkage disequilibrium (Table 4.1, page 88). Plasma was obtained for cytokine/chemokine measurement and at the same visit epidemiological data was collected from each woman including: socio-demographic, sexual behavior, duration of sex work, number of sex clients, condom use, number of regular partners and reproductive history. Cohort participants also underwent a gynecological exam where CVL was obtained for cytokine/chemokine measurement and patients were tested for the presence of bacterial vaginosis (Nugent score), *Nisseria gonorrhoeae* and *Chlamydia* (PCR), syphilis (RPR) and Trichomoniasis. All individuals with positive results for any of the tested infections were excluded from the analysis.

5.4.2 Statistical analysis

Univariate statistical analysis was performed using GraphPad Prism v5.0a, (GraphPad Software, La Jolla, CA) and p values <0.05 were considered to be statistically significant. Its important to note that in the initial analysis no

corrections for multiple comparison were made and p-values are reported and discussed as observed [194]. The D'agostino and Pearson's omnibus normality test was used to test for Gaussian distribution. Data did not follow normal distribution and therefore Spearman nonparametric correlations and Mann Whitney non-parametric tests were used in the analysis.

As we have previously shown that age has a profound effect on systemic and mucosal cytokine/chemokine expression[195], multivariate analysis was performed using SAS version 9.3 program (SAS Institute, Cary NC). We ran linear regression models with IRF1 genotype as a predictor and age as a covariate. In order to fit the regression model some of the outcomes were log transformed (indicated in the table). Additionally, some models had shapes that were suggestive of a bi-modal distribution due to a large number of values that fell below the detection limit. Values below a given limit of detection (LOD) were not fully observed and should be considered censored. Ad hoc methods, such as imputing half the value of the LOD, although common have an inherent bias when the prevalence of censored values is high, as was the case with a number of tested cytokines [196]. Furthermore, data with values below the LOD did not fit the conventional regression model assumptions. To accommodate these data a left-censored regression model with IRF1 genotype as a predictor and age as a covariate was used. This approach is similar to those used in the survival analysis literature, which often deals with right-censored endpoints. Left censored models were fit using the LIFEREG procedure of SAS, with log transformations as appropriate. For all log-transformed models,

comparisons are made in terms of ratios of medians, not differences in means, as is the case in conventional normally distributed models.

5.5 Results

5.5.1 Cohort characteristics

All participants examined in this study were HIV-uninfected female sex workers from the Majengo cohort [107,190]. Study participants were sequenced for 3 different IRF1 polymorphisms (619, 179 microsatellite (MS) and 6516) as described in Section 2.7. All participants who tested positive for the presence of bacterial vaginosis, *Nisseria gonorrhoeae*, *Chlamydia,* syphilis and trichmonoiasis (n=26) were excluded from the analysis as concurrent infection with other sexually transmitted diseases can greatly influence the expression of cytokines/chemokines at the systemic and mucosal level. This resulted in 110 plasma samples and 113 CVL samples that were included in the final analysis. Only 75 plasma and CVL samples were tested for MIP-3β and IL-12p70 due to limitations in sample volume. Sixty-four CVL and plasma samples that were analyzed were date matched. No significant difference in median age was observed between individuals based on the IRF1 genotypes (plasma samples: median age 37 (protective) vs 39 (non-protective), p=0.7702, Man-Whitney Test; for CVL samples: median age 42 (protective) vs 38 (non-protective), p=0.2157, Man-Whitney Test). Selected study cohort characteristics are shown in Table 5.1.

Table 5.1. Study cohort characteristics

Sample type	Plasma			CVL		
Parameter	protective	non-protective	p -value	protective	non-protective	p -value
Age (median, IQR)	42 (30-49.8)	38 (31.3-44.8)	0.2157	37 (38.8-49)	39 (31-44)	0.7702
%Kenyan	88	86.7	-	85.8	84.2	-
%Tanzanian	12	10	-	14.2	14	-
% Ugandan	-	3.3	-	-	1.8	-
Duration of sex work in years (median, IQR)	7 (2-21)	10 (4-17)	0.6538	13.5 (3.3-22)	10.5 (5-19.3)	0.7267

5.5.2 Effect of IRF1 polymorphisms on plasma cytokine/chemokine expression

A Milliplex cytokine/chemokine bead assay was conducted in order to characterize the cytokine/chemokine expression patterns that may be unique to IRF1 haplotype, associated with the HIV-1 resistant phenotype. No significant difference in the median cytokine/chemokine levels was observed between the two groups (protective vs nonprotective IRF1 haplotype) for the majority of the plasma cytokines/chemokines examined, including IL1β, IL2, sIL2Rα, IL8, IL10, IL12p70, sCD40L, Fractalkine, IP10, MCP1, MCP3, MIP1β, TNFα, ITAC, MIG, MIP3α and MIP3β (Table 5.2). However significantly higher IL6 (p=0.0113) and IL15 (p=0.0071) and significantly lower MIP1α (p=0.0431) plasma cytokine expression was observed using univariate analysis in individuals with protective IRF1 haplotype compared to individuals with nonprotective IRF1 haplotype (Figure 5.1, Table 5.2). Although not reaching statistical significance, there was a trend towards higher plasma IFNγ

(p=0.0669) and IL17 (p=0.0672) in individuals with protective IRF1 haplotype (Figure 5.1).

Table 5.2 Plasma cytokine/chemokine expression in individuals with protective (n=50) and non-protective (n=60) IRF1 haplotype.

The Mann –Whitney test was used to determine significance ($p<0.05$), [pg/ml, median, interquartile range (IQR)].

Cytokine/Chemokine	Protective IRF1 haplotype (median, IQR)	Non-protective IRF1 haplotype (median, IQR)	p-value
MIP3β	138.3 (118.1-165.7)	144.3 (115.3-209)	0.4253
MIG	871.3 (550-1253)	896.9 (544.3-1960)	0.6036
MIP3α	14.97 (10.06- 22.39)	16.19 (9.66- 34.57)	0.3921
ITAC	114.2 (76.49-169.9)	120.4 (63.04-251.6)	0.7255
IL1β	0.5 (0.5-0.5)	0.5 (0.5-0.5)	0.1422
IL2	0.8 (0.8-2.08)	0.8 (0.8-1.23)	0.2424
sIL2Rα	10.25 (10.25-33.47)	10.25 (10.25-29.03)	0.8309
IL6	1.83 (0.65-5.14)	0.65 (0.65-2.47)	0.0113
IL8	14.39 (6.34-27.28)	14.05 (8.28-25.89)	0.5168
IL10	0.8 (0.8- 2.06)	0.8 (0.8-0.97)	0.1183
IL12p70	7.78 (4.12-19.21)	8.06 (5.13-13.45)	0.9070
IL15	0.85 (0.85-4.25)	0.85 (0.85-0.85)	0.0071
IL17	3.18 (0.89-7.06)	2.62 (0.6-4.45)	0.0672
sCD40L	3410 (1802-5134)	3948 (1714-7603)	0.4440
Fractalkine	85.92 (48.22-146.2)	78.56 (53.13-108.4)	0.3045
IFNγ	10.20 (4.39-18.53)	7.48 (1.89-12.95)	0.0669
IP10	313 (233.9-478.5)	332.4 (276-589.6)	0.1311
MCP1	125.2 (94.62-159.1)	133.7 (107.6-177.6)	0.2042
MCP3	23.99 (9.85-33.05)	17.90 (7.99-26.85)	0.1721
MIP1α	9.5 (3.18-18.18)	12.87 (6.31-34)	0.0431
MIP1β	25.85 (20-33.18)	26.85 (19.31-44.79)	0.3233
TNFα	9.12 (6.89-11.98)	9.02 (6.43-11.62)	0.6396

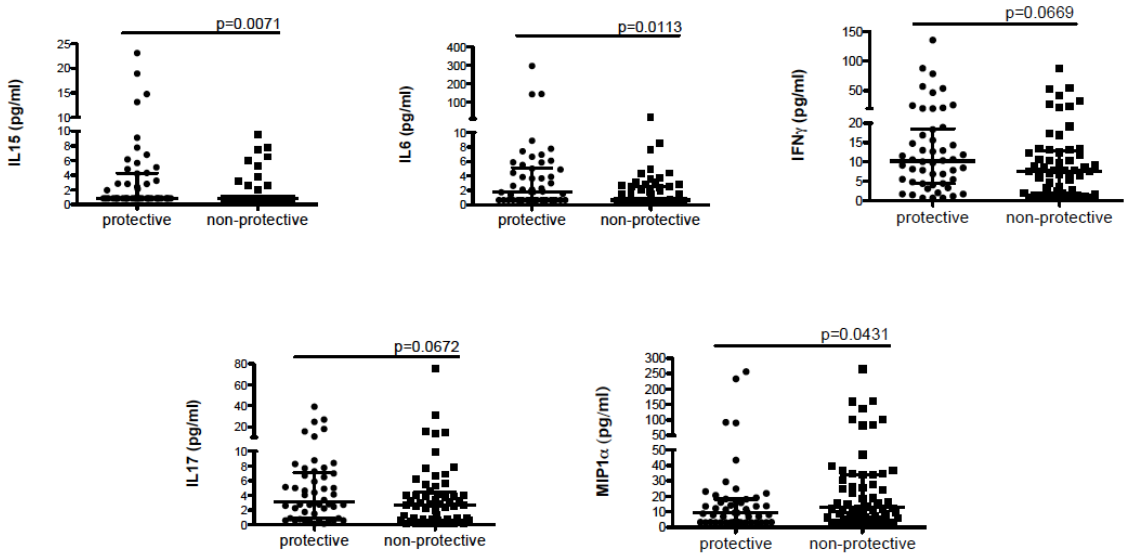


Figure 5.1. Quantification of plasma cytokine/chemokine levels between participants with differing IRF1 haplotypes.

The plasma cytokine/chemokines (pg/ml) differentially expressed in individuals with different IRF1 haplotypes: protective (n=50) vs non-protective (n=60). The Mann-Whitney test was used to determine significance ($p<0.05$), bars represent the median expression and interquartile range.

We had previously shown that age has a profound effect on plasma and CVL cytokine/chemokine expression[195]; therefore regression models with IRF1 genotype as a predictor and age as a covariate were run to further confirm that the observed differences in cytokine/chemokine levels were not due to aging of the immune system. After multivariate adjustment HESN subjects with protective IRF1 haplotype were shown to have significantly higher plasma IFNγ (p=0.0407), in addition to IL15 (p=0.0093) and IL6 (p=0.0085) expression levels, which confirmed the univariate analysis (Table 5.3 and Table 5.4). No significant differences were observed in the expression of other tested cytokines (data not shown), including IL17 and MIP1α differentially expressed between the two groups by univariate analysis (Table 5.3 and Table 5.4). Thus, individuals with protective IRF1 haplotype have significantly higher IL6, IL15 and IFNγ plasma expression when compared to the individuals with nonprotective IRF1 haplotype independent of the effect of age.

Table 5.3. Linear regression model of associations between plasma cytokine/chemokine expression and IRF1 haplotype adjusting for age.
The non-protective IRF1 haplotype was used as the reference category.

Cytokine/chemokine	Parameter	Estimate	p-value	95% CI	
logIFNγ	protective	0.5842	**0.049**	0.0031	1.1652
	non-protective	0.0000	.	.	.
	Age	-0.0134	0.329	-0.0404	0.0137
logIL17	protective	0.4862	0.076	-0.0509	1.0235
	non-protective	0.0000	**.**	.	.
	Age	-0.0015	0.903	-0.0265	0.0234

Table 5.4. Left-censored regression model of association between plasma cytokine/chemokine expression and IRF1 haplotype adjusting for age.
The non-protective IRF1 haplotype was used as the reference category.

Cytokine/chemokine	Parameter	Estimate	p-value	Lower-upper limit	
logIL6	protective	1.2567	**0.0085**	1.3782	8.9597
	non-protective	0.0000	.	.	.
	Age	-0.0110	0.6261	0.9462	1.0339
IL15	protective	5.7716	**0.0093**	1.4216	10.1217
	non-protective	0.0000	**.**	.	.
	Age	0.1927	0.0566	-0.0054	0.3908
MIP1α	protective	-14.8422	0.2223	-38.679	8.9951
	Non-protective	0.0000	**.**	.	.
	Age	0.2217	0.6926	-0.8774	1.3208

As IRF1 has been described to be the main transcriptional regulator of IL6[139] and IL15[140], we next examined the correlation between cytokines shown to be differentially expressed depending on the IRF1 haplotype (IL6, IL15, IL17, IFNγ and MIP1α). A significant, moderate to strong positive correlation (r_s: 0.4859 – 0.8402) was observed between all cytokines except MIP1α (Table 5.5). IL6 positively correlated with IL15 (r_s:0.5896, p<0.0001), IFNγ (r_s:0.5315, p<0.0001) and IL17(r_s:0.5675, p<0.0001), IL15 positively correlated with IFNγ (r_s:0.4859, p<0.0001) and IL17 (r_s:0.4955, p<0.0001), and IFNγ positively correlated with IL17 (r_s:0.8402, p<0.0001). The consistency and the magnitude of the correlations

between IL6, IL15, IFNγ and IL17 cytokines suggests an interdependent and coordinate regulation of their expression, potentially mediated by IRF1.

Table 5.5. Spearman rank correlations between plasma cytokine/chemokine levels differentially expressed between protective and nonprotective IRF1 haplotypes [rs (p-value), n=110]

Cytokine/Chemokine	IL6	IL15	IFNγ	IL17	MIP1α
IL6		0.5896 (<0.0001)	0.5315 (<0.0001)	0.5675 (<0.0001)	0.0734 (0.4461)
IL15			0.4859 (<0.0001)	0.4955 (<0.0001)	0.0481 (0.6177)
IFNγ				0.8402 (<0.0001)	0.0591 (0.5394)
IL17					0.0879 (0.3613)
MIP1α					

5.5.3 Effect of IRF1 polymorphisms on CVL cytokine/chemokine expression

The mucosal environment of the female genital tract represent the first line of defense against HIV in heterosexual transmission and likely contributes to the failure of most infected foci to develop into an established infection site. The expression of 22 cytokines/chemokines in CVL samples was measured in order to examine the effect of IRF1 genotypes on the immune environment of the mucosa. All cytokines except MIP3α were detectable in the CVL samples using the Milliplex cytokine/chemokine bead assay (Table 5.6). Significantly higher IL2 (p=0.0402),

sIL2Rα (p=0.0345), IL15 (p=0.0297) and IFNγ (p=0.0267) levels were observed in CVL from individuals with protective IRF1 haplotype when compared to nonprotective IRF1 haplotype (Figure 5.2). Although not reaching statistical significance, there was a trend towards higher IL17 level in CVL from individuals with protective IRF1 haplotype (p=0.0685). Overall we observed significantly higher CVL levels of IL2, sIL2Rα and IL15 and IFNγ (also shown to be upregulated in plasma) of individuals with protective IRF1 haplotype. However, after multivariate adjustment none of the examined cytokines were shown to be significantly different between the two groups. Due to the nature of CVL sample collection and associated dilution of secretions a significant number of samples had cytokine/chemokine values fell below limit of detection. A left-censored regression model was used to accommodate these data, however this lead to a loss of statistical power and an inability detect differences in cytokine/chemokine expression by this method (Supplemental data, Table 10.1).

Table 5.6. CVL cytokine/chemokine expression in individuals with protective (n=37) and nonprotective (n=76) IRF1 haplotypes.

The Mann –Whitney test was used to determine significance ($p<0.05$), [pg/ml, median, interquartile range (IQR)].

Cytokine/Chemokine	Protective IRF1 haplotype (median, IQR)	Non-protective IRF1 haplotype (median, IQR)	p-value
MIP3β*	-	-	-
MIG	199.4 (36.49-942.6)	253.4 (79.46–889.6)	0.3637
MIP3α	2.87 (2.51-9.04)	3.36 (2.31–24.28)	0.2336
ITAC	0.85 (0.85 -1.25)	0.85 (0.85-1.74)	0.8702
IL1β	4.35 (0.5-27.84)	2.8 (0.5-19.99)	0.5693
IL2	0.8 (0.8 – 3.63)	0.8 (0.8- 0.88)	0.0402
sIL2Rα	10.25 (10.25-20.29)	10.25(10.25-10.25)	0.0345
IL6	3.34 (0.65-12.72)	2.01 (0.65-9.82)	0.7683
IL8	458.6 (61.77-1179)	558.3 (101.7-1768)	0.4703
IL10	0.8 (0.8-9.61)	0.8 (0.8-2.97)	0.2621
IL12p70	0.5 (0.5-5.55)	0.5 (0.5-4.52)	0.8177
IL15	0.85 (0.85-6.44)	0.85 (0.85-1.74)	0.0297
IL17	0.6 (0.6-4.4)	0.6 (0.6-1.43)	0.0685
sCD40L	3.3 (3.3 – 30.1)	4.5 (3.3-10.95)	0.9222
Fractalkine	18.85 (18.85- 146.3)	18.85 (18.85-63.28)	0.2279
IFNγ	2.96 (0.55-11.64)	1.33 (0.55-4.12)	0.0267
IP10	37.3 (7-189.5)	50.26 (7 – 246)	0.6946
MCP1	19.61 (4.52-95.63)	24.07 (4.05-90.02)	0.8829
MCP3	7.28 (3.2- 16.02)	4.36 (3.2- 9.52)	0.1780
MIP1α	3.1 (3.1-33.03)	3.1 (3.1-23.45)	0.7364
MIP1β	12.40 (2.4-32.13)	2.4 (2.4-22.06)	0.1813
TNFα	1.2 (0.55-3.79)	0.55 (0.55- 1.67)	0.0765

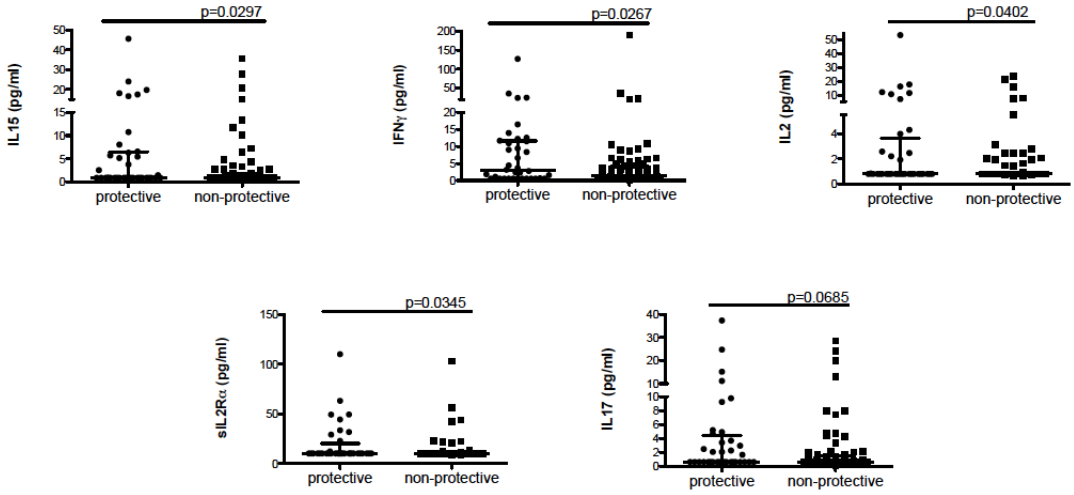


Figure 5.2. Quantification of CVL cytokine/chemokine levels between participants with differing IRF1 haplotypes.

The CVL cytokine/chemokines (pg/ml) differentially expressed in individuals with different IRF1 haplotypes: protective (n=37) vs non-protective (n=76). The Mann-Whitney test was used to examine significance ($p<0.05$), bars represent the median expression and interquartile range.

IRF1 is known to either directly or indirectly regulate the cytokines that are uniquely associated with IRF1 protective haplotype. Therefore we examined the correlation between CVL cytokines that were differentially expressed between different IRF1 haplotypes (IL2, sIL2Rα, IL15, IFNγ and IL17) to test if their expression is regulated by a shared mechanism. There was a significant, moderate positive correlation (r_s: 0.4878 – 0.7863) between all the affected cytokines (IL2, sIL2Rα, IL15, IFNγ and IL17, Table 5.7). IL2 positively correlated with sIL2Rα (r_s:0.6626, p<0.0001), IL15 (r_s:0.7863, p<0.0001), IFNγ (r_s:0.6206, p<0.0001) and IL17(r_s:0.6885, p<0.0001); sIL2Rα positively correlated with IL15 (r_s:0.7307, p<0.0001), IFNγ (r_s:0.4878, p<0.0001) and IL17 (r_s:0.6338, p<0.0001); IL15 positively correlated with IFNγ (r_s:0.6505, p<0.0001) and IL17 (r_s:0.6942, p<0.0001) and IFNγ positively correlated with IL17 (r_s:0.7073, p<0.0001). Observed strong correlations suggest coordinate regulation.

Table 5.7. Spearman rank correlations between CVL cytokine/chemokine levels found to be differentially expressed between protective and nonprotective IRF1 haplotypes.

[rs (p-value), n=113]

Cytokine/Chemokine	IL2	SIL2Rα	IL15	IFNγ	IL17
IL2		0.6626 (<0.0001)	0.7863 (<0.0001)	0.6206 (<0.0001)	0.6885 (<0.0001)
sIL2Rα			0.7307 (<0.0001)	0.4878 (<0.0001)	0.6337 (<0.0001)
IL15				0.6505 (<0.0001)	0.6942 (<0.0001)
IFNγ					0.7073 (<0.0001)
IL17					

Since our data shows significantly higher levels of IL15 and IFNγ and a trend towards higher IL17 in both plasma and CVL samples from individuals with protective IRF1 haplotype, we examined if there was a correlation between the expression of these cytokines between plasma and CVL in matched samples (n=64, Table 5.8). A statistically significant, weak to moderate correlation in IL15 (r_s= 0.2502, p=0.0461), IFNγ (r_s=0.3563, p=0.0042) and IL17 (r_s=0.4482, p=0.0002) levels was observed between plasma and CVL samples. The weak association and the observed differences between plasma and CVL cytokine expression could be contributed to the differences in lymphocyte repertoire and tissue specific IRF1 expression between systemic and mucosal compartments.

Table 5.8. Spearman rank correlations between cytokine/chemokine levels found to be differentially expressed between protective and nonprotective IRF1 haplotypes in matched CVL and plasma samples from the same patient.
[rs (p-value), n=64]

Cytokine/Chemokine	r_s	p-value
IL15	0.2502	0.0461
IFNγ	0.3563	0.0042
IL17	0.4482	0.0002

5.6 Discussion

Studies on natural immunity to HIV-1 infection suggest that early innate immune responses may be crucial in fighting and controlling incoming virus and these responses may lead to the establishment of effective adaptive immunity. Previous work from our group suggests that protective IRF1 genotypes may play a crucial role in preventing initial acquisition of HIV-1. A transient, yet robust IRF1 response, such as the one observed in PBMCs from our HESN cohort[102,107], could significantly limit HIV replication without hindering the induction of the IRF1 mediated antiviral immune responses. IRF1 has been identified as a key regulator of several immune processes including NK cell responses, Th1/Th2 differentiation and MHC class I and II expression[114,115,140,143]. IRFs are also know to induce an antiviral state, by stimulating the expression of ISGs [174]; many of which have direct intrinsic antiviral effects or act to recruit and enhance adaptive immune responses. The results presented here demonstrate that individuals with protective IRF1 haplotype exhibit a unique cytokine expression profile at both the systemic and mucosal compartments. Significantly higher plasma levels of IL6, IL15 and IFNγ and significantly higher CVL IL2, sILRα, IL15 and IFNγ were observed in individuals with protective IRF1 haplotype. Furthermore we observed a moderate to strong correlation between the affected cytokines within the systemic and mucosal compartments suggesting an interdependent and coordinate regulation by a common transcriptional regulator. Interestingly, we observed a low level of correlation between IL15 and IFNγ expression between plasma and CVL, possibly

due to differences in immunobiology, lymphocyte distribution and regulatory mechanisms between mucosal and systemic compartments.

Specifically our data showed increased plasma and CVL levels of both IL15 and IFNγ in individuals with protective IRF1 haplotype. Transcriptionally regulated by IRF1, IL15 plays an important role in regulating NK cell numbers and function. IRF1 knock-out mice exhibit severe NK –cell deficiency and this is in part thought to occur due to the lack of IL15 expression in bone marrow cells [140,145]. IFNγ, shown to be upregulated in both plasma and CVL samples from individuals with protective IRF1 genotypes is a key cytokine that stimulates innate immune responses and directs adaptive T cells toward a Th1 type response. IFNs are known to inhibit HIV replication both in primary cells such as macrophages and PBMCs as well as monocyte and T cell lines[54-56,197]. Furthermore, IL15 was shown to activate IFNγ mRNA and protein production in both NK and T cells[198]. The observed increase in plasma and CVL levels of IL15 and IFNγ from individuals with protective IRF1 haplotype could potentially be a cause and a consequence of increased NK cell activity. A number of different studies have demonstrated that IFNγ production by innate immune cells, particularly NK cells, plays a decisive role in natural protection against HIV-1 infection. NK cell mediated IFNγ production was significantly elevated in a discordant HESN cohort where individuals were exposed to HIV through sexual intercourse with a known HIV+ve partner[89] and in Vietnamese HESN intravascular drug users, compared to healthy controls and HIV+ve individuals [75]. Data from the Majengo HESN cohort also demonstrated

that activated NK cells from HESN women produced higher levels of IFNγ and are capable of more robust degranulation when compared to susceptible uninfected women[90]. Additionally, higher levels of IFNγ production in an unstimulated state [93] and stronger inhibition of HIV-1 replication *in vitro*[94] have been reported in NK cells expressing the protective killer cell immunoglobulin-like receptor (KIR) 3DS1, associated with reduced risk of HIV-1 infection[91]. Data from several HESN cohorts, including the results presented here, demonstrate that increase in IL15 and IFNγ production and the associated changes in NK cell mediated immune responses may represent a key player in natural protection against HIV-1 infection.

In addition to IL-15 and IFNγ, significantly higher levels of IL6 were observed in the plasma of individuals with protective IRF1 haplotype. IFNγ is known to induce IL6 gene expression in monocytic and other cell types through activation of IRF1, which synergistically with NFκB regulates IL6 transcription[139]. The late stages of HIV infection are associated with IL6 overproduction[199] and this is thought to contribute to the overall B cell deregulation[72,200] and an increased risk of all cause mortality in HIV infected patients[201]. However IL6 does not lead to increased HIV replication in HIV infected patients [202] and increasing levels of IL6 in HIV infected patients are considered to be a consequence rather then a cause of disease progression[203]. Interestingly, IL6 was shown to have direct antiretroviral effects and IL6 treatment results in a dose-dependent inhibition of HIV replication in primary human macrophages [203]. The findings presented here suggest that IL6 may be playing a role in protection from early HIV-1 infection.

While HIV mediated overproduction of cytokines/chemokines and general HIV-1 associated immune dysregulation following the establishment of infection is known to be detrimental to the host, a balanced antiviral host immune response could be sufficient in restricting the early HIV replication and allowing for viral clearance.

Although not statistically significant, a trend towards higher IL17 levels was observed in both plasma and CVL samples from individuals with protective IRF1 haplotype when examined by univariate analysis. Additionally there was a moderate to strong, statistically significant correlation between IL17 and the other affected cytokines (Table 5.5. and 5.7.). Predominantly described as a T cell-secreted cytokine, IL17 is also produced by innate immune cells including NK and iNKT cells and plays a key role in mucosal tissue defenses[204]. IL17 was shown to directly promote the release of β defensin 2 (hBD2)[205] in epithelial cells. hBD2 is known to inhibit HIV infection[206] and increased hBD2 levels were reported in HESN individuals [77]. Furthermore IL15 was shown to elicit IL17 production in human PBMCs, and IL17 production was found to be significantly lower in patients with detectable plasma viremia when compared to successfully treated HIV infected patients[207]. The observed trend of higher IL17 levels in individuals with protective IRF1 genotypes could potentially be a consequence of higher IL15 production and increase in NK cell activity.

Although not passing more stringent statistical analysis, we observed significantly higher levels of IL2 and sIL2Rα in CVL samples from individuals with protective IRF1 haplotype. IL2 shares many similarities with IL15, having nearly

identical signaling complexes, they both stimulate proliferation of T and B cells and enhance generation and persistence of NK cells[208]. While IL2 can amplify CD8+T cell responses and drive immune stimulation[209], sIL2Rα-IL2 complex is necessary for maintenance and function of regulatory T cells[210] and immune suppression. Elevated frequency of CD4+CD25+FoxP3+ Regulatory T cells was shown to be associated with resistance to HIV-1 infection in the Majengo cohort[81] and plays an important role in controlling levels of T cell activation and protection for HIV-1 infection by reducing the number of HIV-1 target cells.

The weak association and the observed differences in between plasma and CVL cytokine expression are not surprising considering the differences in immune environment and lymphocyte repertoire between systemic and mucosal compartments. Furthermore, as demonstrated in Section 3 IRF1 expression and IRF1 regulation varies greatly between different human tissues. However, increased levels of IL15 and IFNγ in both plasma and CVL of the individuals with protective IRF1 haplotype potentially point to a better NK cell mediated responses both at the initial site of exposure to the virus as well as systemically.

Although the aberrant activation of immune responses by HIV contributes to disease progression, the beneficial aspects of innate immune responses in preventing initial HIV infection and replication are evident. It is generally believed that the epithelial microenvironment and innate immune responses represent the main barrier that the HIV-1 has to overcome in HESN individuals. These antiviral immune responses provide protection immediately after exposure and potentially contribute to the low

probability of vaginal transmission, the failure of most infected foci to become established and the restricted cellular tropism observed in HIV infection[47,177]. Controlled, transient IRF1 response observed in HESN individuals[102,107], together with heightened antiviral cytokine expression may be sufficient in directly limiting viral transcription at the exposure site while at the same time initiating specific antiviral immune responses and preventing systemic infection.

5.7 Limitations and Opportunities

Depending on sample availability, cell specific IRF1 expression should be examined in matched samples with the obtained plasma and CVL cytokine/chemokine profile in order to establish a direct link between IRF1 regulation and the observed phenotype. The results presented here point to a better NK cell mediated responses and those should be examined in the context of IRF1 polymorphisms.

The current CVL collection method results in dilution of cervical secretions and potentially accounts for the large number of values falling below the LOD. Soft cup method should be considered for the future studies as it collects undiluted cervical secretions. Additionally, longitudinal studies to address cytokine/chemokine expression differences between IRF1 haplotypes can be performed, in order to increase statistical power. This can be difficult to achieve by increasing sample size in the cross-sectional studies due to low frequency of the protective IRF1 haplotype.

6. Effect of IRF1 polymorphisms on HIV disease progression

6.1 Rationale

As discussed in Section 1.7 IRF1 is one of the key players in the HIV infection process, important for early HIV replication, establishment and termination of viral latency and HIV pathogenesis[211]. IRF1 is up-regulated early in HIV infection and together with NFκB activates HIV LTR transcription even in the absence of the viral transactivator Tat [137,212].

Previous studies have shown that PBMCs from individuals with protective IRF1 haplotype exhibited significantly lower basal IRF1 expression[46] and a reduced ability to transactivate the HIV-1 LTR, suggesting a limited ability to support HIV replication[102]. However, the connection between the protective genetic polymorphisms and susceptibility to infection is not absolute. Infection can occur despite pre-existing protective mechanisms due to behavioral factors correlated with increased viral exposure, immune activation due to presence of other genital infections[213], or other risk-related genetic polymorphisms. While the protective IRF1 polymorphisms were shown to restrict HIV replication during the early stages of infection, their impact on disease progression once a person becomes infected remains unknown. This study examined the role of the described IRF1 polymorphisms on HIV-1 disease progression after infection.

6.2 Hypothesis

Protective IRF1 genotypes are associated with decreased disease progression and reduced HIV VL in HIV-1 infected ART-naïve individuals

6.3 Objective

To determine if IRF1 polymorphisms associated with the HIV-resistant phenotype play a role in HIV pathogenesis and disease progression in HIV infected individuals

6.4 Section specific methods

6.4.1 Study cohort

Study participants were HIV-infected antiretroviral therapy (ART)-naïve female sex workers from Nairobi Majengo cohort (n= 847) [107,190] and were sequenced for IRF1 polymorphisms (619, 179 MS and 6516) as described in the Section 2.7. CD4 data collection was performed during bi-annual follow-up from 1990 onwards and CD4 counts were determined using Becton Dickinson Tritest reagents (Section 2.5). Study participants were followed for a median of 1,072 days [interquartile range (IQR) 247-2,472 days], and had a median of 6 CD4 counts during that period (IQR 2-11). The median age at last visit was 37 (IQR 32-43). Viral loads were determined on a randomly selected subset of patients (n=263), as standard of care in Kenya does not routinely include HIV VL. Plasma viral loads were measured using Roche bDNA viral load assay v. 3.0 (Section 2.6).

6.4.2 Statistical analysis

A Kruskal-Wallis test for non-parametric data was used to test differences in age and follow-up time between individuals with different IRF-1 genotypes. In an early study on the effects of IRF1 polymorphisms, Kaplan – Meier survival analysis was conducted to determine if polymorphisms in IRF1 played a role in HIV disease progression [46]. This initial study was limited due to large number of seroincident subjects, an inability to control for CD4 count at enrollment, and a small sample size. Here, we analyzed the slope of CD4 decline using unstructured linear mixed models analysis with random effects (slopes and intercept) in a much larger cohort to unequivocally determine the effects of IRF1 polymorphisms on HIV disease progression. Natural log of the CD4+ T cell count was the dependant variable, as these have been shown to decline linearly with time[214]. Natural log CD4 counts are comparable to square root CD4 counts, since they are comparable in the ranges studied[215], and advantageous since the interpretation of the estimate is more straightforward. IRF1 genotypes were used as independent predictors of CD4 decline, classified into three groups: protective haplotypes associated with decreased susceptibility to HIV infection (619AA, 179+179+, 6516GG); neutral haplotypes (619AC, 179+179-, 6516GT) and haplotypes associated with increased susceptibility (619CC, 179-179-, 6516TT). All three IRF1 loci were analyzed separately, including their interactions with time. Data for the IRF1 619 polymorphism is displayed here, as 619A was the primary allele associated with HIV resistance, and the other two polymorphisms are in strong linkage disequilibrium[46]. Results for the other two IRF1 polymorphisms were identical

unless stated otherwise. As done elsewhere[216,217], only participants with a baseline CD4>350 were included, since this is the threshold for ART initiation as recommended by WHO [218]. CD4 decline near the start is more meaningful for progression, since individuals with CD4<350 have for the most part, progressed. For study participants where viral load data was available, mixed models analysis was used to examine the relationship between identified IRF1 polymorphisms and viral load, controlling for CD4 count at the time of the viral load measure. Statistical analysis was performed using PASW Statistics for Mac version 18.0 (SPSS Inc., Chicago, Illinois, USA).

6.5 Results

6.5.1 Characteristics of study participants

To investigate the role of IRF1 polymorphisms in HIV acquisition and disease progression, we determined the IRF1 genotypes of 1,492 participants. Approximately 60% of sequenced individuals were HIV-1 infected (847/1,492). Only HIV infected participants who had a CD4>350 at baseline (487/847, 57.5%) were analyzed in this study. Out of the final study participants, 8.9% were homozygous for the protective 619 IRF1 allele (AA), 43.1% were heterozygous (AC) and 48 % were homozygous for the non-protective allele (CC). No significant difference in age between individuals based on the IRF1 619 genotype was observed [median 42 (AA) vs 36 (AC) vs 38 (CC), p=0.101, Kruskal Wallis Test]. Participants with AA genotype were followed for a median of 2023 days, compared to 1462 and 1746 days for AC and CC genotypes respectively (p=0.219, Kruskal Wallis Test). The

median follow-up for AA genotype was 9 CD4 counts/participant compared to 7.5 for AC and 8 for CC genotypes (p=0.101, Kruskal Wallis Test). There was no significant difference in baseline CD4 counts between the three groups (p=0.508). Similar characteristics were observed for the 6516, and the 179 MS, which was expected as these 3 polymorphisms are in strong linkage disequilibrium [12]. Summary of baseline characteristics of the study participants are shown in Table 6.1.

Table 6.1. Baseline characteristics of the study participants (HIV positive with CD4+ T cell count>350, n=487)
[219]

Parameter	IRF1 619 genotype			p-value
	AA	AC	CC	
% Total number (n=487)	8.9	43.1	48	-
% Female	100	100	100	-
Nationality (%)				-
Kenya	90	78	69	
Tanzania	10	21	29	
Uganda	-	1	2	
Age (median, IQR)	42 (34-46.5)	36(33-43)	38(33-43)	0.101
Follow-up, days (median, IQR)	2023 (740-3241)	1462 (351.8-3069.3)	1746 (469.5-3261.5)	0.219
CD4 counts at baseline (median, IQR)	508 (407.2-751.5)	590.5 (447.3-794.8)	576 (455-708.5)	0.508
No. of CD4 counts/participant (median, IQR)	9(3.5-15.5)	7.5(3.0-13.8)	8(3.0-14.0)	0.101
Treatment	ART-naïve	ART-naïve	ART-naïve	-
% with VL (n=263)*	10.6	40	49.4	-
Average log copies/ml (median, IQR)	3.1(1.7-4.0)	3.1(2.0 – 4.0)	3.0(1.9-4.2)	0.9797

* Standard of care in Kenya does not include HIV VL; these were analyzed on a random subset of patients (total n=263).

6.5.2 Effect of IRF1 polymorphisms on CD4 decline

In order to assess the influence of IRF1 polymorphisms on HIV disease progression, we analyzed the association between IRF1 619 polymorphism and the rate of CD4+

T cells decline using linear mixed model analyses (Table 6.2). As expected, CD4 decline (and thus disease progression over time) was indeed observed in the study population during longitudinal follow-up ($p<0.001$); however, no association was observed between the rate of CD4 decline and specific IRF1 genotypes. We found that the protective IRF1 genotype 619 AA ($p=0.854$) and the neutral genotype AC ($p=0.391$) did not have a significant difference in CD4 decline compared to those with the non-protective CC genotype (Table 6.2). A similar lack of association was obtained for the other two IRF1 polymorphisms [IRF1 6516 GG ($p=0.955$), GT ($p=0.436$) and IRF1 MS 179+179+ ($p=0.676$), 179+179- ($p=0.472$) compared to their respective non-protective genotypes]. This observation remained true even if the individuals with CD4 count<350 were included or analyzed separately. These results indicate that identified IRF1 polymorphisms do not influence HIV disease progression rate as defined by longitudinal CD4 decline in ART naïve HIV-infected patients.

Table 6.2. Linear mixed models analyses to determine effect IRF1 619 genotypes have on the rate of CD4+T cell decline in Kenyan FSW cohort with baseline CD4count>350.
[219]

Baseline CD4 count	*Parameter*	*Estimate (daily)*	*P value*	*95% Confidence Interval*	
				Lower	Upper
CD4>350	Follow-up (days)	-.000341	.000	-.000410	-.000273
	IRF1 619=AA*follow-up	-1.573174E-5	.854	-.000184	.000153
	IRF1 619=AC*follow-up	-4.424301E-5	.391	-.000146	5.728030E-5
	IRF1 619=CC*follow-up	0[a]	.	.	.

* IRF1 619 genotypes: AA (protective against HIV acquisition); AC, CC (non-protective against HIV acquisition, CC genotype was used as the reference for comparison)

6.5.3 Effect of IRF1 polymorphisms on HIV VL

Next the association between IRF1 polymorphisms and HIV-1 VL was determined. HIV-1 VL is a prognostic marker of HIV-1 disease progression[220], and could potentially associate with differences in IRF1 regulation. In order to account for multiple viral load measures (15 participants had two VL measures and 5 had 3 VL measures at different time points) linear mixed models analysis was performed. As expected, VL significantly correlated with the natural log of CD4 counts (p=0.008, Table 6.3). However, no association was observed between protective and non-protective IRF1 genotypes and HIV VL (p=0.468 for AA and p=0.512 for AC compared to CC genotype, Table 6.3). These data suggest that IRF1 polymorphisms associated with reduced susceptibility to HIV infection have no apparent effect on driving systemic HIV replication *in vivo* in already infected individuals.

Table 6.3. Differences in VL between 619 IRF1 genotypes, and the influence of logCD4 count in linear mixed model analysis.
[219]

Variable	*Estimate*	*Sig.*	*95% Confidence Interval*	
			Lower bound	*Upper bound*
IRF1 619=AA	.181972	.468	-.312139	.676083
IRF1 619=AC	.107070	.512	-.214074	.428214
IRF1 619=CC	0[a]	.	.	.
Natural log CD4 count	-.298256	.008	-.519209	-.077304

* IRF1 619 genotypes: AA (protective against HIV acquisition); AC, CC (non-protective against HIV acquisition, CC genotype was used as the reference comparison)

6.6 Discussion:

HIV-1 susceptibility and disease progression are influenced by a number of distinct host genetic factors such as IRF1, HLA-B and HLA-C loci and CCR5 [221]. Previous data from our group suggest that protective IRF1 polymorphisms play a crucial role in preventing the acquisition of HIV infection. Because PBMCs from individuals with protective IRF1 polymorphisms have decreased IRF1 protein levels, resulting in reduced susceptibility to HIV infection[46,100,102], we hypothesized that these same polymorphisms could associate with differences in HIV disease progression. Our data shows that although specific IRF1 polymorphisms associate with decreased susceptibility to HIV infection they show no effect on disease progression, either measured by HIV-1 RNA levels or the slopes of CD4 decline before treatment initiation. Therefore, in HIV infected subjects, the protective IRF1 polymorphisms do not seem to have any prognostic significance on HIV-1 disease progression.

HIV-1 has evolved various mechanisms that evade and modify various aspects of the innate and adaptive immune response enabling the long-term persistence and survival of the virus. As with many other host factors, HIV commandeers IRF1 activity, using it to modify the immune response and perpetuate viral spread. Recently, it has been shown that HIV is able to regulate IRF1 protein levels and function by controlling IRF2 and IRF8 (known IRF1 antagonists) leading to the induction of specific interferon stimulated genes without detectable induction of antiviral Type I or II IFN responses in monocyte-derived dendritic cells [155,222]. Immune activation could be a potential culprit of seroconversion in resistant women by increasing IRF1 levels and thereby overriding the protective effects of the

IRF1 polymorphisms. The differential expression of IRF1 in activated versus non-activated target cells may play a role in establishment of a productive HIV infection at different tissue sites. It seems likely that at low activation levels in the mucosal tissues the effect of reduced IRF1 expression due to genetic polymorphisms may be sufficient in preventing initial viral replication and establishment of infection. At this stage, most HIV exposures do not lead to productive infection, as evidenced by a severe genetic bottleneck and small foci on HIV-infected cells[70,223]. However, the HESN phenotype is relative and some HIV infections still occur. Once HIV infection is established and spreads into an activated systemic lymphatic system HIV-1 may be able to override the protective mechanisms present at the time of exposure, including the IRF1 polymorphisms studied here. In fact there is evidence that HIV infection contributes to IRF1 stimulation and T cell activation thus creating an environment that favors viral replication and spread[113]. Extracellular Tat was shown to increase IRF1 cellular expression preparing the uninfected cells for viral infection[164]. Additionally once inside the cell Tat can directly bind IRF1 impairing its function and therefore generation of host antiviral immune responses[137,164].

In summary, the previously identified IRF1 polymorphisms shown to protect against HIV infection are not associated with HIV disease progression as defined by CD4 decline and HIV VL. The protective effect of these polymorphisms may not be sufficient in controlling HIV replication once initial infection is established. HIV-1 subverts the IRF1 response enabling viral replication and evasion of the host immune response. Results presented here suggest that IRF1 polymorphisms

associated with HIV-resistant phenotype play a role in preventing primary infection and have no protective effect once infection is established.

6.7 Limitations and Opportunities:

A potential limitation of this study is that the viral load data was only available on a subset of patients on a single time point. Furthermore, a majority of the patients were seropositive on enrollment. Since IRF1 plays a crucial role in early stages of HIV infection, it would be interesting to see if IRF1 polymorphisms have any effect on HIV replication during acute infection and establishment of viral set point. IRF1 interaction with other viral and host factors needs to be examined since HIV is known to manipulate IRF1 response through Tat and IRF1 antagonists (IRF2 and IRF8). Further studies are required to determine tissue specific cellular IRF1 expression during an ongoing HIV infection, and determine if IRF1 regulation by other mechanisms may play a role in HIV disease progression.

7. The effect of IRF1 polymorphisms and HESN phenotype on plasma hormone levels

7.1 Rationale

IRF1 polymorphisms do not account for all cases of resistance to HIV-1 infection in the Majengo cohort, indicating that additional mechanisms of immune and IRF1 regulation could be at play. There is an increasing amount of evidence suggesting that hormones play a crucial role in development and maintenance of host immune responses including interferon responses[136,224], inflammation[225], NK cell activity[226,227] and T cell development[228]. The sex steroids, estrogen and progesterone, have been associated with altered susceptibility to HIV-1 acquisition in a number of human[229,230] and primate studies[59]. Hormonal contraceptives including oral contraceptive pills and long-acting synthetic progesterone formulations have been implicated to associate with increased risk of HIV-1 acquisition in a number of human clinical trials[230-232]. Additionally, there is an increasing amount of evidence showing that pregnancy, a state of high estrogen and progesterone levels, increases the risk of HIV-1 acquisition[229,233]. The peptide hormone, prolactin is a known regulator of IRF1 expression and differences in prolactin expression could contribute to the altered IRF1 regulation observed in the Majengo HESN cohort. Furthermore, the steroid hormone cortisol has been shown to directly inhibit NK cell mediated cytotoxicity[227,234,235], a response previously associated with the HIV-resistant phenotype. Here we investigated the differences

in the systemic prolactin, cortisol, estrogen and progesterone levels with respect to IRF1 polymorphisms and HESN status.

7.2 Hypothesis

The HIV resistant phenotype overall, and IRF1 protective haplotype specifically will be associated with decrease in systemic hormone expression (prolactin, cortisol, estrogen and progesterone).

7.3 Objective

To quantitate systemic hormone levels with respect to IRF1 polymorphisms and the HESN status.

7.4 Section specific methods

Detailed description of methods used to quantify plasma hormone expression can be found in Section 2. 18.

7.4.1 Study cohort

Samples used for this study were obtained from the Majengo Sex Worker Cohort in Nairobi, Kenya [190]. Overall, samples from 157 individuals were utilized in this study. Participants were sequenced for IRF1 polymorphisms as done previously[101] and described in Section 2.7. Based on their IRF1 genotypes, patients were separated into two groups: those who had protective haplotype associated with reduced susceptibility to HIV-1 infection (619AA, 179+179+, 6516GG); and non-protective haplotype (619CC, 179-179-, 6516TT). As shown previously[46] and demonstrated in Section 4.5.1, all three polymorphisms are in strong linkage disequilibrium (Table 4.1, Page 87). Study participants were also

separated into two groups: HESN individuals who were HIV-1 negative at enrollment and remained negative for over 7 years while continuing active sex work; and HIV susceptible (HIV-S), individuals who are HIV uninfected at enrollment but do not fulfill the time requirement to be classified as HESN. Based on epidemiological evidence ~80% of HIV-S women seroconvert to HIV positive within 3 years of follow-up[74,190]. Epidemiological data was collected from each woman including: socio-demographic, sexual behavior, duration of sex work, number of sex clients, condom use, number of regular partners and reproductive history. All cohort participants underwent a gynecological exam where vaginal specimens were obtained and tested for the presence of bacterial vaginosis (Nugent score), Nisseria gonorrhoeae and Chlamydia (PCR), syphilis (RPR) and Trichomoniasis. All individuals that tested positive for any of the tested infections were excluded from the analysis. Information on menopause, last menstrual period and contraceptive use was obtained by a self-report method as part of the collection of the epidemiological data.

7.4.2 Statistical analysis

Univariate analysis was performed using GraphPad Prism (version 5, GraphPad software, La Jolla, CA) and p-values < 0.05 were considered significant. Multivariate analysis was performed using SAS version 9.3 program (SAS Institute, Cary NC). From the sample of 80 unique cases included in the final analysis, only 12 cases had more than one value. Given the small number of cases with longitudinal data available, methods such as generalized estimating equations (GEE) were thought not be appropriate given the relatively large proportion of cases (>80%) with

unique values. However, to address the non-independent structure of the data clustered standard errors were used in multiple linear regression models to correct for clustering by individual cases. IRF1 haplotypes and HESN/HIV-S status were used as an independent variable in linear regression models, with the four log-transformed hormones (estradiol, prolactin, progesterone and cortisol) used as the dependent variable. Due to the large degree of skewness in the distribution of the dependent variables, all dependent variables were log-transformed using their natural logarithms.

For IRF1 haplotype and hormone combination, 4 models were built, starting from a crude model investigating only the association between the independent variable and the dependent variable. Step 1 of the model-building process added age (as a continuous variable), while Step 2 added whether or not women reported the use of hormonal contraceptives. Finally, in Step 3, the menstrual phase (folicular vs. luteal) was added to the regression models. Thus, for each IRF1 haplotype and hormone combination, four models, progressing from simplest to more complex, were fitted to the data. In models where the independent variable was shown to be significantly associated with the dependent variable, the Aikake's Information Criterion (AIC) was used to choose the best model, with lower values indicating better fit. The same analysis was applied to the HESN/HIV-S status and hormone combination analysis.

7.5 Results

7.5.1 Cohort characteristics

Originally, the hormone expression data was collected on 157 individual women. Because some women were assayed more then once, a total of 195 data points were collected on 157 women. All HIV-1 infected participants (n=38) were excluded from the analysis as HIV-1 infection is known to cause multiple metabolic and endocrine abnormalities[237]. As menopause status has a profound effect on hormone expression, 30 post-menopausal women were excluded from the analysis. Finally, 32 observations with missing menstrual cycle information were excluded, leaving 93 observations on 80 individual women. Further reductions in sample size were observed due to the availability of non-missing values under different hormonal level and IRF1 haplotype/HESN status combinations. Additionally, due to sample limitations, prolactin levels were only available from 61 women included in the final analysis. Two women were breastfeeding at the time of the study and were further excluded from the prolactin analysis.

Based on the type of contraceptives, study participants were separated into two groups: hormonal methods (Oral pill and Depo-Provera, n=23) and non-hormonal methods (condom, copper intrauterine contraceptive device, tubal ligation and no contraceptives, n=68). Based on their last menstrual period date, samples were seperated into folicular (0 to 14 days, n=45) and leuteal phase (14 days to menses, n=46). Tables 7.1 to 7.4 show the levels of estradiol, prolactin, progesterone and cortisol by selected demographics, IRF1 haplotype and HESN/HIV-S status.

Table 7.1. Descriptive statistics for prolactin (pg/ml).

	No.	Mean	SD	Median	IQR
TOTAL	59	29,371.1	24,901.2	21,329.4	15,927.5 – 31,010.7
IRF1 haplotype					
Protective	21	20,656.68	12,433.66	20,442.25	11,375.57 - 24,808.33
Non-protective	29	29,986.26	23,359.23	21,288.80	16,077.77 – 31,534.59
Age group					
<35	20	26,553.35	19,853.66	21,335.99	16,455.88 – 31,010.72
35-39	11	25,015.78	27,181.93	16,077.77	11,826.06 – 19,528.34
40-44	15	36,734.40	24,348.96	26,768.61	20,511.57 – 54,097.71
45-49	11	31,431.95	32,608.04	23,273.32	20,364.24 – 26,755.06
50+	2	13,912.81	571.06	13,912.81	13,509.01 – 14,316.61
Status, NN and HESN only					
HESN	27	23,665.82	23,773.32	18,273.36	13,912.81 – 23,762.74
NN	32	34,363.21	25,154.71	26,156.54	19,257.68 – 39,110.86
Contraceptive methods					
None/Condom/Other	46	28,315.69	21,930.83	21,979.53	15,613.08 – 33,670.22
Hormonal methods	13	33,186.77	34,421.63	18,944.50	16,285.44 – 27,682.19
Menstrual Stage					
Folicular	29	31,301.79	28,676.62	20,647.01	16,077.77 – 30,486.86
Luteal	30	27,564.96	21,094.27	22,024.97	15,777.21 – 31,534.59

Table 7.2. Descriptive statistics for cortisol (ng/ml).

	No.	**Mean**	**SD**	**Median**	**IQR**
TOTAL	91	42.2	21.2	32.8	27.9 – 53.4
IRF1 haplotype					
Protective	23	44.64	19.54	40.11	28.33 - 57.70
Non-protective	43	43.39	24.58	32.43	26.84 - 54.57
Age group					
<35	28	47.11	20.61	41.21	29.52 - 58.76
35-39	19	37.99	19.03	30.39	26.86 - 41.10
40-44	26	38.65	23.12	31.23	27.42 - 40.11
45-49	16	44.44	22.02	35.54	29.17 - 57.47
50+	2	40.37	19.29	40.37	26.73 - 54.02
Status, HIV-S and HESN only					
HESN	50	37.75	17.49	30.50	26.83 - 41.10
HIV-S	41	47.73	24.17	40.11	29.82 - 57.70
Contraceptive methods					
None/Condom/Other	68	43.33	22.41	34.83	28.40 - 54.02
Hormonal methods	23	38.80	17.10	30.48	26.27 - 51.64
Menstrual Stage					
Folicular	45	41.94	19.35	35.45	28.33 - 51.64
Luteal	46	42.44	23.05	31.10	27.79 - 55.13

Table 7.3. Descriptive statistics for estradiol (ng/ml).

	No.	Mean	SD	Median	IQR
TOTAL	89	0.2	0.2	0.1	0.1 - 0.2
IRF1 haplotype					
Protective	23	0.15	0.08	0.14	0.09 - 0.23
Non-protective	41	0.19	0.33	0.15	0.06 - 0.21
Age group					
<35	27	0.14	0.10	0.14	0.04 - 0.23
35-39	18	0.10	0.08	0.09	0.03 - 0.15
40-44	26	0.22	0.41	0.13	0.06 - 0.21
45-49	16	0.15	0.13	0.12	0.09 - 0.18
50+	2	0.19	0.04	0.19	0.16 - 0.21
Status, HIV-S and HESN only					
HESN	49	0.12	0.11	0.11	0.03 - 0.18
HIV-S	40	0.21	0.33	0.15	0.09 – 0.21
Contraceptive methods					
None/Condom/Other	68	0.18	0.26	0.15	0.09 – 0.23
Hormonal methods	21	0.07	0.06	0.06	0.01 – 0.12
Menstrual Stage					
Folicular	44	0.14	0.09	0.14	0.08 – 0.17
Luteal	45	0.17	0.32	0.11	0.04 – 0.19

Table 7.4. Descriptive statistics for progesterone (ng/ml).

	No.	Mean	SD	Median	IQR
TOTAL	91	1.6	2.3	1.0	0.5 – 1.8
IRF1 haplotype					
Protective	23	1.64	1.51	1.25	0.58 - 1.72
Non-protective	43	1.89	3.11	1.21	0.44 - 1.96
Age group					
<35	28	1.64	1.50	1.29	0.59 - 1.85
35-39	19	1.34	1.40	0.83	0.44 - 1.82
40-44	26	2.08	3.85	1.06	0.49 - 1.92
45-49	16	1.26	1.04	0.81	0.53 - 1.67
50+	2	0.70	0.16	0.70	0.58 - 0.81
Status, HIV-S and HESN only					
HESN	50	1.06	1.11	0.66	0.41 - 1.34
HIV-S	41	2.30	3.16	1.51	0.75 – 3.03
Contraceptive methods					
None/Condom/Other	68	1.84	2.60	1.31	0.59 – 1.92
Hormonal methods	23	0.92	0.94	0.60	0.34 – 1.23
Menstrual Stage					
Folicular	45	1.31	1.15	1.00	0.53 – 1.60
Luteal	46	1.90	3.05	0.93	0.51 – 1.97

7.5.2 Differences in plasma hormone expression in samples from individuals with protective and nonprotective IRF1 haplotypes

Since prolactin was previously described as an important regulator of IRF1 expression and function, this first study investigated the effect of IRF1 polymorphisms associated with HIV-resistant phenotype on plasma prolactin expression (Figure 7.1 and Table 7.5). Univariate analysis showed no statistically significant differences in estradiol, progesterone, cortisol and prolactin levels between paslma samples from individuals with protective and nonprotective IRF1 haplotypes. No statistically significant association between IRF1 haplotype and logged values of prolactin was detected in bivariate analyses (Crude p=0.092, Table 7.5) and in Model 1 correcting for age only (Model 1 p=0.056). However, prolactin levels were previously shown to be significantly affected by hormonal contraceptive use[238] and menstrual stage[239]. After adjusting for age and use of hormonal contraception (Model 2 p=0.043); and age, hormonal contraception and menstrual cycle (Model 3 p=0.048), the relationship between IRF1 haplotype and prolactin reached statistical significance. In the most stringent model, adjusting for age, hormonal contraceptives and menstrual stage, women with protective IRF1 haplotype had logged prolactin values 0.473 pg/ml lower than their counterparts with non-protective IRF1 haplotype (p=0.048). Prolactin has been previously described as a regulator of IRF1 expression, however data presented here indicate that a bidirectional relationship between IRF1 and prolactin may exist. IRF1 haplotype were not associated with changes in plasma estrogen, progesterone and cortisol levels (Figure 7.1 and Supplemental data, Tables 10.2-10.4).

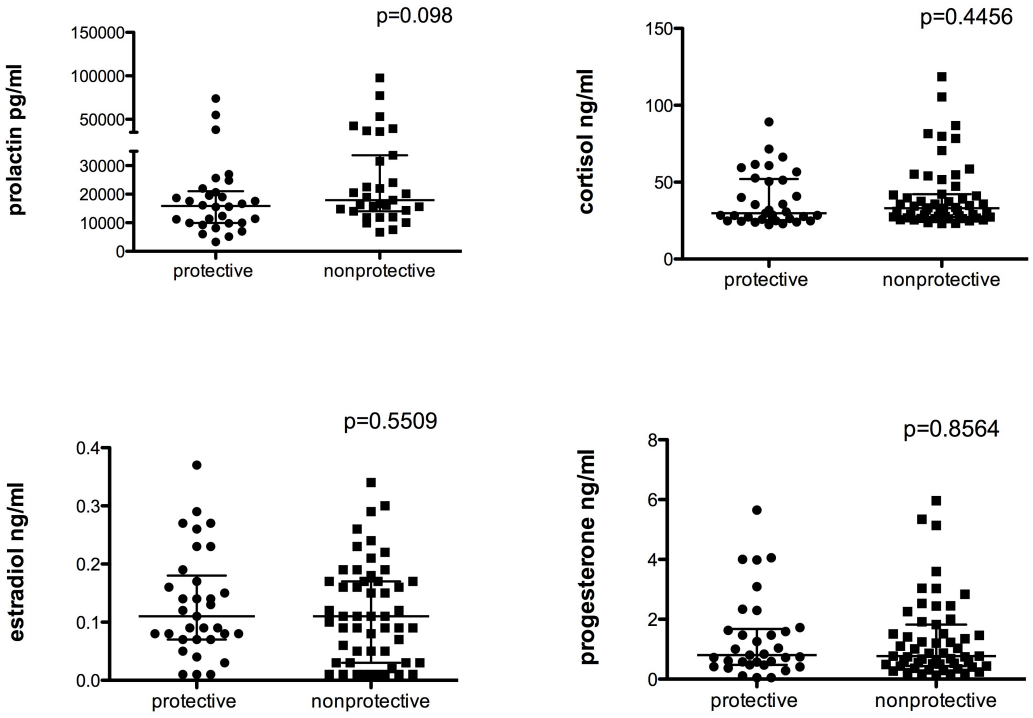


Figure 7.1. Quantification of plasma hormone levels between individuals with differing IRF1 haplotypes.

Prolactin (pg/ml), estradiol (ng/ml), progesterone (ng/ml) and cortisol (ng/ml) levels in plasma samples from individuals with protective and nonprotective IRF1 haplotypes. The Mann-Whitney test was used to examine significance ($p<0.05$), bars represent the median expression and interquartile range.

Table 7.5. Crude and adjusted coefficients of the impact of IRF1 haplotypes on log_prolactin (pg/ml)

	Crude	Model 1	Model 2	Model 3
IRF1 haplotype				
Protective	-0.361	-0.449	-0.475*	-0.473*
	(0.092)	(0.056)	(0.043)	(0.048)
Non-protective	*Ref*	.	.	.
Age		-0.0190	-0.0206	-0.0204
		(0.076)	(0.060)	(0.067)
Use of hormonal contraception				
No			*Ref*	.
Yes			-0.142	-0.138
			(0.450)	(0.477)
Menstrual cycle				
Folicular				*Ref*
Luteal				-0.0442
				(0.824)
AIC[a]	99.80	98.87	100.43	102.47

[a]Aikake's Information Criterion
p-values in parentheses
Number of observations: 50
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

7.5.3 Differences in plasma hormone expression in samples from HESN and HIV-S individuals

Plasma hormone levels were quantified in order to examine their role in HIV-resistant phenotype. All hormones in HESN plasma differed significantly from that of HIV-S controls by univariate analysis (Figure 7.2) as well as in all four statistical models (Table 7.6-7.9). Significantly lower prolactin (p=0.002), cortisol (p=0.002), estradiol (p<0.0001) and progesterone (p<0.0001) levels were observed in plasma samples from HESN individuals by univariate analysis (Figure 7.2). In multivariate analysis, Model 3 correcting for age, hormonal contraceptive use and menstrual stage was biologically most relevant as all hormones including cortisol[240,241] were significantly affected by these factors. Logged estradiol values in HESN women

were 0.984 ng/ml lower compared to HIV-S women (p<.001, Table 7.6). Logged plasma cortisol levels in HESN women were 0.207 ng/ml lower, compared to HIV-S women (p=0.042, Table 7.7). Logged progesterone levels were 0.783 ng/ml lower among HESN women, compared to HIV-S women (p=0.004, Table 7.8). Finally, logged plasma prolactin levels among HESN women were 0.527 ng/ml lower on average than HIV-S women (p=0.025, Table 7.9).

Overall, HESN women have significantly lower plasma levels of sex hormones (estradiol and progesterone), cortisol and prolactin. Prolactin has been previously described as a regulator of IRF1 expression and differences in prolactin expression could contribute to IRF1 regulation in Majengo HESN cohort. Tested hormones are all strong immune-regulators and the observed differences in their expression likely play a major role in HIV-resistant phenotype.

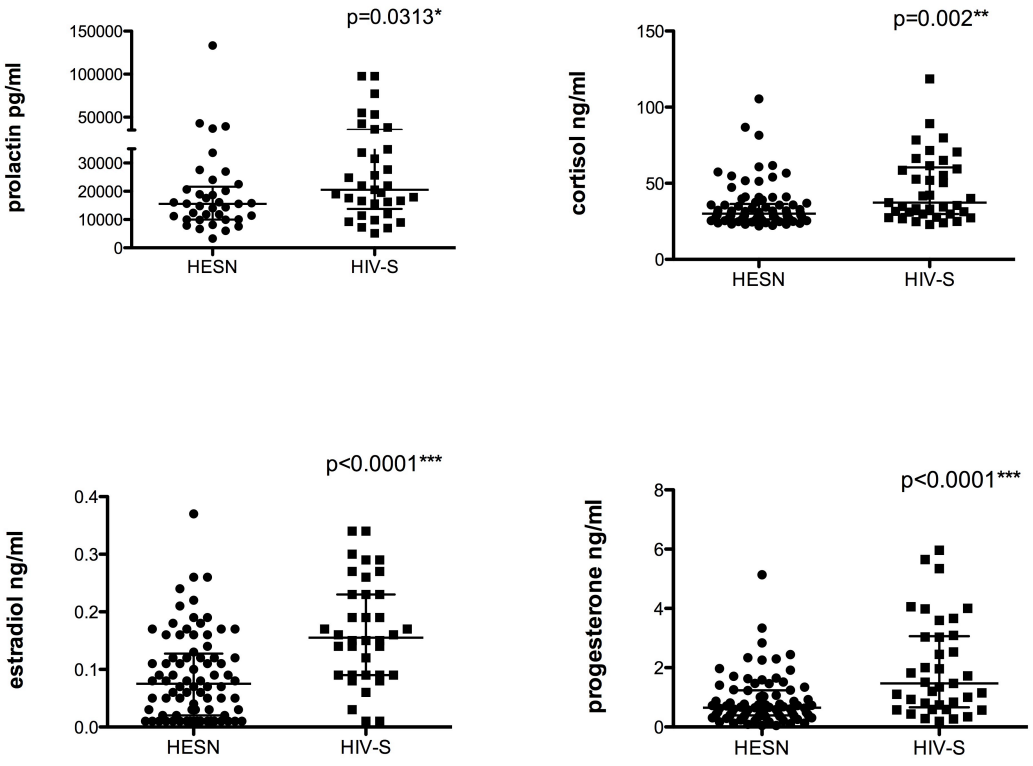


Figure 7.2. Quantification of plasma hormone levels in HESN and HIV-S individuals

Estradiol (ng/ml), cortisol (ng/ml), progesterone (ng/ml) and prolactin (pg/ml) levels in plasma samples from HESN and HIV-S individuals. The Mann-Whitney test was used to examine significance ($p<0.05$), bars represent the median expression and interquartile range.

Table 7.6. Crude and adjusted coefficients of the impact of HESN/HIV-S status on log_estradiol (ng/ml)

	Crude	Model 1	Model 2	Model 3
Status				
HESN	-0.931***	-1.133***	-0.988***	-0.984***
	(0.000)	(0.000)	(0.000)	(0.000)
HIV-S	*Ref*	.	.	.
Age		0.0358*	0.0236	0.0241
		(0.028)	(0.083)	(0.075)
Use of hormonal contraception				
No			*Ref*	.
Yes			-0.880***	-0.865***
			(0.000)	(0.000)
Menstrual cycle				
Folicular				*Ref*
Luteal				-0.0979
				(0.611)
AIC[a]	252.62	249.5	237.44	239.17

[a]Aikake's Information Criterion
p-values in parentheses
Number of groups: 89
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 7.7. Crude and adjusted coefficients of the impact of HESN/HIV-S status on log_cortisol (ng/ml)

	Crude	Model 1	Model 2	Model 3
Status				
HESN	-0.251**	-0.222*	-0.207*	-0.207*
	(0.005)	(0.024)	(0.040)	(0.042)
HIV-S	*Ref*	.	.	.
Age		-0.00479	-0.00620	-0.00621
		(0.458)	(0.341)	(0.340)
Use of hormonal contraception				
No			*Ref*	.
Yes			-0.0848	-0.0850
			(0.296)	(0.307)
Menstrual cycle				
Folicular				*Ref*
Luteal				0.00152
				(0.986)
AIC[a]	91.21	92.62	93.86	95.86

[a]Aikake's Information Criterion
p-values in parentheses
Number of groups: 91
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 7.8. Crude and adjusted coefficients of the impact of HESN/HIV-S status on log_progesterone (ng/ml)

	Crude	Model 1	Model 2	Model 3
Status				
HESN	-0.784***	-0.862**	-0.779**	-0.783**
	(0.000)	(0.001)	(0.004)	(0.004)
HIV-S	*Ref*	.	.	.
Age		0.0130	0.00513	0.00462
		(0.479)	(0.782)	(0.810)
Use of hormonal contraception				
No			*Ref*	.
Yes			-0.477	-0.499
			(0.059)	(0.060)
Menstrual cycle				
Folicular				*Ref*
Luteal				0.150
				(0.477)
AIC[a]	247.32	248.53	246.11	247.48

[a]Aikake's Information Criterion
p-values in parentheses
Number of groups: 91
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 7.9. Crude and adjusted coefficients of the impact of HESN/HIV-S status on log_prolactin (ng/ml)

	Crude	Model 1	Model 2	Model 3
Status				
HESN	-0.414*	-0.518*	-0.527*	-0.527*
	(0.032)	(0.023)	(0.022)	(0.025)
HIV-S	*Ref*	.	.	.
Age		0.0129	0.0143	0.0143
		(0.285)	(0.298)	(0.296)
Use of hormonal contraception				
No			*Ref*	.
Yes			0.136	0.137
			(0.581)	(0.585)
Menstrual cycle				
Folicular				*Ref*
Luteal				-0.00542
				(0.978)
AIC[a]	121.98	122.97	124.54	126.54

[a]Aikake's Information Criterion
p-values in parentheses
Number of groups: 59
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

7.6 Discussion

Hormones have been shown to play a crucial role in development and regulation of both innate and adaptive immune responses[242-245] and contribute to increased prevalence of sexually transmitted infections in women[246]. Hormonal contraceptive use and pregnancy, a state of high estrogen and progesterone levels, have been associated with increased risk of HIV-1 acquisition[59,229-233]. This study examined the estrogen, progesterone, cortisol and prolactin plasma hormone levels with respect to IRF1 polymorphisms and the HESN/HIV-S phenotypes. Significantly lower prolactin levels were observed in plasma samples from women with protective IRF1 haplotype when corrected for age, use of hormonal contraceptives and menstrual cycle. Interestingly, levels of all four hormones were significantly lower in plasma samples from HESN women compared to HIV-S in all statistical models.

The peptide hormone prolactin, is best known as the pituitary modulator of lactation and reproduction. Hyperprolactinaemia is a common immunological phenomena in a wide spectrum of microbial diseases and has been associated with secondary infections in HIV-1 infected patients [247,248]. Significantly lower prolactin levels were detected in plasma samples from women with protective IRF1 haplotype when corrected for age, hormonal contraceptive use and/or menstrual cycle. HESN women were also shown to have significantly lower plasma prolactin levels independent of age, hormonal contraceptive use and menstrual stage. By regulating the JAK/Stat signaling pathway prolactin has been shown to both activate

as well as inhibit IRF1 promoter activity[249]. Prolactin inducible Stat5 is involved in negative signaling to the IRF1 promoter[250,251], while prolactin inducible Stat1 leads to stimulation of IRF1 transcription[136]. Furthermore, 16-kDa prolactin, a NH_2-terminal natural breakdown fragment of intact 23-kDa prolactin, was shown to inhibit IL-1β-induced IRF-1 transcription and signaling through downregulation of Stat1 signaling pathway [252]. While the exact mechanism of IRF1/prolactin regulation remains to be determined, differences in prolactin expression depending on the IRF1 genotypes indicate that IRF1 may control prolactin signaling and/or prolactin receptor by negative feedback mechanism. Lower prolactin levels and a complex prolactin/IRF1 relationship could contribute to the robust but transient response to IFNγ stimulation and HIV-1 infection observed in HESN and women with protective IRF1 haplotype respectively.

HESN women from the Majengo cohort in Kenya, express significantly lower levels of steroid hormones (cortisol, progesterone and estrogen) compared to susceptible controls independent of age, hormonal contraceptive use and menstrual stage. All steroid hormones are produced through a series of enzymatic reactions that modify cholesterol molecules. Female sex hormones, estrogen and progesterone have been shown to significantly influence both susceptibility and immune responses to sexually transmitted infections[246]. There is an increasing amount of evidence showing that pregnancy, a state of high estrogen and progesterone levels, increases the risk of HIV acquisition[60,229]. Pregnant women were shown to have a significantly increased risk of HIV-1 acquisition in HIV-

discordant couples in a clinical trial in Kisumu, Kenya[233]. Increased levels of estrogen and progesterone during pregnancy likely contribute to increased HIV-1 acquisition by inducing immunological changes in the FGT. Furthermore, use of combined oral contraceptives and depot-medroxyprogesterone, in HSV-2 seronegative individuals resulted in increased risk of HIV acquisition compared to the non-hormonal group in general population of Uganda, Zimbabwe and Thailand [232]. In a 10-year Kenyan cohort study, hormonal contraception was associated with increased risk for HIV-1 acquisition in high risk sex worker population independent of the HSV-2 status[253,254]. The exact mechanism of how sex steroids alter HIV acquisition remains enigmatic. Progesterone was shown to be associated with an increase in CCR5 expression in PBMCs and FGT, suggesting that high progesterone states might predispose women to HIV-1 acquisition[255]. The role of estrogen in HIV infection is controversial. Estrogen therapy in the macaque model was shown to protect against vaginal transmission of SIV[256]. However, transient luciferase expression studies showed that estrogen could activate HIV-LTR transcription in HEK293 cells, independent of NFκB[257]. Furthermore, estrogen was shown to inhibit trans-epithelial resistance (TER), thereby reducing epithelial barrier integrity and potentially allowing pathogens such as HIV to cross the physical barrier and gain access to immune target cells[258]. A Decrease in systemic estrogen and progesterone levels in HESN women and the associated decrease in CCR5 expression, a reduction of estrogen mediated HIV-1 transcription and increased epithelial integrity may restrict initial HIV-1 replication and contribute to decreased susceptibility to HIV-1 infection.

The effects of hormones on the host immune system are well documented and could further contribute to the altered susceptibility to HIV-1 infection. Progesterone, estrogen and cortisol were all shown to suppress NK cell function [227,259,260]. The glucocorticoid cortisol, as the end products of the hypothalamic-pituitary –adrenal (HPA) axis affects a number of physiological functions and elevated levels of cortisol have been associated with number of disorders ranging from depression to cancer [261,262]. Cortisol was shown to suppress the immune system and directly inhibit NK cell- mediated cytotoxicity[227,234,235,263]. As discussed previously in Section 1 and Section 5, NK cell responses are thought to play a pivotal role in natural protection against HIV-1 infection. Decrease in cortisol, estrogen and progesterone levels in HESN women may be one of the contributing factors in increased NK cell activity previously described in Majengo cohort[90]. Furthermore, progesterone inhibits TLR9 induced IFNα production by human and mouse plasmocytoid dendritic cells[224]. IFNα induces an antiviral state, prevents productive HIV-1 infection and exerts selective pressure on the transmitted virus pool [53,66,264,265]. Thus, lower progesterone levels observed in HESN women potentially associated with increased interferon responses could play an important role in restricting early HIV-1 replication and preventing acquisition. Prolactin was shown to inhibit the suppressive function of Treg cells in humans[266], and lower prolactin levels in HESN women may contribute to elevated frequency of CD4+CD25+FOXP3+ Treg cells and IQ phenotype observed in Majengo HESN cohort[81,192]. Progesterone and estrogen have been reported to have both pro- and anti-inflammatory function[225,267]. Considering the impact of hormones on

susceptibility to HIV-1 infection, studies looking at the effects of hormones on immune activation, particularly at the FGT are urgently needed. Taken together, lower prolactin, estrogen, progesterone and cortisol levels observed in this study and the associated increase in antiviral interferon responses and perhaps NK cell function may be one of the main contributors to the HIV-resistant phenotype observed in Kenyan Majnego cohort.

To the best of our knowledge this is the first report showing decrease in systemic cortisol, estrogen and progesterone levels in HESN women. These results indicate that hormonal regulation could be one of the key players in HIV-resistant phenotype in the Kenyan Majengo cohort. Manipulating the microenvironment of the FGT with hormones could contribute to the development of improved immunization strategies against HIV-1.

7.7 Limitations and opportunities

With the increase in heterosexual transmission of HIV-1 in women it is important to fully understand the role of hormones in susceptibility and immune responses to sexually transmitted infections. NK cell activity and interferon responses, including IRF1 expression need to be examined in samples with high and low hormone expression in order to establish a direct link between the two. The mechanism of IRF1/prolactin regulation needs to be further examined to address what determines prolactin mediated positive vs negative regulation of the IRF1 expression. One possible explanation could be that IRF1 controls prolactin signaling and/or prolactin receptor by negative feedback mechanism. While estrogen induction of

HIV-1 LTR transcription was shown to be NFκB independent, this process could potentially be mediated by estrogen-induced changes in IRF1 expression. This study examined hormone plasma levels; hormonal regulation should be further examined at the FGT, the site of initial HIV-1 exposure in women.

Our study was conducted in high-risk sex workers and our results may be most applicable to women at high-risk of HIV- 1 infection. Given the widespread use of hormonal contraceptives in areas of high HIV-1 incidence, our findings are concerning. Women that use hormonal contraception, especially in the areas of high HIV-1 transmission rates should be encouraged to use condoms, currently the only method proven to prevent HIV-1 transmission. Further understanding of how hormones influence innate and adaptive immune responses may provide new tools for controlling HIV-1 infection.

8. FINAL DISCUSSION

8.1 Major findings of the thesis

This thesis investigated potential mechanisms of natural protection against HIV-1 infection in Majengo FSW cohort, focusing on IRF1 polymorphisms that are prevalent in individuals with the HESN phenotype. The following main observations were made:

- Intronic IRF1 polymorphisms (619, MS GT repeat, 6516) do not affect the regulation of IRF1 transcription but may act as intronic splicing regulators. IRF1 transcripts from the *ex vivo* PBMCs of individuals with protective IRF1 haplotype have increased inclusion of IRF1 exons 7/8. Furthermore, decreased IRF1 protein stability was associated with the protective haplotype, when compared to samples with nonprotective haplotype. Lower basal IRF1 expression together with the ability for faster IRF1 protein degradation could significantly limit IRF1-mediated HIV-1 transcription and replication, while at the same time allowing for the initiation of IRF1-mediated host immune response.
- Significantly higher plasma levels of IL15, IFNγ and IL6 expression, and significantly higher mucosal IL15, IFNγ, IL2 and sIL2Rα expression, (with univariate analysis only) was observed in individuals with the protective IRF1 haplotype when compared to those with nonprotective IRF1 haplotype. The observed increase in antiviral cytokine expression may drive the

development of protective innate and adaptive immune responses as well as directly limit viral transcription at the exposure site.

- Women with the protective IRF1 haplotype expressed significantly lower levels of plasma prolactin, suggesting a potential bidirectional relationship between IRF1 and prolactin regulation.
- IRF1 polymorphisms, were not associated with HIV disease progression as defined by the rate of CD4 decline and HIV VL, suggesting that the protective effect of IRF1 polymorphisms may be limited to the early stages of HIV pathogenesis, prior to establishment of HIV-1 infection.
- Independent of IRF1 genotypes, HESN women as a whole had significantly lower plasma prolactin, estrogen, progesterone and cortisol levels. These results indicate that hormonal regulation may be one of the key players in natural resistance to HIV-1 infection in the Majengo HESN cohort.

Together, these findings significantly increase our understandings of the possible mechanisms underlying the natural resistance to HIV-1 infection and yield potential to impact the design of future prophylactic and therapeutic strategies.

8.2 Model of protection against HIV infection

Data presented in this thesis, along with the previously described correlates of protection in HESN individuals can be utilized to frame a model of reduced susceptibility to HIV-1 infection (Figure 9.1).

While no single genetic factor or specific protective correlate is likely to account completely for the HESN phenotype, the resulting immunological phenotype is likely to be similar. The data presented here and data from the studies of HESN cohorts around the world indicate that innate immune responses (particularly NK cells and IFN responses) and T-cell immune quiescence likely play a major role in natural resistance to HIV-1 infection[80,192,268]. The proposed model of protection, supported by the results presented in this thesis emphasizes the importance of limiting early HIV-1 replication as a critical mechanism that drives the development of resistance to HIV-1 infection. Reduction in HIV-1 replication at the early stage widens the window of opportunity for the host innate immune responses to develop and halt the establishment of systemic HIV-1 infection.

Our results suggest that HIV-1 replication is restricted at multiple levels by a number of factors. Regulation of IRF1 expression and IRF1-mediated responses have emerged as one of the key regulators of the HESN phenotype in the Majengo FSW cohort. IRF1, estrogen and progesterone have all been shown to increase HIV replication either by direct activation of HIV-1 LTR transcription (IRF1[156] and estrogen[257]) or by increasing expression of the HIV co-receptor CCR5 (progesterone[255]). Therefore, the lower levels of IRF1, estrogen and progesterone expression found in HESN individuals could significantly limit initial HIV replication and prevent HIV spread. Additionally, increased estrogen levels lead to decrease in TER and reduced physical barrier integrity, potentially allowing HIV to gain access to the immune target cells at the FGT[258]. Decreased estrogen levels in HESN women can contribute to stronger epithelial barrier thereby preventing initial HIV

transmission. Furthermore, IL6 and IFNγ, shown to be significantly upregulated in samples from individuals with protective IRF1 haplotype, are known to directly inhibit HIV-1 replication particularly in macrophages and monocytes, which are the main early targets of HIV infection [55,203]. In addition to having anti-viral activity, the observed changes in IRF1 regulation, plasma and CVL cytokine, and reduced hormone levels associated with the HESN phenotype may act individually or together to drive the establishment of a prolonged anti-viral response. This includes activation of innate immune responses, and NK cell function in particular, and an increase in T cell immune quiescence through increased levels of immunosuppressive Tregs. Decreased T cell activation would significantly limit available target cells for HIV replication allowing NK cells and the rest of the innate immune system an opportunity to clear the virus, the infected cells and prevent the establishment of productive HIV-1 infection. Overall, the accumulating data suggests that the HESN phenotype can be defined as a finely controlled balance of basal immune activation with a focused and potent antiviral innate immune response.

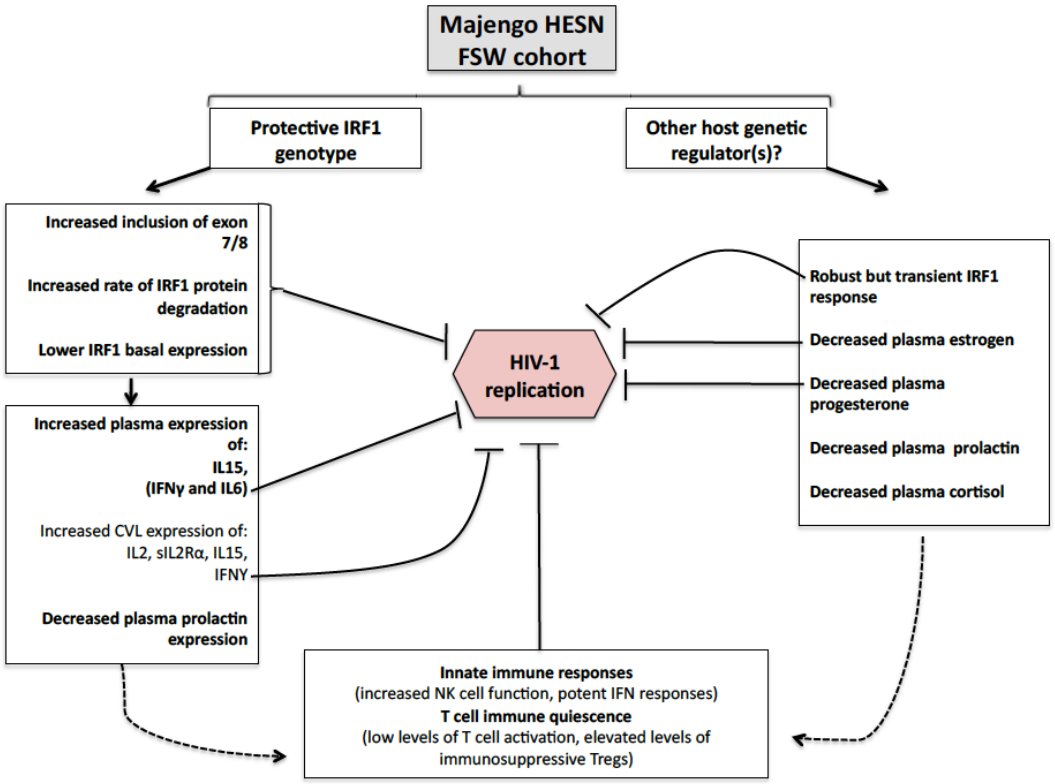


Figure 8.1. Model of protection against HIV-1 acquisition in Majengo FSW HESN cohort.

HIV replication in Majengo HESN cohort is restricted by multiple mechanisms: 1) decrease in expression of known activators of HIV-1 LTR transcription, IRF1 and estrogen 2) increased integrity of the physical barrier through estrogen modulation of TER 3) decrease in progesterone mediated CCR5 expression 4) increase in the expression of anti-retroviral cytokines, IL6 and IFNγ and 5) activation of innate immune responses (increased NK cell function and IFN responses) and increase in T cell immune quiescence mediated by increase in immunosuppressive Tregs.

8.3 Modulating IRF1 as a component of prevention and treatment strategies

HIV's dependence on IRF1 for initial replication, latency and evasion of the host immune responses could ideally be exploited to the host's advantage in the development of both preventative and therapeutic strategies. Virus control requires a balance between IRF1 induced antiviral response and reduced viral replication. Preliminary studies (Su et al, submitted) show that a reduction of IRF-1 expression via siRNA by modest amounts in $CD4^+$ T cells *ex vivo* (~25-60%) resulted in reduced LTR-driven transcriptions when these cells were infected by HIV-1_{VSV-G}, HIV-1_{IIIB} or HIV-1_{BAL}. These data suggest that HIV replication is impaired when IRF1 expression is reduced. However, IRF1 knockdown in the same CD4+ T cells resulted in a normal ability to up-regulate IRF1 regulated immune genes following IFNγ or HIV-1 stimulation. Thus, reducing endogenous IRF1 expression could significantly limit HIV replication without impacting the generation of the antiviral immune responses. Targeting IRF1, as a part of HIV prevention strategies may be a promising approach to limit HIV replication during the early stages of infection, allowing host immunity to contain infection. Furthermore, IRF1, as an important modulator of the host immune responses, represents a good candidate for use as a vaccine adjuvant. *In vivo* administration of plasmid DNA encoding wild type IRF1 or IRF1 lacking the DNA-binding domain, enhanced the efficacy of Tat-DNA vaccination with predominant Th1 responses with increased IFNγ production and CTL responses[269]. Interestingly, the adjuvant effect of the mutated IRF1 (lacking the DNA binding domain) and the wild type form showed similar efficacy. This study

suggests that the mutated IRF1 would retain the ability to enhance protective immune response but not be able to stimulate HIV-1 LTR thereby limiting HIV replication.

Increasing IRF1 expression in HIV-infected patients may also represent a strategy to activate and eliminate the latent HIV reservoir, a key objective of developing a viable cure strategy for HIV[270]. Targeting latent HIV infection requires efficiently activating latent viral cellular reservoirs and inducing local host innate immunity to supplement ART in tissues/cells where bioavailability of drugs is problematic, and to drive appropriate antiviral immune responses without triggering immune activation. Methods that induce IRF1 expression could potentially accomplish this, given IRF1's ability to concurrently drive HIV transcription and induce antiviral immune responses.

Further studies are needed to provide a better understanding of the complex IRF1 regulation in order to optimize antiviral effects and minimize viral replication early in the infection process. Data presented in Section 7 indicate that peptide hormone prolactin may be an important regulator of IRF1 activity. Comprehensive understanding of the complex IRF1/prolactin relationship may potentially allow for prolactin-mediated manipulation of IRF1 responses.

8.4 Modulating innate immune responses as a component of prevention and treatment strategies

Natural protection against HIV infection is associated with enhanced NK cell activity and potent IFN responses[88,89,271]. Given the small founder population that

establishes HIV infection, the first few days following HIV exposure are a period of great vulnerability for the virus and potentially represent a window of opportunity for NK cell and IFN–mediated intervention[61].

The main challenge associated with IFN-mediated therapy is the need to minimize the immunopathological HIV-induced IFN effects while optimizing the beneficial antiviral IFN effects. Several studies have assessed both the use of IFN-inhibitors to reduce IFN-mediated immunopathology and the induction of IFN responses to strengthen antiviral immunity. Due to IFNα's role in HIV immunopathogenesis, IFNα inhibitors have been tested in several clinical trials[272,273]. In order to dampen elevated chronic IFNα levels, chloroquine, an endosomal inhibitor used in the treatment of Malaria and autoimmune disorders for its ability to reduce chronic immune activation[274], was used to treat chronic HIV infected patients. In an *in vitro* model, chloroquine mediated the blockade of IFNα which lead to reduced T and pDC cell activation and the blockade of indoleamine 2,3-dioxygenase (IDO) and programmed death ligand 1 (PDL-1), both negative modulators of T cell function[275]. Chloroqine treatment of participants on ART with CD4+ T cell counts <200 cells/μl was shown to reduce immune activation, as measured by several immunological parameters including decreased circulating LPS levels, decreased T cell activation, reduced production of inflammatory cytokines, reduced IFNα-producing pDC, and increases in Treg numbers[276]. However, recent randomized controlled trial looking at the effect of chloroquine treatment in ART-naïve patients with CD4+ T cell count >400 cells/μl found that the use of chloroquine did not reduce T cell activation and actually led to greater decline in

CD4+ T cell count and increased viral replication[277]. Furthermore, IFNγ treatment in individuals co-infected with HIV and tuberculosis with median CD4 count>350 cells/ml was shown to be safe and efficacious, including increased CD4+ T cell counts and decreased plasma viral load[278]. This study showed that at earlier stages of infection, in ART naïve patients with high CD4 counts, IFN treatment results in restricted HIV replication and a slower rate of CD4 decline, presumably due to more effective IFN-mediated antiviral responses. On the other hand, later in the infection, after CD4 counts have already declined, the benefits of inhibiting immune activation might outweigh the suppression of the IFN-mediated antiviral responses. This could be in part due to the fact that the majority of anti-viral effector cells (pDCs, NK and T cells) are destroyed in later stages of infection and with them the ability to mount any IFN-mediated HIV control.

Results from a small study by Azzoni et al. provide the first clinical evidence showing that natural host defense mechanisms can successfully reduce circulating HIV reservoirs and control HIV replication without continued antiretroviral therapy[265]. The study analyzed the effect of pIFN-α2a treatment in 23 HAART-treated patients with CD4 count > 450 cells/μl and HIV plasma RNA levels of <50 copies/ml. Study participants received pIFN-α2a therapy in addition to HAART for 5 weeks; following that antiretroviral therapy was discontinued and pIFN-α2a therapy was maintained for 12-24 weeks. Results show that 45% of treated patients were able to maintain viral control and showed significantly lower ratio of integrated HIV DNA copies/CD4+ T cell between end point and baseline. Taking into consideration the protective role of IFNs in early stages of HIV replication and the

potential to reduce circulating HIV reservoirs, IFN-therapy could be an important part of the pre- and post-exposure prophylaxis.

It is important to note that adverse side effects associated with IFN therapy and mode of administration could provide further challenges for utilization of IFNs in HIV treatment and prevention. However, advances in drug-delivery research may allow the administration of IFNs via slow-releasing pills or other delivery systems. Furthermore, as discussed in Section 7 progesterone and cortisol have been shown to inhibit IFN and NK cell responses, respectively. Modulation of cortisol and progesterone levels may provide new ways to enhance NK cell activation and IFN antiviral response at the FGT. Induction of NK cell and IFN responses prior to and during the early stages of HIV infection may restrict viral replication enough to allow for HIV clearance.

Manipulation of hormone expression and signaling could also potentially be used to increase the integrity of the physical barrier at the FGT and thereby prevent early HIV infection and spread. Wira *et al.* have shown that treatment of ECC-1 cells with selective estrogen receptor modulators (SERMs), like tamoxifen (clinically relevant for the treatment of breast cancer) and raloxifene (used to prevent and treat osteoporosis) significantly enhanced TER[258]. These results suggest that the use of these clinically approved SERMs as part of the cervical ring may lead to increased epithelial barrier integrity and protection from invading pathogens such as HIV.

8.5 Future directions

To expand this study and conclusively prove our findings following experiments should be performed:

- Examine posttranslational modifications of IRF1 protein (phosphorylation, ubiquitination and SUMOylation) in samples from women with different IRF1 haplotypes. These studies will further describe the mechanism of IRF1 intronic polymorphisms-mediated regulation of IRF1 expression and function.
- Generate IRF1 clones lacking exons 7 and 8 and examine their impact on HIV-1 LTR transcription and IRF1 downstream gene regulation. The resulting IRF1 protein isoforms lacking exon 7/8 are likely to interfere with the wild-type IRF1 induction of antiviral ISGs but could potentially still drive the NFκB/IRF1- mediated HIV-1 transcription.
- Cell type specific IRF1 expression and downstream gene regulation should be examined in samples from women with different IRF1 haplotypes. Observed differences IRF1 splice variants and protein regulation could significantly impact the cell specific IRF1-mediated immune responses.
- Results in Section 3 indicate that IRF1 could be one of the main drivers of tissue-specific early HIV-1 transcription. Therefore depending on feasibility, IRF1 protein levels and regulation should be further examined in different sections of the FGT from women with protective and nonprotective IRF1 haplotypes.
- Examine NK cell function/numbers and interferon responses in samples with different IRF1 haplotypes and samples with high and low plasma hormone expression in order to establish a direct link between IRF1/hormone regulation and protective immune responses.

- Examine the FGT hormone expression levels in HESN and HIV susceptible women. Sex hormones regulate the mucosal immune system within the female reproductive tract and are likely to contribute to differences in HIV-1 replication and rates of acquisition at the exposure site.
- Characterize the mechanism of IRF1-prolactin regulation. Comprehensive understanding of this complex relationship could potentially allow for prolactin-mediated manipulation of IRF1 and IFN responses.
- Explore the potential ways to manipulate IRF1 expression in host cells for use in the development of HIV prophylactic strategies including microbicide design.

8.6 Final conclusion

Thirty years after the discovery of HIV as the causative agent of AIDS, more than 2.3 million new infections occurred in 2012, making the search for an effective vaccine on the forefront of global public health priorities. While antiretroviral therapy is excellent at controlling viral replication in HIV infected patients, a functional cure cannot be achieved due to the establishment of viral reservoirs, highlighting the need for alternative treatment options. IRF1 has been shown to be an important factor in natural protection against HIV in highly exposed seronegative (HESN) individuals and crucial in regulating the initial stages of HIV replication, HIV disease progression as well as establishment of latency. An understanding of how the protective effects of IRF1 responses are controlled in HESN individuals may provide important clues on how to regain control of HIV and tip the balance of immunity in

the favor of the host, or provide new opportunities to eliminate HIV in its host altogether.

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10. Appendices

10.1. Abbreviations:

AIDS: acquired immunodeficiency syndrome

APC: antigen presenting cells

APOBEC: apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like

ART: antiretroviral therapy

ARV: antiretroviral drugs

CCR5: CC chemokine receptor 5

CSW: commercial sex worker

CVL: cervicovaginal lavage

DC: dendritic cell

DMSO: dymethyl sulphoxide

DNA: deoxyribonucleic acid

ELISA: enzyme-linked immunosorbent assay

FBS: fetal bovine serum

FGT: female genital tract

FSW: female sex worker

GALT: gut associated lymphoid tissue

GEE: generalized estimating equations

GI: gastrointestinal

HAART: highly active antiretroviral therapy

HDAC: hystone deacetylase

HESN: HIV-exposed seronegative

HIV: human immunodeficiency virus

HLA: human leukocyte antigen

HRP: horseradish peroxidase

IAD: IRF-association domain

IFN: interferon

IL: interleukin

iNOS: inducible nitric oxide synthase

IP-10: interferon gamma induced protein 10

IRF: interferon regulatory factor

ISG: interferon stimulated gene

ISRE: interferon stimulated response element

IQR: interquartile range

KIR: killer immunoglobulin-like receptors

LC: Langerhans cells

LD: linkage disequilibrium

LMP2: low molecular mass polypeptide 2

LN: lymph node

LOD: limit of detection

LPS: lypopolysaccharide

LTR: long terminal repeat

MCP-1: monocyte chemoattractant protein 1

mDC: myeloid dendritic cell

MHC: major histocompatibility complex

MIG: monokine induced by gamma interferon

MIP-3β: microphage inflammatory protein 3 beta

MS: microsatellite

NK: natural killer cell

NKT: natural killer T cell

PBMC: peripheral blood mononuclear cell

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

pDC: plasmacytoid dendritic cell

PRR: pattern recognition receptor

RM: rhesus macaque

RNA: ribonuclear acid

RT: reverse transcriptase

RT-PCR: real time PCR

sCD40L: soluble CD40 ligand

sIL2R: soluble interleukin-2 receptor

SAMHD1: SAM domain and HD domain containing protein 1

s.d.d: sterile double distilled

SERMs: selective estrogen receptor modulators

SIV: simian immunodeficiency virus

SM: sooty mengabey

STI: sexually transmitted infection

TER: transepithelial resistance

TFA: triflouroacetic acid

$T_{H:}$ T helper

TLR: Toll- like receptor

TNF: tumor necrosis factor

Treg: regulatory T cell

TRIAL: tumor-necrosis

TRIM5α: tripartite motif-containing motif 5 alpha

VL: viral load

VSV: vesicular stomatitis virus

10.2. Supplemental data

Table 10.1. Left-censored regression model of association between CVL cytokine/chemokine expression and IRF1 haplotype adjusting for age.
The nonprotective IRF1 haplotype was used as the reference category.

Cytokine/chemokine	Parameter	Estimate	p-value	95% CI	
logIL2	protective	0.5002	0.4342	0.047	5.778
	non-protective	0.0000	.	.	.
	Age	0.0472	0.1382	0.948	1.116
logsIL2Rα	protective	0.4149	0.4054	0.569	4.023
	non-protective	0.0000	.		
	Age	0.0431	0.0883	0.994	1.097
logIL15	protective	0.6042	0.3192	0.557	6.008
	non-protective	0.0000	.	.	.
	Age	0.0210	0.4769	0.964	1.082
logIFNγ	protective	0.4332	0.3291	0.646	3.681
	Non-protective	0.0000	.	.	.
	Age	0.0077	0.7170	0.967	1.051

Table 10.2. Crude and adjusted coefficients of the impact of IRF1 haplotype on log_estradiol(ng/ml)

	Crude	Model 1	Model 2	Model 3
protective	0.223	0.273	0.0719	0.0716
	(0.323)	(0.245)	(0.754)	(0.760)
Non-protective	0	0	0	0
	(.)	(.)	(.)	(.)
re_age		0.00885	-0.00233	-0.00236
		(0.608)	(0.874)	(0.879)
contracep1			-1.001**	-1.001**
			(0.002)	(0.002)
menstrual2				0.00364
				(0.988)

p-values in parentheses
Number of observations: 64
$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 10.3. Crude and adjusted coefficients of the impact of IRF1 haplotype on log_progesterone (ng/ml)

	Crude	Model 1	Model 2	Model 3
protective	0.0643	0.0302	-0.160	-0.174
	(0.829)	(0.925)	(0.638)	(0.610)
Non-protective	0	0	0	0
	(.)	(.)	(.)	(.)
re_age		-0.00659	-0.0176	-0.0186
		(0.713)	(0.313)	(0.302)
contracep1			-0.775*	-0.807*
			(0.028)	(0.025)
menstrual2				0.220
				(0.441)

p-values in parentheses
Number of observations: 66
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 10.4. Crude and adjusted coefficients of the impact of IRF1 haplotype on log_cortisol (ng/ml)

	Crude	Model 1	Model 2	Model 3
protective	0.0780	0.0176	-0.00499	-0.00508
	(0.428)	(0.861)	(0.965)	(0.965)
Non-protective	0	0	0	0
	(.)	(.)	(.)	(.)
re_age		-0.0117	-0.0130	-0.0130
		(0.097)	(0.068)	(0.068)
contracep1			-0.0918	-0.0920
			(0.448)	(0.464)
menstrual2				0.00156
				(0.989)

p-values in parentheses
Number of observations: 66
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$