GENETIC CORRELATES OF HIV RESISTANCE

by

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Abstract

The Human Immunodeficiency Virus 1 (HIV-1) epidemic continues to claim millions of lives, despite intense research and public health programs. A natural model of resistance is crucial for the development of an effective vaccine. We have identified a group of sex workers in Nairobi, Kenya, who appear to be resistant to infection with HIV.

Research on this cohort has identified numerous immunological and genetic correlates to HIV resistance, but has failed to completely explain the phenomenon. Genetic studies have shown that HIV resistance occurs in families, with both sex worker and non-sex worker relatives of HIV resistant women less likely to be HIV infected. In addition, HIV resistance has been associated with altered innate immune responses, as measured by cytokine production to toll-like receptor stimuli. To test the hypothesis that there is a genetic component to HIV resistance, we will address two specific objectives within this thesis: 1) identify known polymorphisms associated with HIV resistance in the kindred of these women; more specifically interferon regulatory factor 1 (IRF-1) polymorphisms, and 2) identify polymorphisms within toll-like receptors (TLRs) that may be responsible for the altered and apparently successful immune responses in HIV resistant women.

Our findings show an association between HIV resistant kindred and an IRF-1 microsatellite, as well as, with an IRF-1 single nucleotide polymorphism. No associations were found between HIV resistance and the investigated TLR2 and TLR4 polymorphisms. These results also suggest a genetic component to HIV resistance, but do not fully explain the altered immune responses observed within these women.

Dedication

I would like to dedicate this thesis to my parents, Ron and Linda, who supported me every step of the way. Thanks for teaching me to dream.

And to Graham, who never let me quit, and was always there to hug me. Thanks for always believing in me. I love you.

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Table of Contents

List of Figures

List of Tables:

Overview

The Human Immunodeficiency Virus (HIV) epidemic has claimed more than 25 million lives since its identification in 1981. Despite intense research and public health programs, efforts to impede this growing epidemic have not halted transmission. According to UNAIDS, there were more than 4.1 million (3.4-6.2 million) new infections in 2005, with a total of 38.6 million (33.4-46 million) people infected worldwide (UNAIDS, 2006). The majority of these infections occur within developing nations, where access to antiretroviral drugs is limited. Among the most endemic areas is sub-Saharan Africa, which is home to an astounding two thirds of the world's infected population (UNAIDS/WHO, 2005). Growing international pressure and global awareness resulted in the development of an HIV prevention package at the 2005 G8 summit, with the goal of achieving universal access to treatment by 2010 (UNAIDS/WHO, 2005). Irrespective of advances in treatment, the majority of people infected with HIV will develop Acquired Immunodeficiency Syndrome (AIDS) and die within 8-10 years (Hoffmann-Rockstroh-Kamps, 2005). This is all the more realistic for infected populations, such as those in sub-Saharan Africa, who are without access to antiretroviral drugs (ARVs). It is clear that while treatment does have an effect, it is not a cure and a prophylactic vaccine is desperately needed to control this raging epidemic.

A natural model of resistance would be invaluable to the development of a vaccine capable of inducing a protective immune response against HIV. Cases of individuals highly exposed to HIV, who remain uninfected, (Beyrer et al., 1999; Fowke et al., 1996)

and HIV infected long-term non-progressors (LTNPs) (Clerici et al., 1996; Shearer and Clerici, 1996) provide hope of such a model. These documented cases show signs of a cellular immune response to HIV and may provide the information needed for an effective vaccine (Fowke et al., 2000; Haynes et al., 1996; Shearer and Clerici, 1996). The question remains: are these presumably successful immune responses the product of host or viral characteristics? In this thesis, the host genetics of highly exposed uninfected Kenyan sex workers and their families will be explored in an attempt to elucidate the mechanisms of natural resistance to HIV infection.

HIV Discovery and AIDS

In 1981, the US Centers for Disease Control (CDC) reported 5 cases of *Pneumocystis jerovici* (formerly *Pneumocystis carnii* pneumonia (PCP)) in a group of homosexual men in Los Angeles (CDC, 1981). Over the next year and a half, more clusters of otherwise healthy homosexual men presented with PCP and other opportunistic diseases that associate with immunodeficiency. Originally, this illness was thought to be sexually transmitted and restricted to gay men, but was subsequently noted in hemophiliacs and heterosexual IV drug users. This pattern of infection was suggestive of a blood-borne pathogen and thus termed AIDS or Acquired Immune Deficiency Syndrome by the CDC in 1982.

In 1983, scientists at the Pasteur Institute in France isolated the virus that was responsible for AIDS. They called it lymphadenopathy-associated virus (LAV) and thought it

belonged to the family of human T-cell leukemia viruses (HTLV) (Barre-Sinoussi et al., 1983). The isolation of the virus was confirmed by US scientists who renamed the virus human lymphotropic virus type 2 (HTLVII) before the two names were resolved in favor of Human Immunodeficiency Virus (HIV-1) (Coffin et al., 1986). In 1986, HIV-2, an alternate form of HIV was discovered. This related virus causes reduced virulence and is restricted primarily to West Africa (Clavel et al., 1986; Reeves and Doms, 2002).

HIV transmission has been determined to occur through sexual intercourse, intravenous drug use, rare occupational transmission and maternofetal transmission at birth or through breastfeeding (Hoffmann-Rockstroh-Kamps, 2005). The majority of transmission occurs through heterosexual intercourse, the most risky being receptive anal sex (Hoffmann-Rockstroh-Kamps, 2005). Many co-factors can increase this risk, such as other sexually transmitted infections (STIs) (Piot, 1988), trauma to the genital tract (Cameron et al., 1989) and lack of circumcision (Gray et al., 2000; Moses et al., 1998). Additional host factors can also affect transmission such as viral load (Quinn et al., 2000) and douching practices (Gresenguet et al., 1997).

HIV Structure and Replication Cycle

HIV belongs to the *lentivirus* or "slow virus" genus of the *Retroviridae* family (Kingsman and Kingsman, 1996). Retroviruses share three structural proteins; *Gag* (group specific antigen), *Pol* (polymerase) and *Env* (envelope). HIV contains two copies of 9kb positive single-stranded RNA surrounded by a cone shaped p24 protein (*Gag*)

capsid. Also contained within this p24 core are the *Pol* products, reverse transcriptase (RT), integrase (IN) and proteases. The conical core is also surrounded by a p17 matrix (*Gag*) and a plasma membrane, which is formed from the host cell membrane during budding. Embedded in the membrane is glycoprotein gp41 that is non-covalently linked to the exposed gp120. Both of these important glycoproteins are derived from the *Env* encoded gp160. The exposed gp120 and gp41 are responsible for binding to the CD4 receptor and chemokine co-receptor on CD4+ T cells, respectively, initiating viral entry into host cells. In addition to the three structural proteins, HIV also encodes for the accessory proteins, *Tat*, *Rev*, *Vpu*, *Vpr*, *Vif* and *Nef*. Both the 5' and 3' ends of the RNA are flanked by two long terminal repeat (LTR) regions that are important in gene regulation and act in the insertion into the host genome (Hope and Trono, 2000; Kingsman and Kingsman, 1996).

HIV replicates in CD4+ cells, which are mostly lymphocytes but also include monocytes and dendritic cells (Knight et al., 1990; Seligmann et al., 1987). HIV utilizes CD4 as a receptor to gain entry to the cells through an interaction with gp120 and the V1 region of CD4 (Arthos et al., 1989). Although gp120-CD4 binding is necessary for viral entry, it is not sufficient and an additional co-receptor is needed. The main co-receptors are 7 transmembrane G-protein coupled chemokine receptors, found on lymphocytes (CXCR4) and macrophages (CCR5) and are preferentially used by T cell tropic and macrophage tropic HIV strains respectively (Weiss and Clapham, 1996).

During natural infection, the interaction between HIV and CD4+ cells is facilitated by dendritic cells (DCs) that can capture HIV and transport it to lymphoid organs. This is possible through DC expression of dendritic cell-specific intracellular adhesion molecule 3 (ICAM-3)-grabbing integrin (DC-SIGN) and other C-type lectins capable of binding gp120. Once immature DCs are antigen bound, they migrate to draining lymph nodes to initiate a primary immune response. This migration catalyzes the interaction of HIV and CD4+ T cells, thus allowing for systemic distribution of the virus. Infection of DCs also impairs their ability to effectively stimulate T cell responses to other antigens. Direct infection of DCs is also a factor in T cell transmission and may act as a reservoir for HIV (Geijtenbeek et al., 2000; Lekkerkerker et al., 2006).

This high affinity gp120-CD4 binding causes a conformational change in gp120 and exposes the V3 loop, which can subsequently bind to the co-receptor molecules. This additional binding results in a conformational change in gp41 to its "fusion" state, allowing fusion of viral and host membranes and the subsequent entry and uncoating of the viral particle (Chan and Kim, 1998). After viral entry and uncoating, reverse transcriptase (RT) transcribes the viral (+) RNA into its complementary (-) DNA strand (cDNA). A lack of proofreading capacity by the viral RT during this process results in mutations at a rate of approximately 1 nucleotide per 10^3 to 10^4 transcribed. This high error rate is exacerbated by the rapid replication of HIV, resulting in incredible sequence variation. The viral RNase H then digests the RNA component of the DNA-RNA hybrid and transcription into double stranded DNA (dsDNA) can occur (Katz and Skalka, 1990).

This dsDNA is then translocated into the nucleus where it is randomly integrated into transcriptionally active parts of the host genome by viral IN (Schroder et al., 2002). HIV gene transcription is a complex process controlled by both viral and host transcription factors. Most notably, the viral protein Tat, which is produced from short viral transcripts, binds to the viral LTR and facilitates the elongation of the mRNA transcript to full length (Zheng et al., 2005). The viral mRNA is then translated into viral proteins using host machinery. Subsequent to mRNA translation, viral protease cleaves gag and gag-pol precursor products into their "mature" forms that are transported to the plasma membrane. The various structural components assemble at the plasma membrane and the mature infectious HIV virion buds from the cell (Freed, 2001).

The ability of HIV to remain latent within reservoirs throughout the body has presented a major hurdle in HIV treatment and eradication. The most consistent and stable reservoir for HIV is resting memory CD4+ T cells (Koup, 2001). Replication does not occur within these cells due to an absence of host factors, but can be resumed upon activation. Activation occurs when these T cells encounter previously seen antigens and proliferate, becoming permissive to HIV replication. In addition to this reservoir, HIV also maintains more short-term reservoirs such as persistently infected macrophages and follicular dendritic cells in germinal centers of the spleen and lymph (Blankson et al., 1999). The sequestration of virus in these reservoirs prevents complete viral eradication and allows restoration of infection in the absence of treatment.

HIV Pathogenesis

There are three clinical stages of HIV infection; primary infection, clinical latency and progression to an AIDS defining illness. Primary infection is characterized by mononucleosis-like symptoms such as fever, headache and lymphadenopathy. This stage lasts for approximately six to eight weeks and is accompanied by high viremia and an initial drop in CD4+ T cells. The subsequent downregulation of viremia coincides with the detection of both humoral and cellular immune responses, although antibodies produced at this time are most likely non-neutralizing (Pantaleo and Fauci, 1996). Cellular CD8+ T cells (CTL) are thought to function to reduce the initial viremia through elimination of infected cells or suppression of viral replication. Resolution of symptoms and downregulation of viremia characterize the transition to the clinically latent stage. This decline in viremia following the resolution of the acute infection occurs until a viral set point is reached. This set point is a strong predictor of disease progression rate (Hoffmann-Rockstroh-Kamps, 2006). Although viremia is reduced, immune responses of the host fail to completely eliminate the virus enabling detection throughout all stages of infection. During the 8-10 years of this stage, the host remains relatively healthy though immune control of HIV replication. Over time, however, in addition to immune exhaustion, viral escape from neutralizing antibodies and CTL responses diminish the effect of host immune responses, leading to uncontrolled HIV replication and a gradual loss of CD4+ T cells (Levy, 2006). This massive destruction and disabling of CD4+ T cells eventually overwhelms the immune system's capacity to regenerate.

T cell loss is thought to occur through a number of mechanisms, most notably apoptosis. Apoptosis can be induced in HIV infected cells through a variety of mechanisms: 1) activation induced cell death (AICD) through the upregulation of FasL and thus Fas-mediated apoptosis by HIV proteins, 2) formation of cytopathogenic syncytia in CXCR4-tropic HIV strains and 3) cytotoxic T cell (CTL) induced apoptosis. Direct cell killing also takes place in HIV infected cells as a result of cell membrane disruption during budding and interference with cellular machinery during replication. HIV uninfected cells are also subject to apoptosis through Fas-mediated AICD and HIV protein induced apoptosis (Alimonti et al., 2003; NIAID, 2004). Eventually the level of CD4+ T cells drops so low (200cells/μl) that individuals easily acquire opportunistic infections and eventually succumb to these AIDS-related illnesses (Fauci et al., 1985).

In addition to the "typical" HIV progressors described above, studies have identified rapid progressors who progress to AIDS within 2-3 years following primary infection; long-term non-progressors, those who do not experience progressive disease for many years, and long term survivors; those who progress to AIDS, but remain relatively stable after progression (Pantaleo and Fauci, 1996).

Innate Immune Response to HIV

The host immune response to HIV involves both innate and adaptive immunity. Innate response mechanisms discriminate self from non-self through the recognition of pathogen associated molecular patterns (PAMPs) (Medzhitov et al., 1997). Innate immunity is a crucial response mechanism in humans, as it is a rapid general response to pathogens that

instructs the antigen-specific adaptive immune response. Many components of the innate immune system are present at mucosal sites and thus fundamental in the initial response to HIV. The innate immune system is composed of a variety of cell types, including dendritic cells (DCs), macrophages, natural killer cells (NK) and γδ T cells, as well as many soluble components such as cytokines, chemokines and lectin-binding proteins (MBLs) (Alfano and Poli, 2005; Levy, 2001). Critical to this innate immune response is the initial recognition of pathogens by pathogen recognition receptor (PRR) mechanisms. These PRRs include toll-like receptors (TLRs), NACHT-leucine-rich repeat proteins (NLR proteins), and the RNA helicases RIG-1, Mda5 and LGP2. The more recently described NLR proteins and RNA helicases are found in the cytoplasm and are capable of detecting bacterial components and viral RNA, respectively. The ten known TLRs are the most well studied PRRs, and are capable of detecting bacterial, fungal and viral components (Mitchell et al., 2006).

Subsequent to pattern recognition, a variety of signaling pathways are initiated in an attempt to induce immune responses that will eradicate a pathogen. Depending on which PRR is involved, various adaptor molecules and pathways are triggered. All pathways do, however, result in the nuclear translocation of either the transcription factor NFκB, or interferon regulatory factor-3 (IRF-3). These transcription factors can then initiate the transcriptional regulation of various cytokines involved in the adaptive immune response. PRRs are thus an important link between adaptive and innate immunity (Mitchell et al., 2006).

Plasmacytoid dendritic cells (pDC) are also critical in the relationship between innate and adaptive immunity. pDCs function not only as part of the innate immune system through type I interferon (IFN α and β) secretion, but may also be the primary drivers of the adaptive immune response by differentiating into antigen presenting DCs. pDCs amplify the innate immune system in the early stages of viral infection through IFN-mediated activation of natural killer (NK) cells. pDCs then differentiate into mature DCs that regulate the function of T cells and other antigen presenting cells (APCs) such as B cells, myeloid DCs and monocytes. In addition, IFN production by pDCs can also induce bystander maturation of DCs in response to HIV *in vitro.* These functions illustrate the vital role of pDCs in antiviral innate and adaptive immunity and also as a fundamental bridge between the two systems (Mitchell et al., 2006)

The importance of other innate immune system factors in HIV infection was recently confirmed with the discovery of host antimicrobial peptides. These secreted proteins of the innate immune system offer cells natural mechanisms to limit HIV replication. Proteins involved in the post-entry block of HIV include tripartite motif protein 5α (Trim5α) and the apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G (APOBEC-3G). APOBEC-3G is a cytosine deaminase that creates mutations within the HIV genome, while Trim5α is thought to block HIV capsid uncoating (Levy, 2006). In addition to these molecules, β-defensins secreted by epithelial cells have been shown to block HIV infection in a dose dependent manner and are thought to contribute to the lack of oral HIV transmission (Sun et al., 2005). Soluble HIV suppressive factors known as β chemokines are also secreted by activated CD8+ T cells (Cocchi et al., 1995). These

chemokines; RANTES, MIP-1α and MIP-1β, are natural ligands of the CCR5 chemokine receptor and are thus capable of blocking HIV through competitive binding (Garzino-Demo et al., 1998). The vast repertoire of the human innate immune system allows for a variety of immune responses against HIV. One of the most crucial functions of innate immunity is, however, the capacity to direct and initiate a successful adaptive immune response.

Adaptive Immune Response to HIV

The adaptive immune response consists of lymphocytes capable of proliferating upon antigen recognition. There are two major types of lymphocyte: B lymphocytes (B cells), which secrete antibodies, and T lymphocytes (T cells). T cells can be further divided into T helper cells (CD4 T cells), which activate B cells and direct CD8+ T cell responses, and cytotoxic T cells (CTLs or CD8+ T cells), which kill cells presenting non-self antigens. Antigens are presented by antigen presentation cells (APCs) via human leukocyte antigen (HLA), with HLA class II alleles presenting to CD4+ T cells and class I alleles presenting to CD8+ T cells.

The innate immune system is crucial in the initiation and direction of adaptive effector mechanisms. This direction is mediated through cytokine and chemokine secretion and the overall induction of an inflammatory response. Adaptive effector mechanisms can be broadly categorized into cellular (Th1) and humoral (Th2) responses. Differentiation of CD4+ T cells into Th1 or Th2 cells occurs during the initial response to antigen and is

influenced by the cellular environment created by the innate immune system. While the presence of IFNγ and IL-12 cytokines stimulate the development of Th1 cells, the presence of IL-4, IL-6 or IL-10 result in Th2 development. Th1 responses are characterized by cell-mediated immune responses, which involve the destruction of cells presenting non-self antigens by CTLs; while Th2 responses result in the production of certain antibody classes. The overall composition of the cellular environment during the initiation of an immune response influences whether Th1 or Th2 cells develop and can bias the immune response as cellular or humoral. This Th1/Th2 balance often determines whether the pathogen is eliminated or allowed to persist within the host (Janeway C.A., 2001).

HIV infected individuals mount both HIV-specific humoral and cellular immune responses, albeit with varying success. Neutralizing antibodies are effective against free virus particles, but have little effect against cell-associated virus, such as virus infected cells. CTL responses are effective against infected cells yet have no effect on free viral particles. Although it appears that the cooperation of these responses would yield a desirable result, neither antibodies nor CTLs are effective against latently infected cells. This prevents sterilizing immunity and allows for HIV reservoirs. In addition, HIV viral escape from neutralizing antibodies and CTL responses diminish the efficacy of the host immune response. It is evident that we must determine the components of a successful sterilizing immune response to HIV in order to develop a viable vaccine. Current knowledge has concluded that, at the very least, the vaccine must be capable of inducing

high titers of broad neutralizing antibodies, as well as virus-specific CD4+ and CD8+ T cells (Pantaleo and Koup, 2004).

Although an extensive immune response is mounted by the host, neither innate, cellular nor antibody responses act to stop disease progression. This lack of protection only reinforces the need for a natural model of resistance to HIV infection. Research on those individuals able to mount a successful immune response is vital to the design and development of an effective vaccine.

Immunology and HIV Resistance

Literature throughout the years has supported the hypothesis that there is variability in both susceptibility to HIV and HIV disease progression. Individuals have been identified who progress rapidly to AIDS, those who do not experience progressive disease for many years (long-term non-progressors) and those who appear to be resistant to infection (Pantaleo and Fauci, 1996). It is the latter group which offers a great deal of optimism, as a natural model of resistance would greatly influence the design of a prophylactic vaccine.

The existence of these apparently resistant individuals is supported by HIV specific immune responses in exposed subjects, even in the absence of detectable HIV by PCR and viral culture (Clerici and Shearer, 1996; Shearer and Clerici, 1996). Studies have detected HIV-specific CD8+ CTLs in newborns of HIV+ mothers (Cheynier et al., 1992;

Rowland-Jones et al., 1993), the HIV negative partner in discordant couples (Bernard et al., 1999; Langlade-Demoyen et al., 1994; Promadej et al., 2003), HIV negative health care professionals exposed to HIV via needlestick injuries (Pinto et al., 1995), and sex workers exposed to HIV via their occupational practices (Rowland-Jones et al., 1995; Rowland-Jones et al., 1998; Sriwanthana et al., 2001). These findings suggest that immunological memory to HIV developed after exposure to the virus and that a cell mediated immune response (Th1) may have a role in preventing infection (Clerici and Shearer, 1996).

A cohort of commercial sex workers (CSW) in Nairobi, Kenya remains one of the few studied adequately to describe absence of HIV infection, despite sufficient HIV exposure. The Pumwani cohort (ML cohort, named after Malaya, the Swahili word for prostitute) members are the subjects of a prospective study that has enrolled 2200 CSW since 1985 with 600 still in active follow up as of 2006. The enrolled women are resurveyed twice yearly when biological samples, epidemiological and behavioral information is collected. On average, these women have 5-6 sexual encounters per day, with the average minimum yearly unprotected sexual exposures being 24 in 1984 and 64 in 1994. Despite this intense exposure, approximately 5% of these women remain persistently seronegative and are classified as HIV resistant upon meeting the following epidemiologically defined definition: 1) HIV seronegative by serology and PCR, 2) still active in sex work and 3) followed for greater than 3 years (Fowke et al., 1996).

Since the identification of these HIV resistant women, much work has been done in an attempt to elucidate the cause of this "natural" immunity. HIV resistance in the Nairobi cohort is not due to altered cellular susceptibility to HIV, systemic β chemokine levels (Fowke et al., 1998) or differences in sexual behavior, condom use or other sexually transmitted infections (Fowke et al., 1996). Resistance has, however, been correlated with systemic HIV-1 specific T-helper responses (Fowke et al., 2000), systemic and mucosal CTL responses (Alimonti et al., 2006; Fowke et al., 2000; Kaul et al., 2000), HIV-1 specific mucosal IgA (Kaul et al., 1999), elevated RANTES levels in the genital mucosa (Iqbal et al., 2005) and hyporesponsive IL-4 production (Trivedi et al., 2001). Although the exact mechanism of resistance is not known, these correlations suggest that these resistant women may have a primarily cellular (Th1) biased immune response and are thus capable of preventing infection.

Genetics and Resistance to Infectious Disease

It is clear that not every individual responds in an identical fashion to infectious agents. Some resist infection entirely, and of those who become infected, symptoms can vary considerably. This observation, along with family studies, suggests that host genetics play an important role in the development and outcome of most infectious diseases.

The most convincing studies to examine the genetic contribution to infectious disease susceptibility have been twin and adoptee studies (Hill, 1998). A premature death study found that adoptees with a biological parent who died before the age of 50 from an

infectious disease had a 5.8 relative risk of dying from an infectious disease, while there was no increase in risk from the same situation with the adoptive parent (Sorensen et al., 1988). Twin studies have also found a higher concordance between monozygotic twins than dizygotic twins in the context of hepatitis B persistence (Lin et al., 1989), tuberculosis (Comstock, 1978) and *Helicobacter pylori* (Malaty et al., 1994). Interestingly, a similar study conducted on Gambian twins, revealed that dizygotic twins had a higher concordance in the presence of malarial parasites than monozygotic twins. Identical twins did, however, concur more frequently on the presence of fever, suggesting that the genetic component has more weight on the development of malarial disease as opposed to malarial infection (Jepson et al., 1995).

Human Leukocyte Antigen (HLA/MHC) is an important component of the immune system that allows presentation of viral epitopes to the host. This function has made HLA a staple in the study of host genetic diversity in the context of infectious diseases. Allelic variants of HLA influence the immune response by determining which epitopes are presented to T cells, thus the presence of certain HLA alleles can affect disease susceptibility. Certain HLA genes have been linked to altered susceptibility to malaria (Hill, 1998), tuberculosis (Singh et al., 1983), hepatitis viral persistence (Thursz et al., 1995), leprosy (Todd et al., 1990) and AIDS (Scorza Smeraldi et al., 1986). Genetic variants that effect gene expression and thus the immune response are likely an important mechanism of altered susceptibility to infectious diseases.

Knockout mice are important tools in the determination of potential genetic regulators of the immune response. This "mouse-to-human" strategy is often employed to determine potential gene candidates responsible for altered immune responses. Various cytokine and chemokine receptor mutations have been studied using these mouse models. Mutations in the IFNγ receptor gene have been associated with an increased risk of atypical mycobacterium infection (Newport et al., 1996) and a TNF-α promoter mutation has been linked with persistent hepatitis B virus infection (Hill, 1998), asthma (Moffatt and Cookson, 1997), meningitis mortality (Nadel et al., 1996), and scarring trachoma (Conway et al., 1997).

Murine models have also provided additional clues into the necessary immunological background for infection control. The development of phenotypically distinct T cells (Th1 vs. Th2) has been implicated in the management of *Leishmania major* infection. C57BL/6 phenotypic mice are able to mount a protective Th1 immune response, while BALB/c mice develop a Th2 immune response and fail to control the infection (Campos-Neto, 2005). This evidence clearly demonstrates the necessity of an effective immune response and the importance of genetics in determining such a response. As it is thought that HIV resistant individuals are more proficient at mounting a Th1 response, it is reasonable to hypothesize that genes controlling the formation of this immunological response would be important in susceptibility and resistance to HIV.

Genetics and HIV

Many genetic variants have been linked with the ability of HIV to infect cells or with subsequent disease progression. Due to its immune function, HLA has been an intensely studied genetic variable in HIV disease susceptibility. MHC class I genes HLA-B*27 and B*57 have been associated with a delay in HIV progression, while HLA-B*35 has been associated with accelerated progression (Carrington and O'Brien, 2003; Gao et al., 2005). The HLA A2/6802 supertype family (A*0202, A*0205, A*0214, and A*6802) and HLA-A*11, have been associated with decreased frequency of HIV-1 seroconversion among sex workers (MacDonald et al., 2000; Sriwanthana et al., 2001). MHC class II allele DRB1*13 has been reported to confer a protective effect against HIV (Chen et al., 1997), while the DRB1*13-DQB1*06 haplotype has been noted in individuals able to maintain viral suppression (Malhotra et al., 2001).

Polymorphisms in chemokine receptors can also influence HIV infection and disease progression. A 32 base pair deletion in CCR5 (delta 32) has been found in some highly exposed, uninfected individuals. Individuals homozygous for this allele appear to have slower disease progression as the mutant allele results in a truncated version of CCR5 that fails to be expressed on the cell surface. Curiously, this allele is common in the Caucasian population with a frequency of 0.0808, but has not been found in people of African or Asian ancestry (Liu et al., 1996; Smith et al., 1997). A variant in the minor HIV co-receptor CCR2 shows no effect on HIV infection, but individuals with this variant progress to AIDS 2-4 years later than individuals homozygous for the common allele (Smith et al., 1997). In contrast, a variant affecting two amino acids in CX3CR1 has been shown to accelerate HIV disease progression (Faure et al., 2000). The other

main chemokine receptors used by HIV for entry into the cell, CCR5 and CXCR4, also possess polymorphisms that affect the development of disease. As CXCR4 is vital to cells that express it, mutations in CXCR4 itself are rare and instead mutations have been noted in its ligand, SDF-1. The SDF-1-3'A mutation has been thought to increase the production of SDF-1 and thus increase the competition for T-tropic HIV binding of CXCR4 (Soriano et al., 2002). RANTES, a ligand for CCR5, also has a promoter mutation that is thought to increase its production and affect HIV infection in a similar fashion to SDF-1(Liu et al., 1999).

From the sheer number of genetic variations associated with changes in HIV infection and disease progression, it is evident that both immunological environment and host genetic makeup have pivotal roles in HIV disease outcome.

Genetics and HIV Resistance

Given the number of known genetic associations with HIV resistance, it was only logical to apply genetic studies to the ML cohort. Resistance within this cohort is not due to known chemokine polymorphisms (Anzala et al., 1998; Fowke et al., 1998; Kaul et al., 1999) , but has been linked to HLA class I and II alleles (MacDonald et al., 2000). Specifically, the HLA A2/6802 supertype family (A*0202, A*0205, A*0214, and A*6802), and the allele DRB1*01, have been associated with resistance (MacDonald et al., 2000). Resistance has also been associated with reduced IRF-1 expression (Ji, 2006). IRF-1 or interferon regulatory factor-1 is a transcription factor that functions as an

important immunregulatory gene. It is induced by viral infection and can bind to the LTR of HIV to upregulate its transcription.

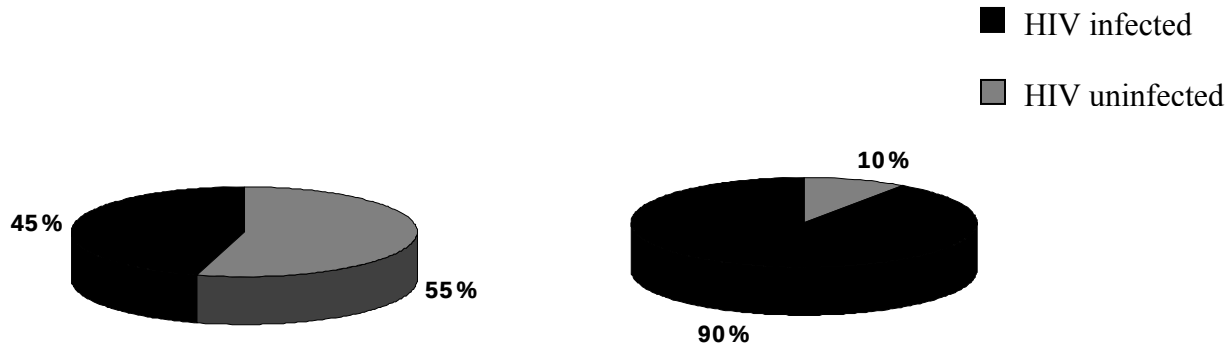
This genetic component of resistance is given more credence with the observation that CSW relatives of HIV resistant women have a decreased risk of contracting HIV (OR 0.1, $p<0.00001$), as compared to relatives of HIV susceptible women (Ball, 2001). This observation led to the development of the Kindred cohort to examine the extent of this association in non-CSW relatives. After analysis, it was determined that non-CSW relatives of HIV resistant women were also less likely to be infected with HIV (OR 0.31, $p<0.0001$) (Figures 1a and 1b).

Although resistance has been associated with numerous immunological and genetic components, the mechanism and composition of this "natural" resistance remains a mystery. Understanding what makes HIV resistant women resistant, is a critical key for the development of a prophylactic vaccine and therefore research knowledge gained from this cohort is invaluable.

Kindred cohort

The Kindred cohort was established to assess the risk of HIV acquisition in relatives of the ML cohort. It is composed of first to third degree non-sex worker relatives of both resistant (cases/HIV resistant kindred) and susceptible women (controls/HIV susceptible kindred) in the ML cohort. Cohort recruitment was conducted between June 1997 and

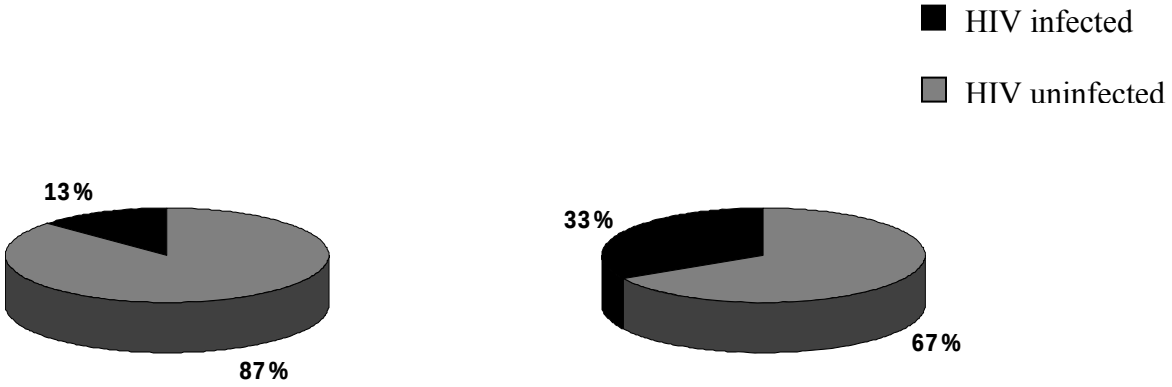
1a.



CSW related to HIV resistant women
(33 women related to 55 index cases)

CSW related to HIV susceptible women
(103 women related to 89 index cases)

1b.



HIV resistant kindred
(279 women related to 58 index cases)

HIV susceptible kindred
(303 women related to 108 index cases)

Figure 1a: HIV prevalence in sex worker relatives of HIV resistant and HIV susceptible women
Figure 1b: HIV prevalence in non-sex worker relatives (kindred cohort) of HIV resistant and HIV susceptible women
Figure used with permission from (Ball, 2001)

June 1998 and resulted in the enrollment of 58 case and 108 control families. Members were given a unique number (Kindred number) that identifies both their samples and the related index ML. All members gave informed consent and were either visited in their home or invited to attend the Majengo clinic where samples were obtained. Epidemiologic data was collected to assess exposure to HIV and other potential confounding risk factors. Analysis of the variables showed that HIV resistant kindred were less likely to be infected with HIV than HIV susceptible kindred. No other study parameters resulted in a significant difference between the two groups (Kimani, 1999).

To test the hypothesis that there is a genetic component to HIV resistance, we will address two specific objectives within this thesis:

1. Based upon the identification of the strongest non-HLA marker of HIV resistance in the IL-4/Th2 gene cluster, we will determine if the previously described polymorphisms in this gene cluster are associated with decreased HIV prevalence in the Kindred cohort.
2. Based upon the important role of the innate immune response, specifically TLRs, we will examine known polymorphisms in TLR2 and TLR4 previously identified as important in altered susceptibility to other infectious diseases.

SECTION I

The Th2 gene cluster has been identified as containing several genes important in the development of the adaptive immune response. Polymorphisms within one of these genes, interferon regulatory factor-1, have been previously associated with HIV resistance. In this section the first objective of the thesis hypothesis will be addressed as we determine if these polymorphisms are associated with decreased HIV prevalence in the Kindred cohort.

Interleukin 4/Th2 Gene Cluster

The IL-4/Th2 gene cluster is located on chromosome 5q31.1 and contains several genes important in the regulation of Th1 and Th2 immune responses (Gorham et al., 1996; Marsh et al., 1994). The pathogenesis of many diseases, including altered susceptibility to infectious diseases, can be linked to a predominant type-1 or type-2 immune response. Th1 cytokines have been implicated in the genesis of several autoimmune diseases such as Crohn's disease, multiple sclerosis and type-1 diabetes mellitus (Romagnani, 1997), where as a predominant Th2 response has been implicated in immunologic pathologies such as atopy (Marsh et al., 1994), asthma and allergy (Romagnani, 1994). A biased Th1 response has also proved necessary in the elimination of both *Listeria monocytogenes* (Gorham et al., 1996) and *Leshmania major* (Guler et al., 1996; Heinzel et al., 1989; Howard, 1986). This delicate balance in the Th1/Th2 paradigm suggests that genetic differences in this gene cluster could prove to be important in the pathogenesis of various infectious diseases, including HIV.

Previous work done on the Pumwani cohort found a global hyporesponsiveness in IL-4 production among HIV resistant sex-workers (Trivedi et al., 2001). This observation, along with evidence of CMI responses, the lack of a systemic antibody response, and a compartmentalized IgA response, suggests a Th1 bias in these women. To further examine the genetic effects of polymorphisms in this gene cluster and its potential impact on HIV susceptibility, six different microsatellites (MS) throughout this region were genotyped (D5S1984, IRF-1 MS, D5S666, 1L-4 MS, D5S2115 and D5S399). There appeared to be a difference in allele distribution between HIV resistant and HIV susceptible women in both the IRF-1 and D5S1984 MS marker. The IRF-1 MS had one particular allele (179) that appeared to be associated with HIV resistance, showing a decreased risk of seroconversion, but no effect on disease progression. The presence of the 179 allele is also associated with an increased IFNγ response to HIV antigen. Although the D5S1984 MS had two alleles (215 and 225) that were decreased in the HIV resistant women and two alleles (219 and 227) that were increased in frequency, no particular allele seemed to be directly associated with resistance (Ball, 2001). These findings led to more extensive characterization of the IRF-1 gene as a possible candidate to explain the hyporesponsive IL-4 production in HIV resistant women.

Interferon Regulatory Factor-1

Interferon Regulatory Factor-1 (IRF-1) was the first described member of the IRF family of transcription factors and functions as an important immunoregulatory gene (Mamane et al., 1999; Taniguchi et al., 1998). IRF-1 is induced by viral infection or IFNγ

stimulation (Harada et al., 1989; Miyamoto et al., 1988) . All members of the IRF family share homology in the first 115 amino acids or the DNA-binding domain (Mamane et al., 1999). Through this DNA-binding domain, IRF-1 can bind to a specific sequence in the upstream regulatory region of type 1 interferon (IFN) genes (IFNα and IFNβ), termed the IFN-stimulated response element (ISRE) (Lohoff and Mak, 2005; Mamane et al., 1999). This binding allows IRF-1 to act as a transcriptional regulator of IFNs and IFN-inducible genes that are key for an effective antiviral response (Harada et al., 1989; Miyamoto et al., 1988). Expression of IRFs also affects antigen presentation, nitric oxide production, cell cycle, and Th1/Th2 differentiation, as discussed below (Hobart et al., 1997; Kamijo et al., 1994; Lohoff and Mak, 2005; Taniguchi et al., 1998).

Many IRF functional studies have been completed using mouse knockout (KO) models. Studies have shown that IRF (-/-) KO mice have an inability to produce IL-12 and express increased levels of the antibodies IgG1 and IgE, suggesting a predominant Th2 immune response. As well, when CD4+ T cells from these IRF (-/-) mice are transferred into IRF (+/+) mice, a Th1 response is successfully mounted, providing convincing evidence that functional IRF-1 is an important factor in a Th1 immune response (Lohoff et al., 1997; Taki et al., 1997). IRF (–/-) KO mice also have natural killer (NK) cell deficiencies and defects in the development of thymic CD8+ T cells (Matsuyama et al., 1993). From this animal model evidence, it is apparent that IRF-1 is an important factor in the development of the host immune response and T cell differentiation, specifically the development of a Th1 immune response. Furthermore, this evidence suggests an

important role for IRF-1 in cellular mediated immune responses and perhaps CTL responses in HIV resistant women.

IRF-1 and HIV

Transcription and replication of HIV is controlled by the complex interaction of both host and viral proteins. The expression of the HIV Tat transactivator protein is essential for viral replication; however, because it is a viral protein, its expression requires HIV promoter activation (Cullen, 1991). It was recently discovered that HIV promoter activation can be accomplished through members of the cellular IRF family. This activation is achieved through binding of the IRFs to a sequence spanning +200 to +217 in the HIV 5'LTR region that is homologous to the ISRE (Sgarbanti et al., 2002). As previously discussed, IRF-1 can be induced by viral infection; therefore HIV can induce IRF-1 expression, which can in turn activate the HIV promoter and induce Tat. IRF-1 can also bind to Tat and work in cooperation to activate transcription (Marsili et al., 2003). Due to its discrete levels in activated T cells, IRF-1 can aid in the re-activation of HIV from latency, even in the absence of Tat. Adding to this, proinflammatory cytokines such as IFNγ, IL-6 and TNFα, which induce cell activation, also induce IRF-1 (Harada et al., 1994). It is evident that IRF-1 appears to be an important host cellular factor in the regulation of HIV replication, specifically, in early viral replication and reactivation of HIV from latency and thus is likely a factor for resistance to infection by HIV.

IRF-1 and HIV Resistance

Based upon this background and the association of a microsatellite in the IRF-1 gene, we hypothesized that IRF-1 would be a good candidate gene for further study. Full gene sequencing of the IRF-1 gene from 507 individuals was conducted and led to the discovery of 53 SNPs (26 novel), one deletion and two insertion polymorphisms present among individuals within the ML cohort (Ji et al., 2004). Two of these polymorphisms, at positions 619 (A→C) (rs17848395) and 6516 (G→T) (rs17848424), located in intron 1 and 9 respectively, were found to be differentially represented in HIV resistant women as compared to HIV susceptible women. Due to the intronic and therefore non-coding nature of these SNPs, various functional studies were performed in an attempt to understand their contribution to resistance.

Both the 619A and 6516G alleles appear to be associated with HIV resistance ($p=0.00073$ and 0.030, respectively). When the presence of any of these protective alleles (179, 619A or 6516G) was assessed, it was noted that the occurrence in HIV resistant women was 78.8% where it was only 55.6% in the HIV infected population ($p=0.0003$). Data also suggest that possession of one of these three protective alleles leads to an increase in time to seroconversion compared to cohort members lacking the protective alleles ($p=0.0001$).

Due to linkage disequilibrium (LD), the three protective alleles (619A, 6516G and 179) tend to co-segregate. This non-random association requires Log Linear multivariate analysis to reduce possible confounding factors. This analysis identified the SNP at position 619 to have the only independent and significant association with HIV

resistance. The association between HIV resistance and the other two polymorphisms is most likely an artifact of the LD (Ji, 2006).

Although these polymorphisms are located in non-coding regions, functional experiments have shown that peripheral blood mononuclear cells (PBMCs) from women with homozygous wildtype genotypes (619AA or 6516GG) show significantly lower basal expression of IRF-1, as well as responsiveness to IFNγ stimulation, when compared to PBMCs of women with either homozygous mutant or heterozygous genotypes. An HIV pseudovirus construct able to infect cells indiscriminately was used to assess early events in HIV replication in PBMCs from patients with the protective and non-protective IRF-1 genotypes. This early infection assay demonstrated a significant decrease in HIV transcription in PBMCs from patients who were homozygous for the wildtype allele at all three loci (619AA+6516GG+179/179) when compared to other haplotype combinations (Ji, 2006). This functional and genetic evidence strongly suggests that IRF-1 plays an important role in HIV susceptibility, possibly through the development of a Th1 immune response, but most likely via a direct effect on HIV viral replication. Based upon these findings we decided to investigate if the IRF-1 polymorphisms exist and affect infection rates in members of the kindred cohort.

Hypothesis & Objectives

We hypothesize that the protective IRF-1 genotype in part explains the decreased susceptibility to HIV observed in the Kindred cohort.

To investigate this hypothesis, two specific objectives were formulated:

1. To type microsatellite markers from members of the Kindred cohort for both the D5S1984 and IRF-1 MS and correlate previously identified protective genotypes with HIV resistant kindred or HIV susceptible kindred. Genotypes will also be correlated with the kindred's actual HIV status to determine if IRF-1 plays a role in their altered susceptibility.
2. To sequence segments of the IRF-1 gene in the Kindred cohort to genotype patients for SNPs (619, 6516) previously associated with altered susceptibility to HIV infection and correlate these genotypes with HIV status and relation.

Materials and Methods

Materials

Source of Biological Material

Previous work discovered that many of the HIV resistant women in the ML cohort were related to each other, thus suggesting a genetic component to such resistance. This intriguing fact led to the establishment of the Kindred cohort described earlier in this thesis. The following experiments were conducted using DNA samples obtained from members of this cohort with informed consent.

DNA Extraction

DNA was extracted using a Qiagen DNA extraction kit (Qiagen, Inc., Mississauga, ON, Canada) from peripheral blood mononuclear cells (PBMCs) or directly from the whole blood of Kindred cohort members. PBMCs were obtained by extraction from whole blood using a Ficoll-Hypaque protocol. Samples were maintained at -20ºC.

Commercial Reagents

1. Betaine (Sigma-Aldrich, Oakville, ON, Canada)
2. Big Dye® 3.1 terminator V3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA)
3. DNA ladder, 100 bp (Invitrogen, Burlington, ON, Canada)
4. Ethidium Bromide (10 mg/ml) (Sigma-Aldrich, Oakville, ON, Canada)
5. GeneScan™ -500 TAMRA™ Size Standard (Applied Biosystems, Foster City, CA, USA)
6. Hi-Di ™ Formamide (Applied Biosystems, Foster City, CA, USA)
7. POP-4™ Performance Optimized Polymer 4 (Applied Biosystems, Foster City, CA, USA)
8. POP-6™ Performance Optimized Polymer 6 (Applied Biosystems, Foster City, CA, USA)
9. Taq DNA polymerase recombinant (Invitrogen, Burlington, ON, Canada)
10. UltraPure™ Agarose (Invitrogen, Burlington, ON, Canada)

Laboratory Prepared Solutions

1. d.d. H_20
    - distilled and deionized water
2. 10x TBE buffer
    - 108 g Tris Base
    - 55 g Boric acid
    - 7.44 g EDTA
    - Add d.d. H_2O until volume is 1 liter.
3. TE buffer (pH 8.0)
    - 10 ml 10 mM Tris-Cl (pH 8.0)
    - 2 ml 1mM EDTA (pH 8.0)
    - 988 ml d.d. H_2O
4. 6x PCR Gel Loading buffer:
    - 0.25 bromophenol blue
    - 0.25 Xylene Cyanol
    - 30% Glycerol in H_2O
5. PCR 2x master mix

Conc. in Storage Buffer	Quantity	Conc. in 2x Mixture
1) 833 mM (or 1M) Tris-HCl (pH 9.0)	144 µl	120mM Tris-HCl (pH 9.0)
2) 50 mM $MgCl_2$	60 µl	3 mM $MgCl_2$
3) 1M $(NH_4)_2$ SO_4	30 µl	30 mM $(NH_4)_2$ SO_4
4) 1mM dNTP Mix	200 µl	200 µM dNTP Mix

5) 1% Gelatin	200 µl	0.2% Gelatin
6) d.d. H_20	276 µl	
Total volume	910 µl	

Methods

Microsatellite Typing

Microsatellite typing was performed to assess the variability within two microsatellites located in the IL-4/Th2 gene cluster. These two dinucleotide repeat regions (IRF-1 and D5S1984) were previously associated with the HIV resistant phenotype within the ML cohort (Ball, 2001). The techniques used were adapted from previously described methods (Hughes, 1993). Specific primers were designed for each microsatellite, where one of each primer set was labeled with either 6-FAM or HEX at the 5' end (Table 1). This incorporation of fluorescence into the PCR product allows for laser size discrimination on an ABI Prism ® 310 genetic analyzer.

The region around each microsatellite marker was amplified in separate 50µl reactions for each sample: 19.75µl 2x PCR Master Mix, 0.1µM each primer, 1.25U Taq polymerase, 23µl d.d.H_20 and 1.5µl DNA sample. PCR conditions were carried out on a thermocycler (MJ Research, Waltham, MA, USA) as follows: 94ºC for 3 min, 94ºC for 30s, 55ºC (IRF-1 and D5S1984), 72ºC for 2 min, go back to step 2, 34 times and 72ºC for 10 min.

Table 1: IRF-1/D5S1984 microsatellite primers

Microsatellite Marker	Custom Oligonucleotide Primers	Annealing Temperature (ºC)
IRF-1	(f) **6-FAM**-ATGGCAGATAGGTCCACCGG (r)TCATCCTCATCTGTTGTAGC	55ºC
D5S1984	(f) **HEX**-CCAGCCCGCTTAGTGT (r) TAGGAGGCTTCCCACATCT	58ºC

Using the amplified PCR product the following 13.5µl mixture was resolved: 1µl D5S1984 PCR product, 1µl IRF-1 PCR product, 0.5µl 500-TAMRA™ Size Standard and 11µl formamide. The samples were then loaded onto an ABI 310 (Applied Biosystems, Foster City, CA, USA) The number of GT repeats were determined using Genescan analysis software. The use of different fluorescent labels for IRF-1 and D5S1984 enabled size determination of both microsatellites simultaneously. This is possible due to the different emission wavelengths of the dyes and thus their appearance as different colors during analysis.

Sequencing

Two single nucleotide polymorphisms in the IRF-1 gene, at locations 619 (segment 11) and 6516 (segment 8) were recently found to be associated with HIV resistance within the ML cohort (Ji, 2006). The IRF-1 gene was broken down into overlapping segments to make sequencing more manageable. The specific primers designed for each segment are outlined in Table 2. Initial PCR amplification consisted of the following 50µl reaction: 19.75µl 2x PCR Master Mix, 0.5µM of each primer, 1.25U Taq polymerase, 23.5 d.d.H_20 (18.5µl d.d.H_20 and 5µl Betaine for segment 11) and 1.5µl DNA sample. PCR was carried out in a thermocycler (MJ Research, Waltham, MA, USA) as follows: 94ºC for 3 min, 94ºC for 30s, 55ºC (segment 11) or 53ºC (segment 8) for 30s, 72ºC for 2 min, go back to step 2, 34 times and 72ºC for 10 min.

Table 2: IRF-1 gene sequencing primers

IRF-1 Segment	Custom Oligonucleotide Primers	Annealing Temperature (ºC)
Segment 8 Initial PCR	(f) AGGGTGAGTCTGCACTGGAA (r) CTTGGCAGTGGGGTCACA	53ºC
Segment 8 Sequencing PCR	(f) AGGGTGAGTCTGCACTGG (r) CTTGGCTGTTGAGGGGC	53ºC 53ºC
Segment 11 Initial PCR	(f) GCTCGCCACTCCTTAGTCG (r) CAGTAAGCAGCCCTTGCC	55ºC
Segment 11 Sequencing PCR	(f) CTCCTTAGTCGAGGCAAGACG (r) AGCCCTTGCCACCAGCACA	53ºC 55ºC

Following initial amplification, the PCR product was transferred to a multiscreen plate (Applied Biosystems, Foster City, CA, USA) for purification. A vacuum of 24″ Hg was applied to the plate for 5 min and then 100µl of TE buffer was added to each well. Following the addition of TE buffer, the vacuum was then reapplied for another 5 min. The plate was then blotted with a paper towel, 50µl of d.d. H_20 added to each well and the plate put on a shaker for 5 min at 200rpm. The purified DNA was then retrieved and transferred to a sterile storage plate.

After the DNA was purified, sequencing PCR was set up as follows: 2µl Big Dye®, 2.7µM primer (forward or reverse) and 2µl purified DNA. PCR conditions were once again carried out on a thermocycler as follows: 96ºC for 3 min, 96ºC for 30s, 53ºC for 30s, 60ºC for 4 min and go to step 2, repeat 79 times.

Precipitation was then performed by adding a mixture of 1µl NaOAc and 20µl 95% ethanol to each sample and letting the samples sit for 2-24 hours in the dark. The samples were then spun in a centrifuge at 3800rpm for 1 hour. Subsequent to spinning, the ethanol was dumped out and the samples received an inverted quick spin to remove all liquid. One hundred and fifty microliters of 70% ethanol was then added to each sample and they were again spun at 3800rpm for 10 min. The samples were then inverted and given a quick spin to remove excess ethanol. The samples were then heated at 90ºC for 1.5 min.

The samples were reconstituted in 20µl formamide and heated for a further 1.5 min at 90ºC. The samples were then immediately put on ice and transferred to an ABI Sequencing plate (Applied Biosystems, Foster City, CA, USA) and resolved on an ABI 3100. Samples were subsequently analyzed using the Sequencher program by comparison to the published GenBank IRF-1 sequence (L05072). This method instills confidence in the findings as sequence is obtained in both the forward and reverse directions.

Results

IRF-1 179 MS is Associated with HIV Resistant Kindred

Previous research has established an association between IRF-1 179 and the HIV resistant phenotype. In an attempt to provide further evidence of genetic correlates to HIV resistance, we attempted to confirm this association in the HIV resistant kindred. Microsatellite typing was performed on 147-210 subjects from the Kindred cohort. The number of subjects varied due to lack of biological material. After genotyping, the subjects were segregated based on whether they were HIV resistant kindred or HIV susceptible kindred, and the allele frequencies compared for both microsatellites (Table 3a and Table 3b). Chi-square analysis was performed on both the D5S1984 and IRF-1 MS to determine the difference in allelic distribution between the two groups ($p=0.2443$ and $p=0.026$ respectively). A significant difference in allele distribution was noted between the two groups for the IRF-1 MS. However, Chi-square analysis is not

Table 3a: IRF-1 MS allele frequencies in HIV resistant and HIV susceptible kindred
Table 3b: D5S1984 MS allele frequencies in HIV resistant and HIV susceptible kindred

3a.

IRF-1				
	related ML			
	susceptible		resistant	
allele	n	%	n	%
**179	64	35.96	113	46.69
181	46	25.84	62	25.62
183	10	5.62	8	3.31
185				
*187	2	1.12	10	4.13
189	35	19.66	40	16.53
*191	5	2.81	1	0.41
*193	1	0.56		
*195	3	1.69		
*197	1	0.56	1	0.41
*199	5	2.81	2	0.83
*201	1	0.56	2	0.83
*203	5	2.81	2	0.83
*205			1	0.41
Total	178		242	
Chi-sqr	12df, X^2=23.18, p=0.026			
*Chi-sqr	4df, X^2=7.02, p=0.1350			
**Chi-sqr	1df, X^2=4.42, p=0.036			

3b.

D5S1984				
	related ML			
	susceptible		resistant	
allele	n	%	n	%
*209	1	0.78	2	1.20
211	23	17.97	15	9.04
213	33	25.78	39	23.49
215	13	10.16	31	18.67
217	8	6.25	5	3.01
*219		0.00	1	0.60
221	7	5.47	11	6.63
223	12	9.38	16	9.64
225	24	18.75	41	24.70
*227	3	2.34	3	1.81
*229	1	0.78	1	0.60
*231	3	2.34	1	0.60
Total	128		166	
Chi-sqr	11df, X^2=13.79, p=0.2443			
*Chi-sqr	7df, X^2=11.43, p=0.1211			

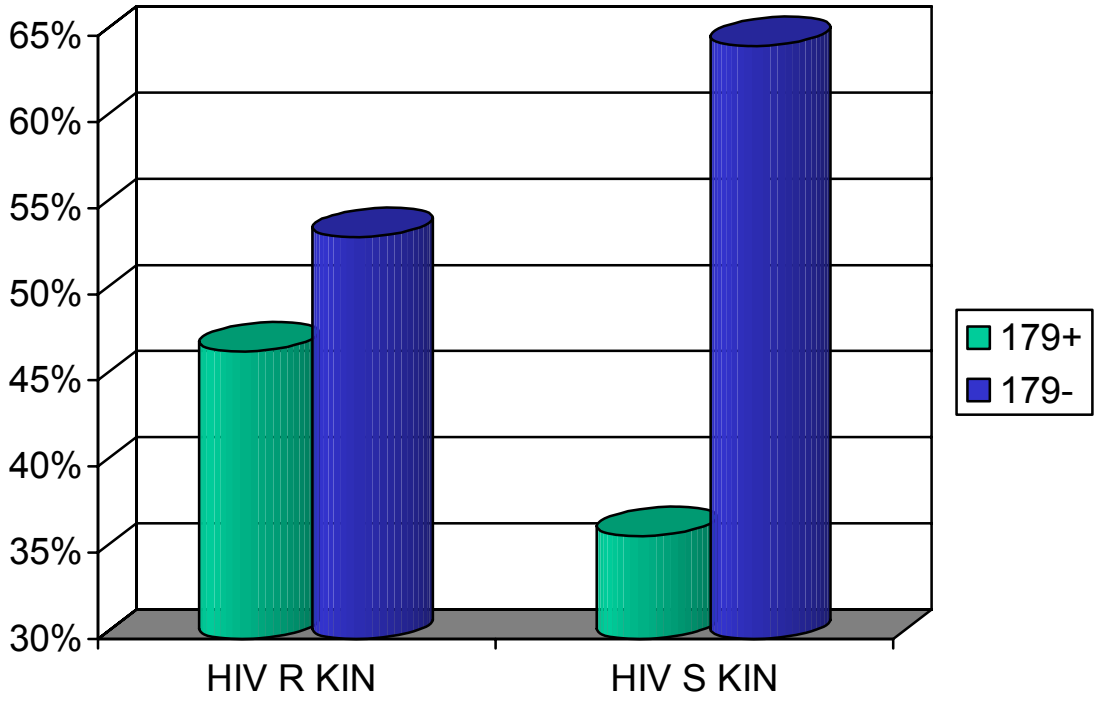
Table 3a: *Allele frequencies of the IRF-1 MS in HIV resistant and HIV susceptible kindred. Allele frequencies show distribution on either chromosome (2n), therefore the study number is the total/2. Chi-square was used to analyze the entire table and repeated by grouping less frequent alleles(*). Analysis was also completed for the 179 MS alone(**).*

Table 3b: *Allele frequencies of the D5S1984 MS in HIV resistant and HIV susceptible kindred. Allele frequencies show distribution on either chromosome (2n), therefore the study number is the total/2. Chi-square was used to analyze the entire table and repeated by grouping less frequent alleles(*).*

appropriate for observed or expected values of less than 5, therefore infrequent alleles were grouped together and the data re-analyzed for both MS (p=0.1211 and p=0.1214 respectively). This more appropriate statistical method fails to demonstrate a significant statistical difference in the distribution of alleles between the two groups, although a slight trend was observed.

Due to the previously established significance of the 179 IRF-1 MS and its association to HIV resistance, another contingency table was constructed specifically comparing the 179 MS allele distribution between the two groups (p=0.036) (Figure 2). A clear association was established between HIV resistance and the presence of the 179 allele. The data were then reanalyzed and separated based on HIV status of the kindred, irrespective of the HIV status of the CSW relative. Subsequent analysis demonstrated that there is no significant difference in allelic distribution between HIV negative and HIV positive kindred in either the D5S1984 or IRF-1 MS. When the distribution of the 179 allele alone also was conducted, this also failed to yield an association with HIV status (Table 4a and Table 4b).

Due to the polymorphic nature of microsatellites, an additional analysis was performed to assess the 179 MS lineage. Specific microsatellite lengths may be inherited or can be caused by "slippage" during DNA replication (slippage may result in an extra repeat, essentially forming a 179 allele from an intended 177 allele) (Li et al., 2002). A



	susceptible	Resistant
179+	**64**	**113**
179-	**114**	**129**

Chi-sqr 1df, X^2=4.42, **p=0.036**
(OR: 0.641 95%CI: 0.422-0.972)

Figure 2: IRF-1 179 MS allele frequency in HIV resistant (HIV R) and HIV susceptible (HIV S) kindred

Table 4a: IRF-1 MS allele frequencies in HIV positive and HIV negative kindred
Table 4b: D5S1984 MS allele frequencies in HIV positive and HIV negative kindred

4a.

IRF-1				
	kindred HIV status			
	positive		negative	
allele	n	%	n	%
179	38	50.00	120	42.25
181	16	21.05	73	25.70
*183	3	3.95	14	4.93
*187			9	3.17
189	12	15.79	51	17.96
*191	2	2.63	3	1.06
*193	1	1.32		
*195	2	2.63	1	0.35
*197			2	0.70
*199			5	1.76
*201			3	1.06
*203	2	2.63	3	1.06
205				
total	76		284	
Chi-sqr df 11, X^2=16.33, p=0.13				
*Chi-sqr df 3,X^2=1.53, p=0.67				

4b.

D5S1984				
	kindred HIV status			
	positive		negative	
allele	n	%	n	%
*209		0.00	3	1.50
211	8	13.79	28	14.00
213	14	24.14	51	25.50
215	5	8.62	34	17.00
217	1	1.72	11	5.50
*219	1	1.72		0.00
221	3	5.17	9	4.50
223	7	12.07	20	10.00
225	15	25.86	38	19.00
227	3	5.17	1	0.50
*229	1	1.72	1	0.50
*231		0.00	4	2.00
total	58		200	
Chi-sqr df 11, X^2=17.44,p=0.096				
*Chi-sqr df 8,X^2=11.13,p=0.195				

Table 4a: *Allele frequencies of the IRF-1 MS in HIV positive and HIV negative kindred. Allele frequencies show distribution on either chromosome (2n), therefore the study number is the total/2. Chi-square was used to analyze the entire table and repeated by grouping less frequent alleles(*).*

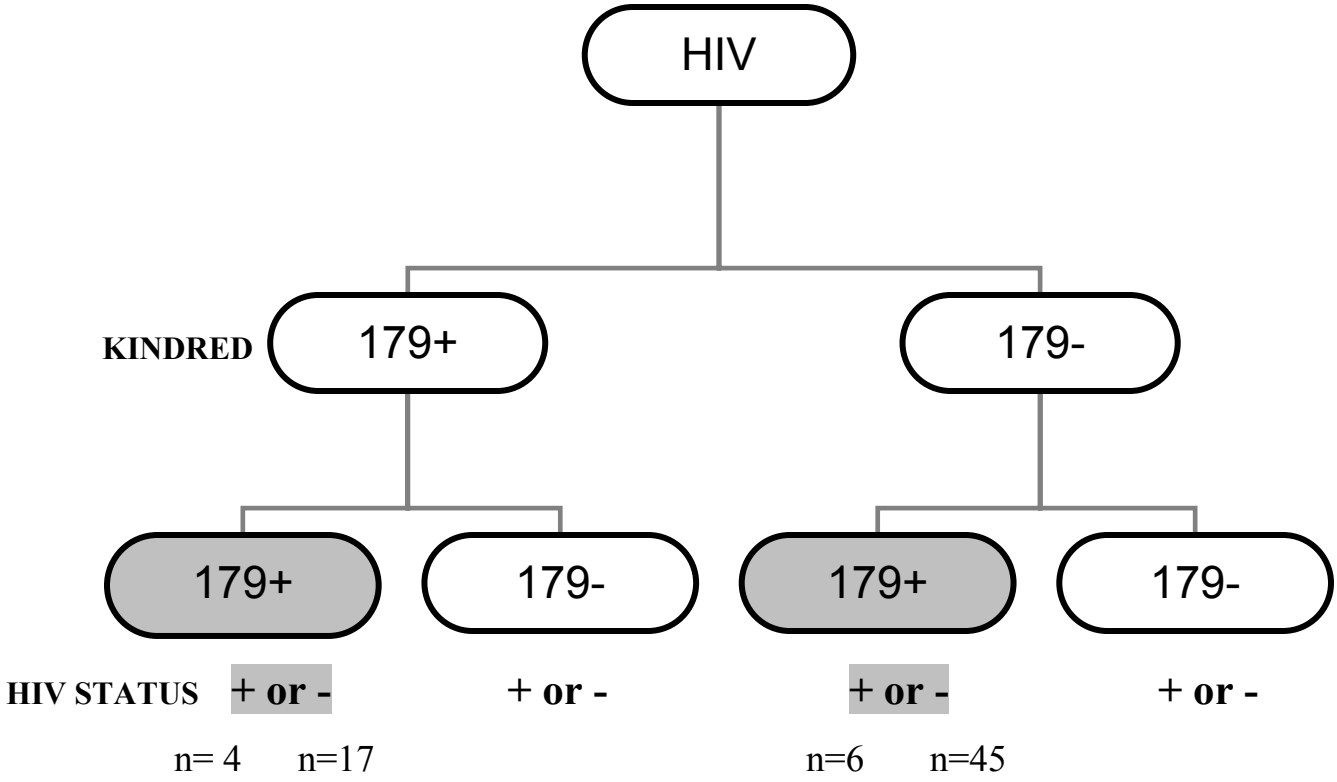
Table 4b: *Allele frequencies of the D5S1984 MS in HIV positive and HIV negative kindred. Allele frequencies show distribution on either chromosome (2n), therefore the study number is the total/2. Chi-square was used to analyze the entire table and repeated by grouping less frequent alleles(*).*

contingency table was constructed comparing the HIV status of kindred possessing the 179 allele whose ML relatives also possess the 179 allele (ie: would have the 179 allele through inheritance), against those kindred possessing the 179 allele whose ML relatives do not have the 179 allele (ie: possession of the 179 allele is due to "slippage") (Figure 3). If those with 179+ relatives are more likely to be HIV negative, it suggests that the microsatellite is most likely in LD with another marker on the gene as this genotype could be directly inherited as opposed to the microsatellite changing during meiosis. After analysis with Fisher's Exact Test, however, this did not seem to be the case ($p=0.321$). There appears to be no significant difference between possessing the protective 179 allele through presumed inheritance as opposed to possession via apparent mutation during meiosis.

IRF 6516G Allele is Associated with HIV Resistant Kindred

Sequence based mutation detection was performed on 191-210 members of the Kindred cohort for both the 619 and 6516 SNP. The number of samples genotyped varied due to the lack of biological material available. The samples were separated based on HIV status of the CSW relative, contingency tables were constructed and Chi-square analysis performed on the distribution of both the 619 and 6516 SNPs ($p=0.156$ and $p=0.229$ respectively) (Figure 4a and 4b). No associations were found between the SNPs and HIV resistant kindred.

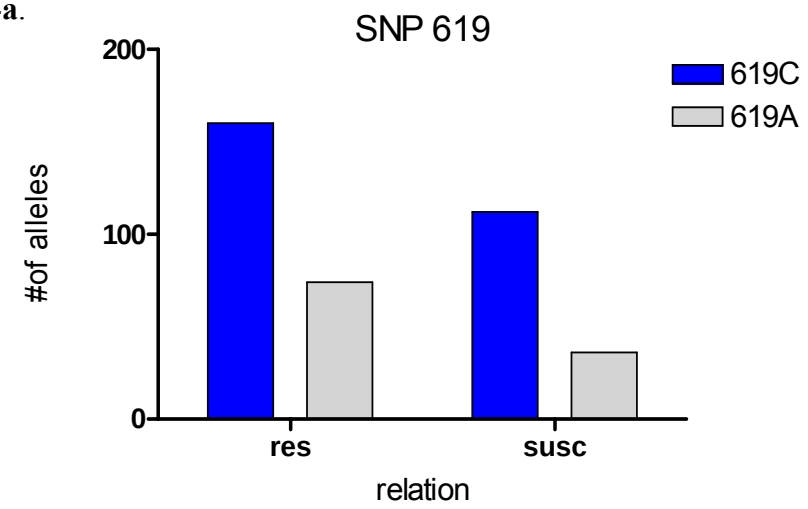
The distribution of alleles was reanalyzed using Chi-square with subjects segregated



	Resistant Relative 179+	Resistant Relative 179-
HIV positive	4	6
HIV negative	17	45

Figure 3: IRF-1 179 MS lineage
(highlighted groups are the populations in question)

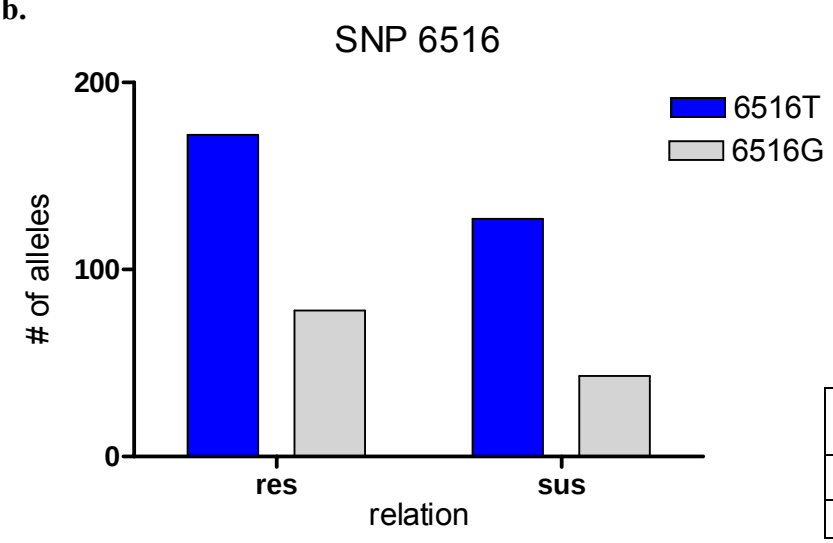
4a.



	Mutant alleles	Wildtype alleles
Related to res	160	74
Related to sus	112	36

X^2=2.013, p=0.1559, OR=0.695

4b.



	Mutant alleles	Wildtype alleles
Related to res	172	78
Related to sus	127	43

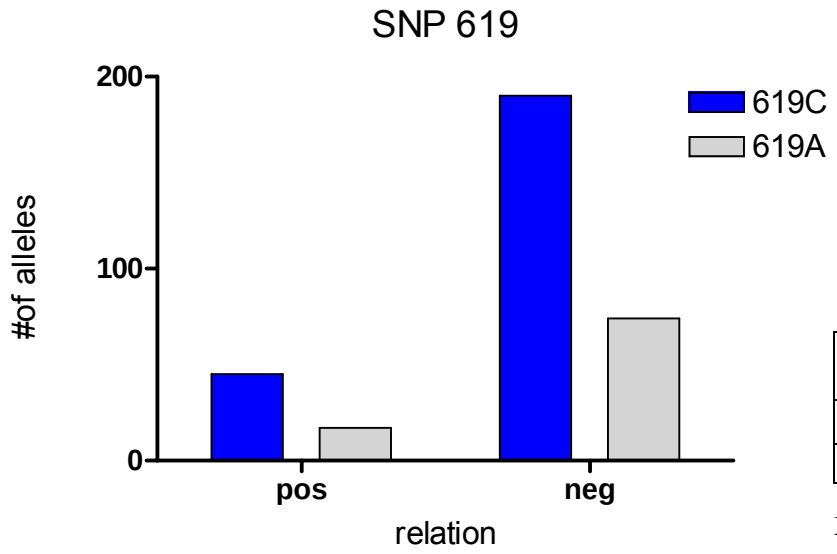
X^2=1.445, p=0.2293, OR=0.747

Figure 4a: IRF-1 SNP 619 frequencies in HIV resistant and HIV susceptible kindred
Figure 4b: IRF-1 SNP 6516 frequencies in HIV resistant and HIV susceptible kindred

based upon their HIV status and not relation (p=0.9515 and p=0.9311 respectively) (Figure 5a and 5b). This analysis also yielded no significant association between the SNPs and HIV status of the kindred. The SNPs have previously been shown to be in LD, however haplotype analysis was unavailable because of the small sample size with predictable haplotypes. Analysis was thus performed based on the presence of at least one advantageous allele and subjects were separated based on relation and HIV status (p=0.1892 and p=0.4767 respectively). The presence of one advantageous allele was not found to be associated with being related to an HIV resistant woman.

Because Chi-square measures the observed versus expected values, the appropriateness of the traditional Chi-square analysis on our data was in question. The Kindred cohort is composed of first to third degree relatives and analyzing them as one group is inaccurate due to the differing genetic similarity per degree. We conducted an additional analysis in which we segregated the kindred on the basis of degree of relationship according to the following guidelines: 1) first degree relative as parents, children or siblings (50% genetic similarity) 2) second degree relatives as aunts, uncles, nieces, nephews, grandparents or half-siblings (25% genetic similarity) 3) third degree relatives as first cousins (12.5% genetic similarity) 4) unknown: members of the kindred cohort who were related to MLs, but it was unknown to which degree (UUHSC, 2005). A contingency table comparing HIV resistant and HIV susceptible kindred, was then compiled for each degree. This Mantel-Haenszel Chi-square provides a pooled odds ratio across all strata while reducing confounders and thus provides a more accurate look at associations. When this more appropriate test was performed on the 6516 SNP there appeared to be an association

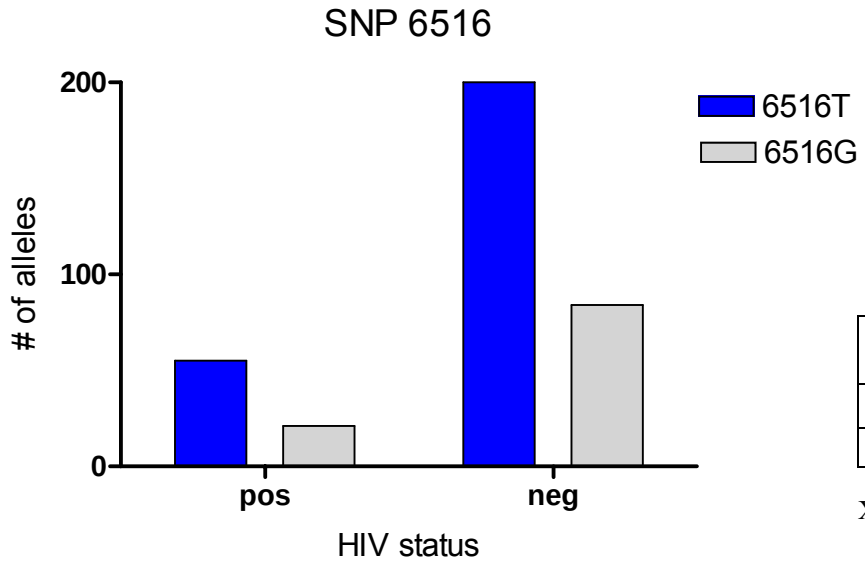
5a.



	Mutant alleles	Wildtype alleles
Related to res	45	17
Related to sus	190	74

$X^2=3.697x10^{-3}$, p=0.9515, OR=1.031

5b.



	Mutant Alleles	Wildtype alleles
Related to res	55	21
Related to sus	206	84

$X^2=7.466x10^{-3}$, p=0.9311, OR=1.06

Figure 5a: IRF-1 SNP 619 frequencies in HIV positive and HIV negative women
Figure 5b: IRF-1 SNP 6516 frequencies in HIV positive and HIV negative women

between the presence of the 6516G allele and HIV resistant kindred (p=0.0419, df=1, X^2=4.1404). When this analysis was performed excluding the group of unknown relationship, the p value was no longer significant, likely due to the significantly smaller sample size. When analysis was done on the 619 SNP, including and excluding the unknown group, the p values did show a trend, but were not statistically significant. The p values found for each situation are summarized in Table 5. After utilizing the appropriate statistical tools, there is a significant association between HIV resistant kindred and the 6516G allele and a trending association with 619A.

After determining that associations between IRF-1 polymorphisms and HIV resistance were not strongly replicated in the Kindred cohort, we resolved no clear role for IRF-1. The overall lack of association led us to investigate other possible mechanisms for HIV resistance, more specifically polymorphisms in innate immune genes. Components of the innate immune system are among the first host factors to interact with HIV and are also crucial in the direction of the host adaptive response. Innate gene variants could thus be important in mounting a successful immune response to HIV and should be investigated in the context of HIV resistance. Due to the difficulty assessing exposure to HIV in the Kindred cohort to determine HIV resistance, we returned to the CSW cohort for further study.

Table 5: Summary of p values found in the association analysis of HIV resistance and 619A and 6516G alleles using Mantel-Haenszel chi-square statistical analysis

	1st degree		2nd degree		3rd degree		Unknown degree	
SNP	619		619		619		619	
Nucleotide	A	C	A	C	A	C	A	C
HIV resistant kindred	45	75	14	44	5	21	14	30
HIV susceptible kindred	24	62	3	13	1	11	8	26
Total alleles (n=total/2)	206		74		38		78	

	1st degree		2nd degree		3rd degree		Unknown degree	
SNP	6516		6516		6516		6516	
Nucleotide	G	T	G	T	G	T	G	T
HIV resistant kindred	52	94	17	43	6	20	13	31
HIV susceptible kindred	29	93	7	11	2	16	8	32
Total alleles (n=total/2)	268		78		44		84	

	619A	6516G
Mantel-Haenszel Chi-square (including unknown)	p=0.0642 (df=1,X^2=3.4266)	p=0.0419 (df=1, X^2=4.1404)
Mantel-Haenszel Chi-square (excluding unknown)	p=0.0950 (df=1, X^2=2.7876)	p=0.0754 (df=1, X^2=3.1618)

Table 5: *Upper tables show actual data used to construct the contingency tables for Mantel-Haenszel Chi-square analysis. Lower table summarizes resulting p values.*

SECTION II

Innate immunity & TLRs

In any organism, the recognition of invading pathogens and the appropriate immune response is critical for survival. This initial recognition and immune response is carried out by the innate immune system, which is characterized by an evolutionarily conserved, germline encoded set of receptors that recognize pathogen associated molecular patterns (PAMPs). These pattern recognition receptors (PRRs) allow an organism to mount a quick and accurate immune response, while instructing the adaptive immune response, which maintains immunological memory. The family of toll-like receptors (TLR) are the most well described family of PRRs (Cook et al., 2003; Iwasaki and Medzhitov, 2004; Staros, 2005).

The human mechanism of the initial receptor driven initial self and non-self discrimination was not unearthed until the 1990's. Much research had been conducted to evaluate the immune system of *Drosophila melanogaster,* an organism which lacks an adaptive immune response, yet is still able to mount a rapid and efficient response to foreign pathogens (Takeuchi, 2005). The induction of an immune response in *Drosophila* is instructed by one of two systems, *Imd* or *Toll*. The *Toll* pathway consists of transmembrane receptors that are activated in response to fungi and gram positive bacteria, while the *Imd* pathway is responsible for the recognition of gram negative bacteria. These pathways allow *Drosophila* the ability to distinguish between invading pathogens and ultimately initiate an appropriate signaling cascade and the production of antimicrobial peptides (Takeda et al., 2003; Takeuchi, 2005). In 1997, human

homologues to these *Toll* receptors were identified and implicated in the immune response of humans. Due to their homology, these receptors were termed Toll-like receptors or TLRs (Medzhitov et al., 1997). To date, there have been 11 separate TLRs identified, ten of which recognize a distinct PAMP (TLR 11 is likely non-functional, but has been found to bind uropathogenic *E.coli* in mice) (Takeda et al., 2003; Takeuchi, 2005). Recognition is also limited by accessibility, as TLRs 3,7,8 and 9, are located intracellularly and thus detect viral/intracellular pathogen nucleic acids (Heil et al., 2003; Matsumoto et al., 2003) (Figure 6).

These receptors consist of three different domains: 1) an extracellular leucine rich region (LRR), which is responsible for ligand recognition, 2) a transmembrane region and 3) a cytoplasmic Toll/IL-1R (Interleukin 1 receptor) homologous domain or TIR, which is essential in triggering cellular pathways (Medzhitov et al., 1997). TLRs are expressed on almost all cell types, including B cells, dendritic cells (DCs), endothelial, and epithelial cells (Zarember and Godowski, 2002). The level of expression determines the strength of the response and is affected by growth factors and cytokines. Studies have also shown that increased expression levels can affect clearance and survival following bacterial infection (Bihl et al., 2003) and can be associated with several chronic inflammatory diseases such as atherosclerosis (Vink et al., 2002), inflammatory bowel disease (Mori et al., 2003), and periodontitis (Mori et al., 2003).

After TLR ligand binding, a signaling pathway is activated with the help of various adaptor molecules. These adaptor molecules vary, depending on the TLR, and affect the

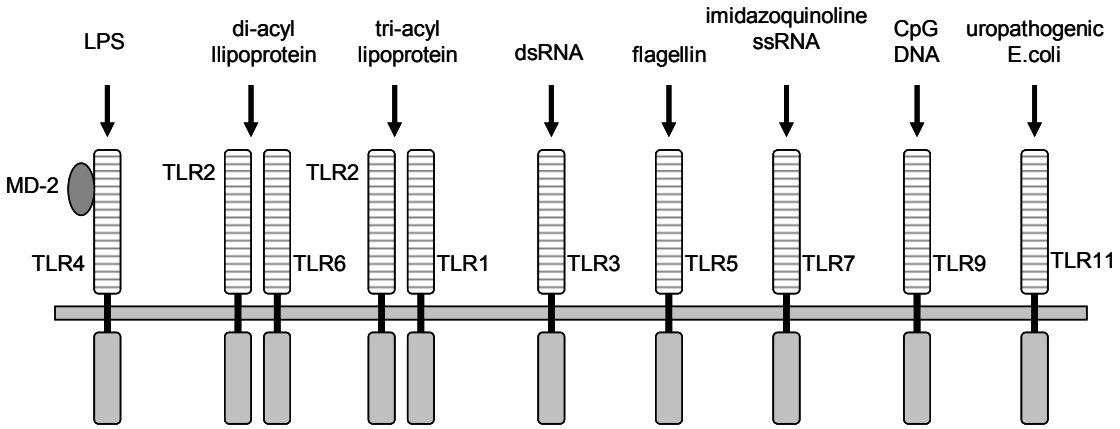


Figure 6: Toll-like Receptors (TLR) and their respective ligands

TLRs recognize various PAMPS. TLR2 specificity varies depending on whether a heterodimer is formed with TLR1 or TLR6. TLR11 has been shown to detect uropathogenic E.coli in mice, but is most likely non-functional in humans.

affinity of the ligands, allowing broad ligand specificity (Schuster and Nelson, 2000). The specific signaling pathway varies with adaptor molecules, but eventually leads to the nuclear translocation of NFκB and the activation of MAP kinases. This nuclear translocation allows NFκB to mediate the expression of proinflammatory cytokines and thus the recruitment of immune cells to the site of infection(Cook et al., 2003; Iwasaki and Medzhitov, 2004; Takeda et al., 2003).

TLR signaling is also essential to the maturation of DCs by regulating costimulatory molecule expression, cytokine production and phagosome maturation. This is an important link between innate and adaptive immunity, as DCs are crucial in T cell activation and differentiation into Th1 or Th2 cells (Cook et al., 2003; Huang et al., 2001). TLR agonists are also well described as vaccine adjuvants used to increase the immunogenicity of antigens. Due to the close link with the adaptive immune system, early innate responses by adjuvants lead to more effective adaptive immune responses (Rezaei, 2006). This intricate influence on the adaptive immune response and their described variability within humans has made TLRs interesting candidates for observed differences in susceptibility to disease. TLR2 and TLR4 have been the most extensively studied TLRs, both functionally and genetically, in the context of disease susceptibility.

TLR2 & TLR4

TLR2

Toll-like receptor 2 is most often recognized for binding peptidoglycan from gram positive bacteria, but can bind a variety of ligands, including glycosylphosphatidylinositol (GPI) from *Trypanosoma cruzi*, bacterial lipoprotein and *Saccharomyces cerevisiae* zymosan (Cristofaro and Opal, 2006; Takeda et al., 2003; Turvey and Hawn, 2006). The wide variety of ligands recognized by TLR2 is one of its unique attributes that makes it a prime target for therapeutic intervention. Its ability to recognize such a wide array of PAMPs is in part due to its capacity to not only form homodimers, but to form heterodimers with both TLR1 and TLR6. The TLR1-2 complex has the ability to bind triacyl peptides such as the ones found in bacterial outer membranes, while the TLR2-6 complex binds diacyl peptides such as those found in *Mycoplasma* and zymosan (Cristofaro and Opal, 2006).

After recognition of the ligand, the TIR domain of TLR2 recruits TIR domain-containing adaptor molecule (TIRAP), which aids in myeloid differentiation factor 88 (MyD88) recruitment. This MyD88 recruitment leads to the activation of IL-1-R associated kinase 4 (IRAK4), which associates with and phosphorylates IRAK 1, triggering recruitment of tumor necrosis receptor-associated factor 6 (TRAF6). This activation of TRAF6 leads to the nuclear translocation of NFκB through the phosphorylation and subsequent degradation of the NFκB inhibitor (IκB) (Bowie and Haga, 2005; O'Neill et al., 2003; Suzuki et al., 2002; Takeuchi, 2005). The activation of NFκB leads to the upregulation of proinflammatory genes such as tumor necrosis factor (TNF), interleukin 6 (IL-6) and IL-1, but not the activation of Type 1 IFN genes (Cook et al., 2003; Takeuchi, 2005).

This may describe the difference in TLR stimulation and the induction of a polarized Th1 or Th2 response.

TLR4

TLR4 is the most extensively studied TLR and perhaps one of the most complicated from a signaling perspective. TLR4 binds lipopolysaccharide from gram negative bacteria but has recently been found to bind respiratory syncytial virus fusion protein and mouse mammary tumor virus envelope protein. As well as these foreign products, TLR4 has the capacity to bind endogenous products such as heat shock protein 60 and fibrinogen (Takeuchi, 2005).

The binding of LPS requires other molecules in addition to TLR4. LPS-binding protein (LPB) binds to LPS in serum and this complex is then recognized by CD14, a GPI-anchored protein located on the surface of monocytes/macrophages and neutrophils (Takeda et al., 2003). Another extracellular protein, MD-2, enhances LPS responsiveness and has proven necessary for surface expression of TLR4 (Cristofaro and Opal, 2006; Takeda et al., 2003; Takeuchi, 2005). This initial recognition of LPS requires the cooperation of various proteins and gives insight into the complexity of TLR4 signaling.

In addition to the pathway described for TLR2, TLR4 has an additional MyD88-independent signaling pathway (Kawai et al., 1999). This alternate pathway results in slow and delayed NFκB translocation and also induces IFN-mediated host responses and

interferon regulatory factor-3 (IRF-3) (Cristofaro and Opal, 2006). Upon recognition of lipopolysaccharide (LPS), the TIR domain of TLR4 associates with TRIF-related adaptor molecule (TRAM), which can then interact with TRIF (TIR domain- containing adaptor inducing IFN-β) (Takeda et al., 2003; Takeuchi, 2005; Yamamoto et al., 2003b). TRIF contains a TRAF6 binding motif and a receptor interacting protein (RIP) domain, which allows interaction with these proteins and results in NFκB translocation. TRIF is also able to associate with TBK1, which phosphorylates IRF-3 and enables it to translocate to the nucleus and induce Type 1 IFN genes (Takeuchi, 2005; Yamamoto et al., 2003a)(Figure 7). This induction of Th1 cytokines may have relevance to the altered adaptive immune response seen in HIV resistant women.

TLRs and HIV

The interaction between TLRs or TLR signaling pathways and HIV is a relatively new field, as the concept of PRRs has only emerged within the last few years. Evidence has shown, however, that HIV gene expression can be activated as a consequence of TLR signaling due to a non-HIV coinfecting pathogen. The mechanism of this upregulation through TLRs is a result of the complex signaling pathway, initiated by ligand recognition. It is thought to occur through either direct interaction of NFκB with the HIV LTR or by the enhancement of the LTR through pro-inflammatory cytokines (TNF, IL-1) regulated by NFκB translocation (Bafica et al., 2004). This interaction could prove to be

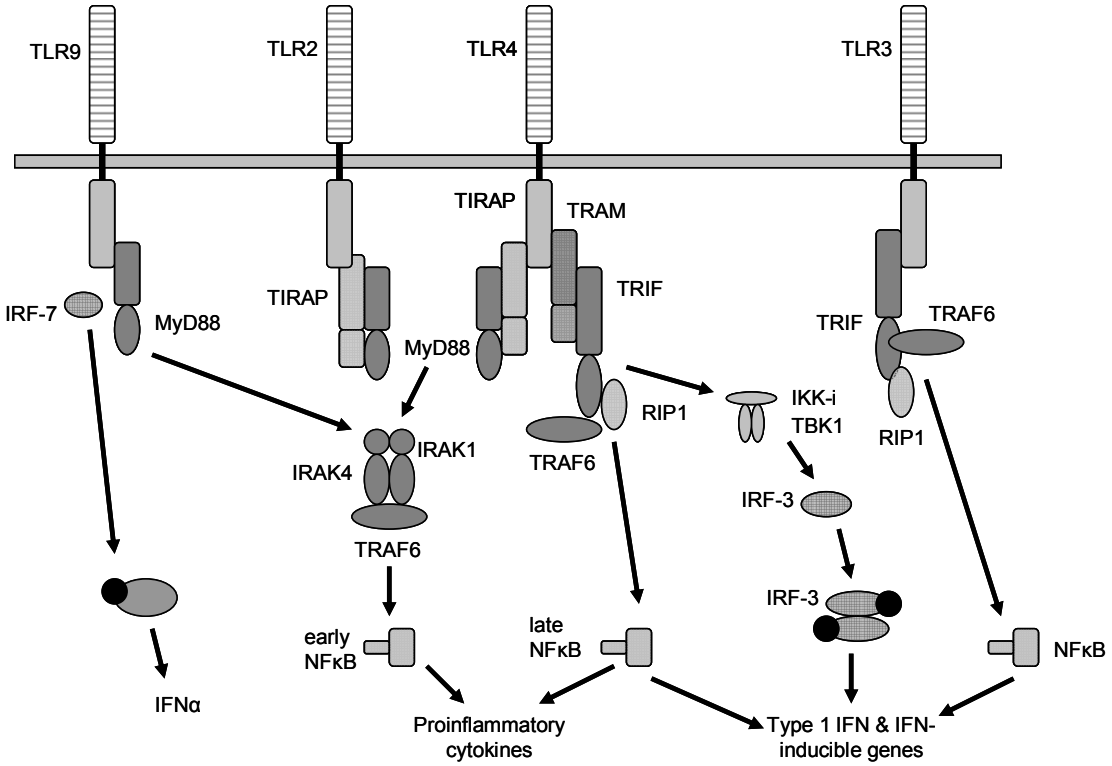


Figure 7: TLR signaling pathways

Signaling pathways induced by TLRs. Through the help of adaptor molecules, the pathways lead to IFN expression and/or the nuclear translocation of NFκB and IRF-3. The black circle on IFNα and IRF-3 represent phosphorylation.

of huge consequence in HIV endemic areas, such as Sub-Saharan Africa, where treatment for such coinfections may be absent.

There is also evidence that signaling through TLRs 2, 4 and 9 can increase HIV replication in latently infected human mast cells (Sundstrom et al., 2004). Overexpression of TLR2 has also been shown on monocytes from HIV positive patients and this correlated with enhanced HIV replication (Heggelund et al., 2004). These observations provide evidence for the role of TLRs in the response to HIV infection and the regulation of its transcription. It can thus be speculated that an altered innate response through TLRs may affect the downstream adaptive immune response. Differences in TLR function may be responsible for altered innate immune responses and should thus be explored in HIV resistant women.

TLR Polymorphisms and Disease

Variety in innate immune genes, such as TLRs, is important for a number of reasons: 1) these changes would be inheritable and therefore most likely provide an evolutionary advantage, 2) due to their initiation of an adaptive immune response, variation in innate genes may alter these cues, and 3) due to their function, variations in innate immune genes are likely to alter downstream responses (Staros, 2005). These variations or SNPs may alter the amino acid composition, or affect promoter characteristics (Schroder and Schumann, 2005). To date, 44 SNPs have been identified in TLR4 and 28 in TLR2.

Genetic variability may be important in altered susceptibility to infectious diseases (Lazarus et al., 2002) and should be further explored in the context of HIV.

TLR4

Two non-synonymous SNPs in the LRR region have been extensively studied in TLR4, Asp299Gly and Thr399Ile. The A→G nucleotide change at +896 from the translational start site results in an aspartic acid to glycine amino acid change (Asp299Gly), while a C→T nucleotide substitution at +1196 nucleotides from the translational start site results in a threonine to isoleucine amino acid change (Thr399Ile). There is some discrepancy in the literature as to whether these SNPs are in complete LD (Michel et al., 2003; Montes et al., 2006) or whether they are often co-segregating (Arbour et al., 2000; Imahara et al., 2005).

The presence of the Asp299Gly allele seems to have a greater functional impact than that of Thr399Ile and has been associated with a blunted response to inhaled LPS (Arbour et al., 2000), increased susceptibility to infectious disease, including septic shock, bacterial infections and gram negative osteomyletis (Lorenz et al., 2002; Montes et al., 2006). The presence of these two SNPs has also been associated with increased susceptibility to RSV infection (Tal et al., 2004), brucellosis (Rezazadeh et al., 2005) and an increased risk of myocardial infarction (Edfeldt et al., 2004). As well, these SNPs have been associated with a predisposition to severe malaria in African children (Mockenhaupt et al., 2006). Decreased susceptibility to various infectious diseases and conditions has also been

associated with the presence of these SNPs, including carotid stenosis (Kiechl et al., 2002), Legionnaire's disease (Hawn et al., 2005) and acute allograft rejection (Palmer et al., 2003).

This evidence seems to support the hypothesis that the Asp299Gly and Thr399Ile alleles confer a reduced inflammatory response. Regardless of the mechanism, it is clear that these polymorphisms exhibit a functional role in the degree of susceptibility to various diseases and that TLR genetics may play an important role in the host immune response.

TLR2

Studies on polymorphisms in TLR2 have been less extensive then those on TLR4 and thus the full scope of their functional abilities remain unknown. There is, however, a microsatellite within the 5' untranslated region, 100bp upstream of the TLR2 translational start site in intron 2, which appears to be highly polymorphic. The length of this (GT)*n* (*n*=12 to 28) repeat differs between human populations and has an effect on promoter activity. The longest (28) and shortest alleles (12) have higher promoter activity after stimulation with IFNγ as compared to medium length alleles (20) as measured by luciferase activity in constructs of these lengths (Yim et al., 2004). The observation that TLR2 deficient mice are susceptible to *Mycobacterium* infection led to a study by the same group in which they showed that short length (13) alleles of this microsatellite are associated with lower expression of TLR2 and the development of tuberculosis in Koreans (Yim et al., 2006). As a result of the association between TLR

polymorphisms and altered susceptibility to infectious disease, a further association with HIV becomes a conceivable possibility.

HIV Resistant Women and their Responses to TLR Stimuli

Previous adaptive immunity studies conducted on the ML cohort, suggest that HIV resistant women have a Th1 biased immune response (Fowke et al., 2000). Due to its influence on adaptive immunity, we hypothesized that an altered innate immune response was the source of the altered adaptive responses. A study was thus conducted to measure innate immune responses in PBMCs from members of the ML cohort, under different innate stimulation conditions. With the above information, it was theorized that HIV resistant women would be better able to produce Th1 cytokines (IL-12 and IFNγ) in response to innate stimuli, thus more likely resulting in a primarily Th1 response. PBMCs from HIV resistant women, HIV negative women (NN) and low-risk controls (LR), were stimulated with TLR2, 4 and 7 ligands (peptidoglycan (PG), lipopolysaccharide (LPS) and an imiquimod analog, respectively) for 18 hours. Following stimulation, cell culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA) for cytokines important in the Th1/Th2 balance (IL-10, IL-12p40, IFNα and IFNγ).

Comparison of cytokine responses for each ligand between groups, revealed some interesting differences. The two most intriguing differences between the three groups were: 1) an increased IL-10 response to a TLR2 ligand (PG 0.004%) in HIV resistant

women compared to NN (p=0.0001) and LR (p= 0.006) and 2) a decreased IFNγ response to TLR4 stimulation (0.5μg/mL LPS) in HIV resistant women compared to NN (p=0.009) and LR (p=0.02) (Figure 8a and 8b).

Although the expected Th1 bias in cytokine responses was not apparent and elicitation of CMI responses in HIV resistant women appears to be more complicated, these altered responses to TLR stimulation in HIV resistant women suggest differences in their ability to direct adaptive immune responses. All of the subjects in this study were HIV negative, thus the differences in their first line of defense against invading pathogens suggests a genetic or inherent difference. The possibilities for differences in TLR signaling and instruction were the focus of this portion of the study.

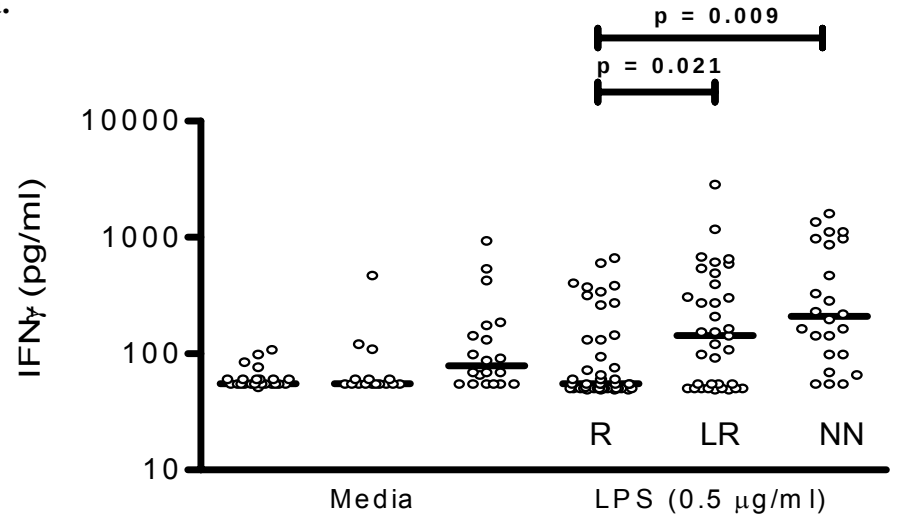
Hypothesis & Objectives

As IRF-1 alone did not clearly associate with HIV resistance, we hypothesized that HIV resistance might also be explained by other genetic polymorphisms. TLR polymorphisms are potential candidates as HIV resistant women have altered immune responses to TLR stimuli and these responses are genetically derived.

To investigate this hypothesis, three specific objectives were formulated:

1. Develop a novel, high-throughput genotyping system for the detection of TLR polymorphisms.

8a.



8b.

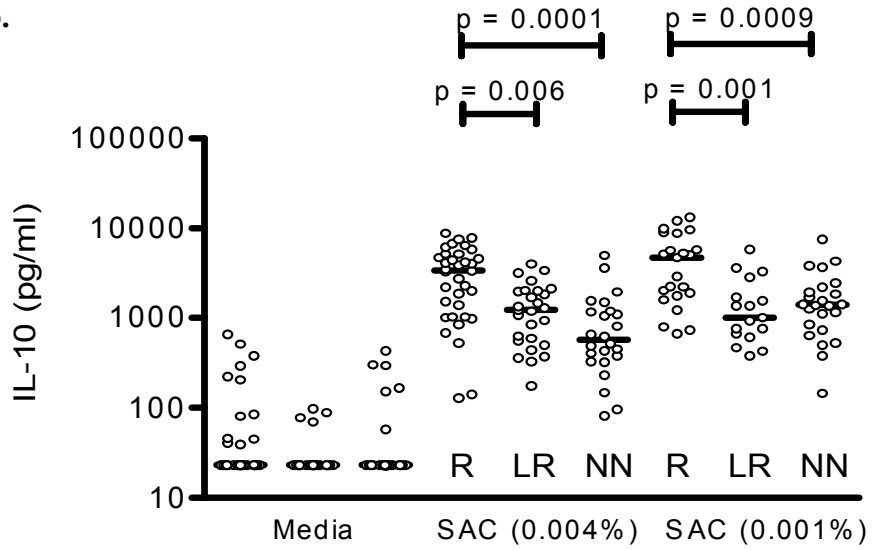


Figure 8a: Mean IFNγ production in response to the TLR4 ligand LPS
Figure 8b: Mean IL-10 production in response to two concentrations of the TLR2 ligand peptidoglycan

***R**=HIV resistant women, **LR**=HIV negative low risk controls, **NN**=HIV negative CSWs*

2. Genotype ML cohort members for both Asp299Gly and Thr399Ile TLR4 polymorphisms and correlate these genotypes with immune responses to TLR4 stimulus and HIV status.

3. Genotype members of the ML cohort for the TLR2 MS and correlate MS lengths with IFNγ production, immune response to TLR2 stimuli and HIV status.

Materials and Methods

Materials

Source of Biological Material

Female sex-workers (CSWs) who attended the Majengo clinic in the Pumwani area of Nairobi, Kenya were recruited to participate in these studies (ML cohort). Women from this clinic have been studied since 1985 (Kreiss et al., 1986; Simonsen et al., 1990) and are invited to return twice a year, when samples are obtained and health care provided. Informed consent was obtained from these women and ethics approval was granted by both the University of Manitoba and the University of Nairobi. All women were given a unique study number (ML number) that identifies them at future visits. The women were further classified into 3 categories for the purpose of these studies: 1) HIV resistant: women who continue to be active in sex work and are HIV negative by both serology and

PCR after 3 years of follow-up 2) HIV negative: women who are HIV negative, but fail to meet the definition of HIV resistance 3) HIV positive or susceptible: women who are HIV positive by serology and PCR.

DNA Extraction

DNA was extracted using a Qiagen DNA extraction kit (Qiagen, Inc., Mississauga, ON, Canada) from separated peripheral blood mononuclear cells (PBMCs) or directly from the whole blood of ML cohort members. PBMCs were obtained by extraction from whole blood using a Ficoll-Hypaque protocol. Samples were stored at -20ºC.

Commercial Reagents

1. 100mM dNTP set PCR Grade (Invitrogen, Burlington, ON, Canada)
2. Big Dye® 3.1 terminator V3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA)
3. DNA ladder, 100 bp (Invitrogen, Burlington, ON, Canada)
4. Ethidium Bromide (10 mg/ml) (Sigma-Aldrich, Oakville, ON, Canada)
5. GeneScan™-500 ROX™ size standard (Applied Biosystems, Foster City, CA, USA)
6. HinfI restriction enzyme (Invitrogen, Burlington, ON, Canada)
    a. React® 3 Buffer

7. LightCycler FastStart DNA MasterPLUS Hybprobe (Roche Mississauga, ON, Canada)
8. NcoI restriction enzyme (Invitrogen, Burlington, ON, Canada)
    b. REact® 2 Buffer
9. Starters Package Probes (Eurogentec, San Diego, CA, USA)
    c. 1x Lithos kit for plastic capillaries
    d. 1x Lithos plastic capillaries
    e. 1x Lithos rotor
10. Taq DNA polymerase recombinant (Invitrogen, Burlington, ON, Canada)
    f. 10x PCR Buffer (-Mg^{2+})
    g. 50mM $MgCl_2$
11. UltraPure™ Agarose (Invitrogen, Burlington, ON, Canada)

Laboratory Prepared Solutions

1. d.d. H_20
    - distilled and deionized water
2. 5mM dNTP mixture
    - 25ul 100mM dGTP
    - 25ul 100mM dCTP
    - 25ul 100mM dTTP
    - 25ul 100mM dATP
    - 1900ul d.d.H_20

3. 10x TBE buffer

    - 108 g Tris Base
    - 55 g Boric acid
    - 7.44 g EDTA
    - Add d.d.H_2O until volume is 1 liter.

4. TE buffer (pH 8.0)

    - 10 ml 10 mM Tris-Cl (pH 8.0)
    - 2 ml 1mM EDTA (pH 8.0)
    - 988 ml d.d. H_2O

5. 6× PCR Gel Loading buffer:

    - 0.25 bromophenol blue
    - 0.25 Xylene Cyanol
    - 30% Glycerol in H_2O

6. PCR 2x master mix

Conc. in Storage Buffer	Quantity	Conc. in 2x Mixture
1) 833 mM (or 1M) Tris-HCl (pH 9.0)	144 µl	120mM Tris-HCl (pH 9.0)
2) 50 mM $MgCl_2$	60 µl	3 mM $MgCl_2$
3) 1M $(NH_4)_2$ SO_4	30 µl	30 mM $(NH_4)_2$ SO_4
4) 1mM dNTP Mix	200 µl	200 µM dNTP Mix
5) 1% Gelatin	200 µl	0.2% Gelatin
6) d.d. H_20	276 µl	
Total volume	910 µl	

Methods

Hybridization Probe Assay

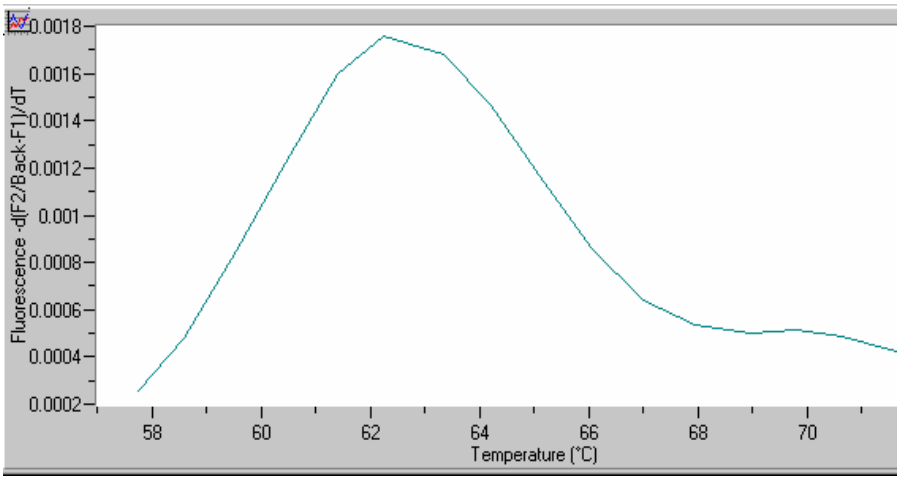
In an attempt to develop a novel typing system, specific probes and amplification primers (TIB molbiol, Adelphia, NJ, USA) were designed to detect two previously described polymorphisms in the Toll-like Receptor 4 gene (Asp299Gly (rs4986790) and Thr399Ile (rs4987233)). These polymorphisms are separated by 100 amino acids and each required their own specific set of amplification primers and fluorescent probes (Table 6). This method of Fluorescence Resonance Energy Transfer (FRET) requires that one probe be 3' labeled with a donor fluor (fluorescein) and the corresponding probe of the set be labeled with a 5' acceptor fluor (LC RED 640 or 705). FRET will only occur when these corresponding probes are in close proximity to one another.

Various conditions were utilized in an attempt to optimize this method in newly developed plastic capillaries, including altered $MgCl_2$ concentration, annealing temperature and numerous combinations of primer and probe concentrations. After numerous failed attempts to obtain fluorescence or DNA amplification, this method was abandoned in favor of the more standard glass lightcycler capillaries. This switch to the more cumbersome and traditional system resulted in the establishment of suitable reaction conditions (Figure 9a and 9b). The failure of the plastic capillaries was not restricted to our research group alone and the product was promptly withdrawn from the market by Eurogentec.

Table 6: PCR primers and fluorescent probes used in the detection of the TLR4 polymorphisms Asp299Gly and Thr399Ile

TLR4 antisense	5'-CCAAGAAGTTTGAACTCATGGTAA-3'
TLR4-sensor-1	5'-CTACTACCTCGATGATATTATTGACTTATT-3'-FL
TLR4-anchor-1	LC RED 640-5'-AATTGTTTGACAAATGTTTCTTCATTTTCC-3'
TLR4-sensor-2	LC RED 705-5'-ATTTTGGGACAACCAGCCTAAAGTAT-3'
TLR-anchor-2	5'-CTTGAGTTTCAAAGGTTGCTGTTCTCAAAGT-3'-FL

9a.



9b.

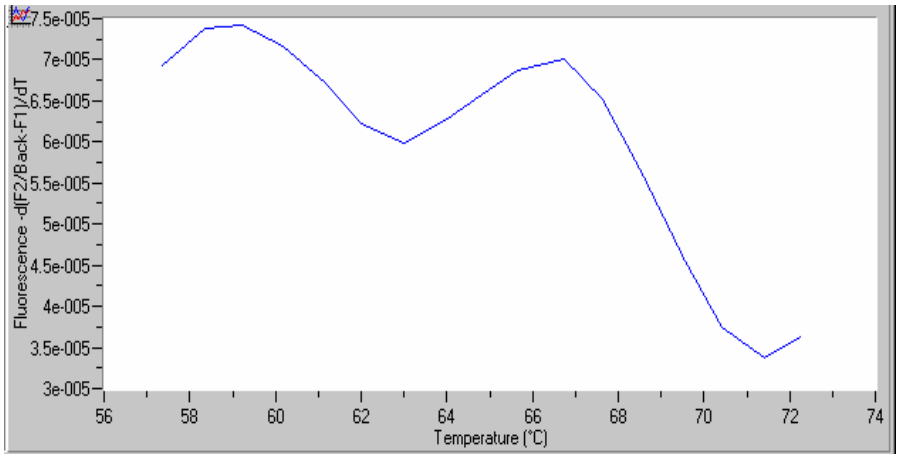


Figure 9a: FRET results on ML 313
Figure 9b: FRET results on ML 1694

Figure 9a: *The negative inverse of the measured fluorescence is used to determine T_m. In this case, one T_m at 62°C indicates that ML 313 is homozygous for the mutation with an Asp299Gly genotype of GG. The AA genotype would be indicated by a single higher T_m around 67°C.*

Figure 9b: *The negative inverse of the measured fluorescence is used to determine T_m. In this case, the presence of two distinct T_m (59°C and 67°C) indicates that ML 1694 is a heterozygote with an Asp299Gly genotype of AG.*

Both polymorphisms were detected simultaneously in a 20µl PCR reaction: 2µl LightCycler® FastStart DNA Master HybProbe Mix, 3mM $MgCl_2$, 6.6µl d.d. H_20, 0.4µM sensor-2 probe, 0.4µM anchor-1 probe, 0.2µM sensor-1 probe, 0.2µM anchor-2 probe, 0.5µM sense primer, 0.5µM antisense primer and 1µl sample DNA. The resulting mixture was then put into the LightCycler® 1.0 instrument (Roche Applied Science, Laval, Quebec, Canada) under the following conditions: 95°C for 10 min, 50 cycles of denaturation (95°C for 5 s, 20°C/s), 54°C for 20s, 72°C for 25s, melting curve analysis: 1 cycle at 95°C for 5s, 52°C for 40s, followed by an increase of temperature to 80°C slope of 0.2 °C/s. This slow increase in temperature allows melting temperature (T_m) determination, which is used to identify each sample's genotype. T_m is determined by monitoring fluorescence, which decreases as denaturation occurs, due to probe separation. This method is useful as the presence of a mutation decreases the T_m (and thus fluorescence) due to the reduced ability of the probes to anneal to template DNA.

Restriction Fragment Length Polymorphism

Due to the irreproducibility of the FRET method, another method was employed to detect the polymorphisms. Two previously described single nucleotide polymorphisms (Asp299Gly (rs4986790) and Thr399Ile (rs4987233)) in the Toll-like Receptor 4 gene (TLR4) were detected by restriction fragment length polymorphism (RFLP) after the induction of splice sites using mutagenic primers. The induced mutations allowed the distinction between wild-type and mutant alleles as the splice sites were only completed upon the presence of these previously described polymorphisms (Figure 10). The

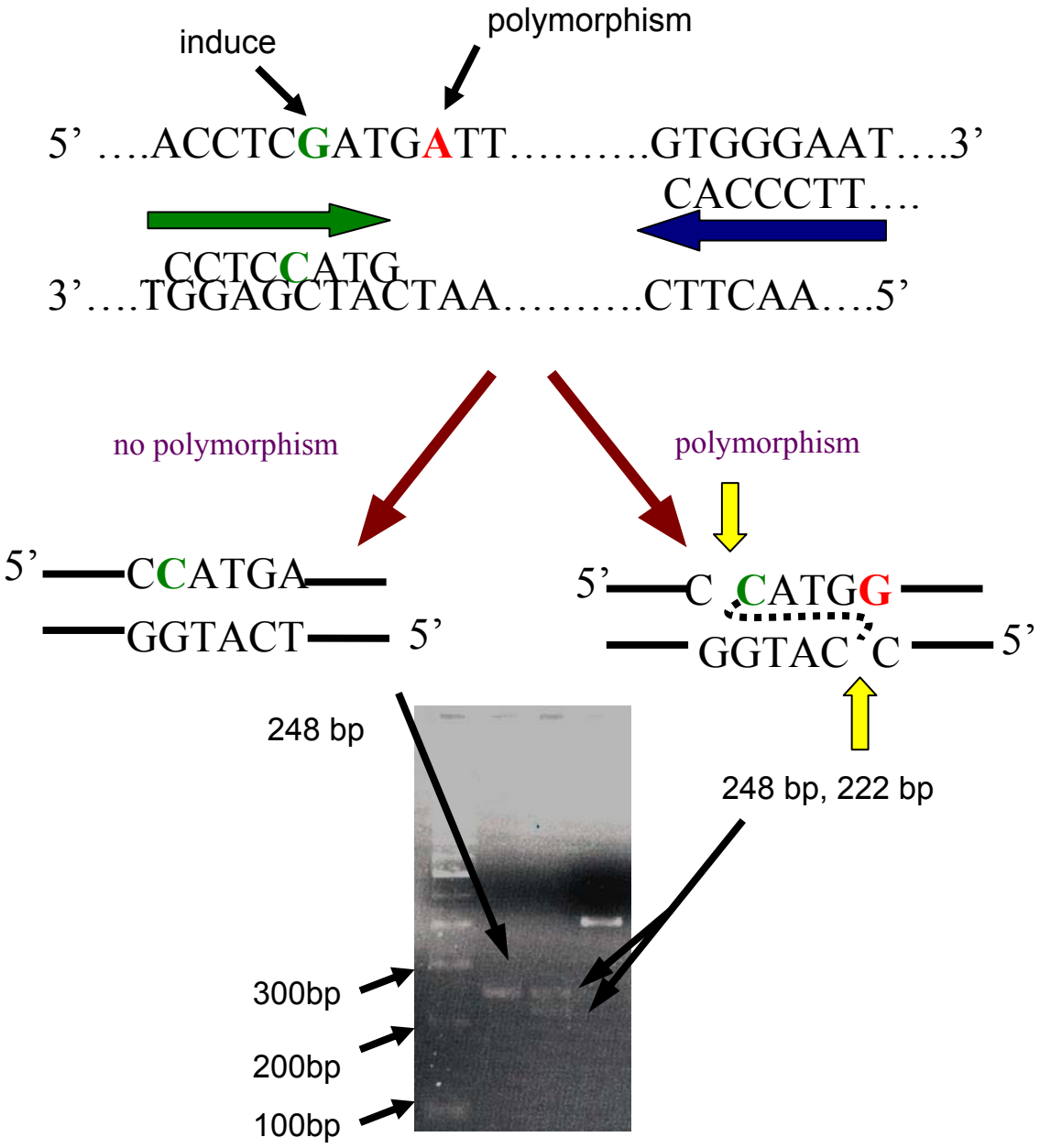


Figure 10: RFLP method employed for TLR4 polymorphism detection

specific primers used in these experiments are detailed in Table 7. PCR conditions for each of the 50µl reactions were as follows: 5µl 10x PCR buffer (-Mg^{2+}), 0.2mM dNTP mix, 1.5mM $MgCl_2$, 0.5µM forward mutagenic primer, 0.5µM reverse primer, 1.25U Taq polymerase, 34.25µl d.d. H_20 and 2µl of each DNA sample. PCR was performed in a thermocycler (MJ Research, Waltham, MA, USA), under the following conditions: 94ºC for 45s, 55ºC for 30s, 72ºC for 1 min 30s, go back to cycle 2, 34 times and 72ºC for 10 min.

Using the above PCR product, the restriction digest was set up as follows: 8µl PCR product, 1µl enzyme (NcoI or HinfI), 2µl REact® Buffer (REact® 2 for NcoI, REact® 3 for HinfI) and 9µl H_20. The digest was carried out at 37ºC for 1.5 hours to ensure complete cutting.

Twelve microliters of the digested product was then mixed with 2µl 6x PCR loading buffer and resolved on an EtBr stained 3% agarose gel in 1x TBE buffer. The gel was then exposed to UV light and the resulting genotype determined based on the visualization of banding patterns.

Two clear bands were present for heterozygote samples and a single band for homozygous samples. Genotyping was performed blind to the HIV status of the patients and a portion of samples were confirmed by gene sequencing. An example of a completed gel is shown in Figure 11.

Table 7: RFLP primers

TLR 4 Polymorphism	Custom Oligonucleotide Primers	Annealing Temperature (ºC)
Asp299Gly	(f) GATTAGCATACTTAGACTACTACCTC**C**ATG (r) GATCAACTTCTGAAAAAGCATTCCCAC	55ºC
Thr399Ile	(f)GGTTGCTGTTCTCAAAGTGATTTTGGGA**G**AA (r) ACCTGAAGACTGGAGAGTGAGTTAAATGCT	55ºC

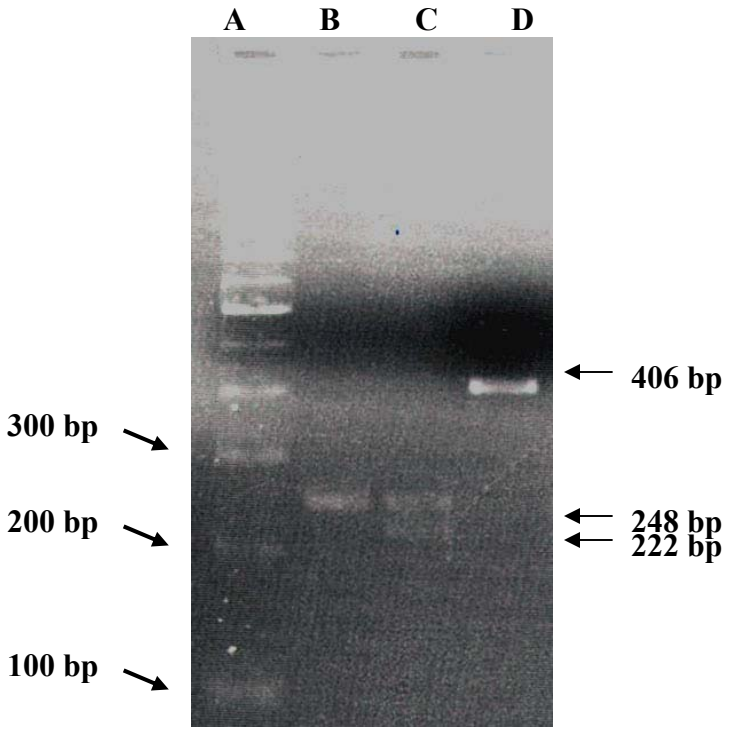


Figure 11: Genotyping of TLR4 Asp299Gly and Thr399Ile on an agarose gel

A. *DNA ladder,* ***B.*** *Homozygous Asp299Gly genotype,* ***C.*** *Heterozygous Asp299Gly genotype,* ***D.*** *Homozygous Thr399Ile genotype*

Sequencing

Sequencing was also performed on a portion of samples to confirm the results from the FRET protocol and RFLP. The primers used in both PCR reactions are outlined in Table 8. Initial PCR amplification was set up as follows: 19.75µl 2x PCR Master Mix, 2mM $MgCl_2$, 1.25U Taq polymerase, 0.5µM of each primer, 21.5µl d.d. H_20 and 1.5µl of DNA sample. This mixture was then amplified in a thermocycler (MJ Research, Waltham, MA, USA), under the following conditions: 94ºC for 3 min, 94º for 30s, 49º for 30s, 72º for 2 min, go back to step 2, 34 times and 72ºC for 10 min.

Following the initial amplification the DNA was transferred to a multiscreen plate for purification. Following the transfer, a vacuum was applied at 24″ Hg for 5 min. One hundred microliters of TE buffer was then added to each well and the vacuum once again applied for 5 min. The plate was blotted with paper towel before 50µl of d.d. H_20 was added to each well and the plate put on a shaker for 5 min (200rpm). The resulting purified DNA was then retrieved and transferred to a sterile storage plate.

Sequencing PCR was then prepared as follows for each sample: 2µl Big Dye®, 2.7µM primer (forward or reverse) and 2µl purified DNA. PCR conditions were carried out on a thermocycler as follows: 96ºC for 3 min, 96ºC for 30s, 53ºC for 30s, 60ºC for 4 min and go to step 2, repeat 79 times. The sample was then precipitated.

To each sample, 1µl of NaOAc and 20µl 95% ethanol was added and mixed with a

Table 8: TLR4 sequencing primers

TLR4 Polymorphism	Custom Oligonucleotide Primers	Annealing Temperature (ºC)
Initial PCR	(f) ATTTAAAGAAATTAGGCTTCATAAGCT (r) CCAAGAAGTTTGAACTCATGGTAA	49ºC
Sequencing PCR	(f) GCTGACTTTAAGAAATAATTTTG (r) GTTTGAACTCATGGTAATAAC	53ºC

vortex. The samples were then left in the dark for at least 2 hours (up to 24 hours) and then centrifuged for 1 hour at 3800 rpms. The plate was then inverted and given a quick spin to remove the NaOAc-ETOH cocktail. One hundred and fifty microliters of 70% ethanol was then added to each sample and centrifuged at 3800rpms for 10 minutes. The plate was then inverted to remove the ethanol and once again underwent a quick spin. The DNA was then heated for 1.5 minutes at 90ºC.

Each sample was then combined with 20µl formamide and heated at 90º for 1.5 minutes and immediately placed on ice. The samples were then transferred to an ABI Sequencing Plate (Applied Biosystems, Foster City, CA, USA) and resolved on an ABI 3100 Sequencer. The samples were subsequently analyzed with Sequencher software and compared to the published GenBank TLR4 (AF177765.1) sequence.

Microsatellite Typing

A previously described polymorphic GT repeat in the 5' untranslated region of Toll-like receptor 2 was detected by adapting previously established microsatellite detection techniques (Hughes, 1993). Approximately 150 base pairs surrounding the repeat region were amplified, using a specific primer set where the forward primer was fluorescently labeled with 6-FAM at the 5' end. The incorporation of fluorescence into the PCR product allowed for size discrimination. PCR conditions for each of the 50µl reactions were as follows: 5µl 10x PCR buffer (-Mg^{2+}), 0.2mM dNTP mix, 1.5mM $MgCl_2$, 0.5µM of each specific primer (TLR2-forward 6-FAM-GCATTGCTGAATGTATCAGGGA,

and TLR2-reverse CCTGAGAAATGTTTTCTAGGC), 1.25U Taq polymerase, 34.25µl d.d. H_20 and 2µl of each DNA sample. PCR was performed in a thermocycler (MJ Research, Waltham, MA, USA), under the following conditions: 94º for 45s, 55º for 30s, 72º for 1min 30s, go back to cycle 2, 34 times and 72º for 10 min.

Using the amplified PCR product, the following 13.5µl reaction was set up: 2µl undiluted PCR product, 11µl formamide and 0.5µl GeneScan™-500 ROX™ size standard, which was then loaded onto the ABI 3100 Sequencer (Applied Biosystems, Foster City, CA, USA). The size of the PCR products and thus the number of GT repeats was then determined using Genescan Analysis software.

Results

Assay Development

In order to develop a quick, reliable, high-throughput typing system, we attempted to use a real-time PCR system developed by Roche, the lightcycler. Initially, these lightcycler techniques were employed using the newly developed lithos kit. This kit contained plastic capillaries, as opposed to the more standard glass, and was developed by Eurogentec as a more "user-friendly" and durable option. Extensive optimization was carried out in an attempt to develop a reliable assay. However, various configurations in both product concentrations and experimental conditions failed to achieve fluorescence or product amplification. Amplification could, however, be achieved using similar

conditions in a regular thermocycler. This fact led us to abandon the plastic capillaries in favor of the traditional glass ones. After optimization with the new glass capillaries, proper amplification and fluorescence was obtained, allowing genotype resolution. The lithos kit was later removed from Eurogentec's product list due to unacceptable performance.

Assay Comparison

In order to test the validity of the Lightcycler dual probe hybridization technique, we compared the results with those of RFLP and sequencing techniques. We used several different primer sets in an attempt to sequence the area containing the two TLR4 polymorphisms. Despite this, sequencing of the Thr399Ile polymorphism still proved problematic. In an attempt to increase reproducibility and increase sample throughput, other viable methods were researched. RFLP analysis was subsequently employed and clear gene sequencing results were compared with their RFLP counterparts (Table 9). RFLP and sequencing results concurred on all samples, however, lightcycler results failed to coincide in 2/3 of the samples. Interestingly, neither RFLP genotyping nor sequencing identified any individuals heterozygous for the Thr399Ile polymorphism. It is not known whether this was due to problems such as enzyme activity, although literature would suggest that, due to its allele frequency and linkage with Asp299Gly, several heterozygous individuals should have been present. Due to this fact, all results were calculated using the Asp299Gly polymorphism and the Thr399Ile results were omitted from further analysis.

Table 9: Assay comparison of the three methods used to genotype the TLR4 Asp299Gly polymorphism *(discrepancies are highlighted)*

	Methods		
ML number	**lightcycler**	**sequencing**	**RFLP**
1500	AG	AG	AG
1694	AG	AG	AG
1250	AA	AA	AA
1598	AG	AA	AA
1837	AG	AA	AA
1378	AG	AA	AA
1337	AG	AA	AA
1804	GG	AG	AG
313	GG	--------	AG

Asp299Gly Allele is not Associated with HIV Resistance

RFLP analysis was performed on 269 DNA samples from members of the ML cohort to determine whether TLR4 polymorphisms are associated with HIV resistance. After genotyping was complete, Chi-square analysis was used to compare the presence of the Asp299Gly allele between HIV resistant and HIV susceptible subjects. Analysis comparing the frequency of the mutation between HIV resistant and HIV susceptible members found no association between HIV resistance and the presence of the polymorphism ($p=0.3718$, $X^2=0.7975$). The allele frequency in HIV resistant and HIV susceptible women was 3.3% and 5.7% respectively. This analysis was repeated with the non-HIV resistant women further subdivided into HIV positive and HIV negative ($p=0.3181$ and $p=0.7883$, respectively). This analysis is important as the HIV negative, non-resistant phenotype is an intermediate step. Individuals with this phenotype will eventually either seroconvert or meet the definition of HIV resistance.

Although the distribution of this SNP varies, statistical analysis showed no association between HIV resistance and the presence of one or more Asp299Gly alleles (Figure 12).

TLR4 Asp299Gly Polymorphism and IFNγ Production to LPS Stimulus

In an attempt to determine how the presence of the Asp299Gly polymorphism affects the ability of TLR4 to respond to LPS, previous IFNγ ELISA levels were compared to the

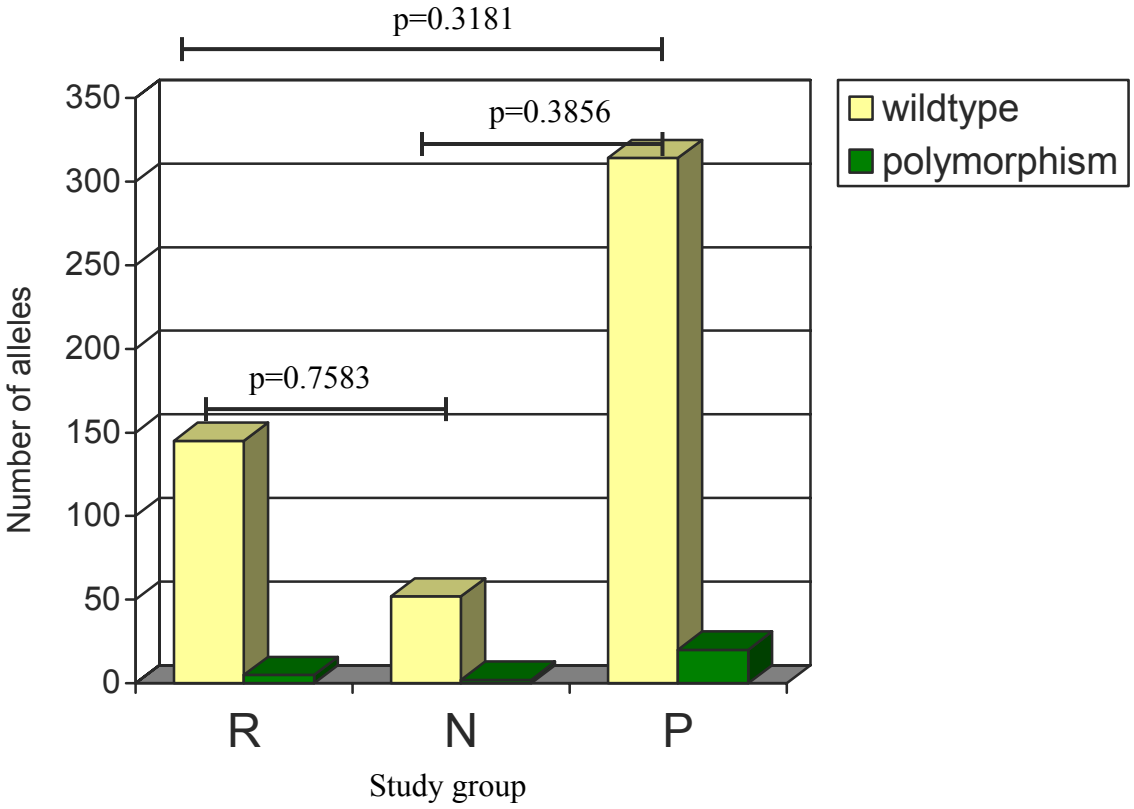


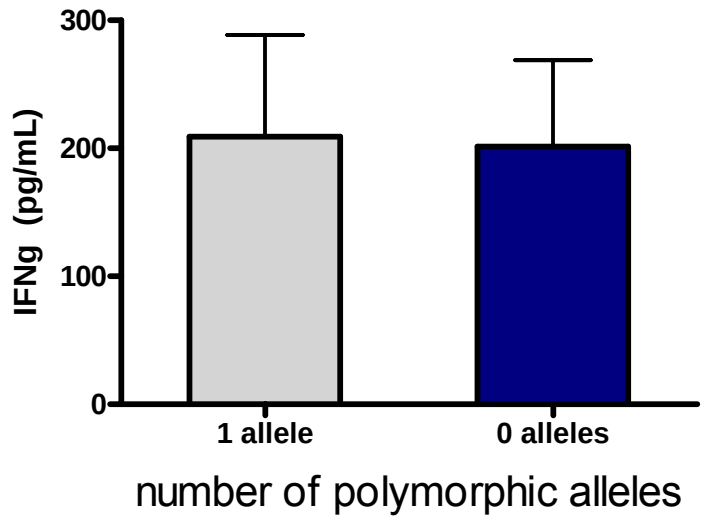
Figure 12: Number of Asp299Gly alleles in HIV resistant (R), HIV susceptible (S) and HIV positive (P) women

genotypes of the respective samples. Mean IFNγ production in response to LPS in HIV resistant women with the 299A polymorphism was compared to the mean production in HIV resistant women without the polymorphism, using an unpaired t test. There was no significant difference in IFNγ production between HIV resistant women who had the 299A allele and those who did not (p=0.7698). Although 85 HIV resistant women were genotyped for the TLR4 polymorphism, cytokine data was only available on 15 of them and more could not be tested due to lack of appropriate biological sample. This analysis was also done on all samples with available cytokine data, which included HIV resistant, HIV negative and HIV positive samples (p=0.9584) (Figure 13a and 13b). It is evident from this analysis that, although HIV resistant women have altered responses to TLR4 ligands, it is not due to the presence of the Asp299Gly polymorphism.

TLR2 Microsatellite Typing and Association with HIV Resistance

To determine if variants of a previously described TLR2 MS are associated with HIV resistance, microsatellite typing was performed on 158 members of the ML cohort. The MS lengths ranged from 10 repeats (129 bp fragment) to 24 (157 bp fragment), with three lengths (135,143 and 145) combining to represent over 66% of the total alleles (Figure 14). Chi-square analysis was conducted to compare the allelic frequencies between HIV resistant and susceptible women, but no significant difference was found in the overall distribution (p=0.47, X^2=12.67). As Chi-square is not appropriate for low numbers in a contingency table, alleles with a frequency less than 5% were combined and Chi-square analysis repeated (p=0.21, X^2=8.37). One allele, 151bp or 21 repeats, had a significantly

13a.



13b

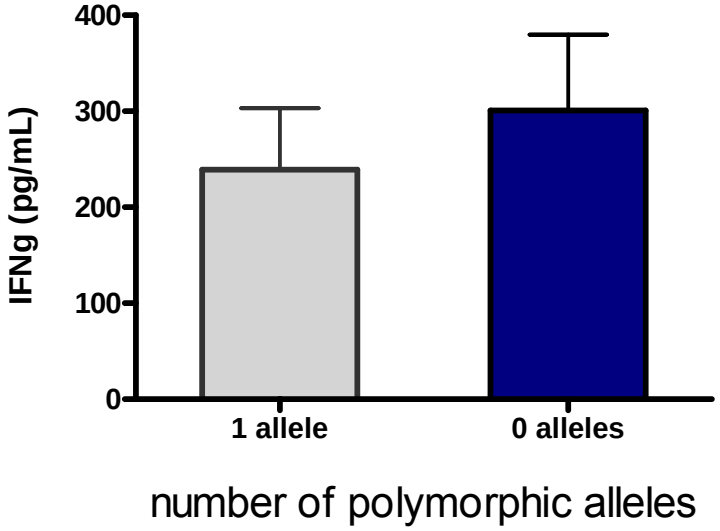


Figure 13a: Asp299Gly genotype and mean IFNγ production to LPS in HIV resistant women
Figure 13b: Asp299Gly genotype and mean IFNγ production to LPS in all individuals

Figure 14: TLR2 MS allele frequency in HIV resistant and HIV susceptible women

different distribution between HIV resistant women and non HIV resistant women ($p=0.047$, $X^2=3.95$), with only 1% of resistant women having the allele compared to 7.27% of susceptible women (Table 10). Taking into account the number of analyses performed, at a 95% confidence interval, this is most likely a statistical artifact and not a true significant result.

Literature suggests that longer and shorter lengths of the repeats have higher promoter activity to IFNγ stimulation then those of medium length (Yim et al., 2004). In this study, luciferase incorporated plasmid constructs of three MS lengths (12, 20 28 repeats) were used to assess their effect on TLR2 promoter activity. Promoter activity was indicated by luciferase activity, which was measured after stimulation with IFNγ for each construct. Due to the use of constructs in this study, only 3 MS lengths were tested. Comparing these results with our variable human population, was therefore difficult.

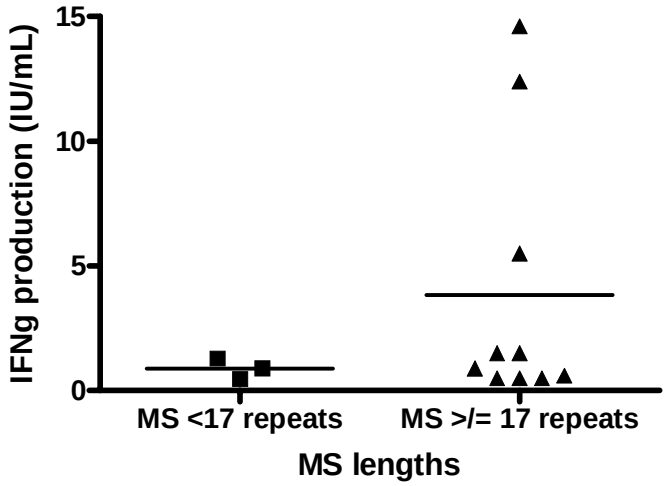
As a measure of TLR2 function, we compared peptidoglycan driven IFNγ and IL-10 production in patients with different TLR2 lengths. Adequate information was only available on 13 subjects, who were divided into the arbitrary categories of those with a MS allele length of less than 17 repeats and those with a MS allele length equal to or greater than 17 repeats. The mean IFNγ production was then compared between the two groups using an unpaired t test, resulting in no significant association between MS length and IFNγ production ($p=0.3706$) (Figure 15a). A similar analysis was also done for IL-10 production in response to peptidoglycan, but was hampered by the low number of subjects in each group. In an attempt to overcome this, Welch's correction was applied.

Table 10: TLR2 MS frequency distribution

		HIV status			
		susceptible		resistant	
# of repeats	allele	n	%	n	%
10	*129	8	3.64	2	2.08
11	131	14	6.36	4	4.17
12	*133	1	0.45	1	1.04
13	135	24	10.91	10	10.42
14	*137	0	0.00	0	0.00
15	*139	6	2.73	3	3.13
16	*141	3	1.36	3	3.13
17	143	30	13.64	20	20.83
18	145	90	40.91	37	38.54
19	147	15	6.82	9	9.38
20	*149	8	3.64	3	3.13
21	151	16	7.27	1	1.04
22	*153	3	1.36	1	1.04
23	*155	1	0.45	2	2.08
24	*157	1	0.45	0	0.00
	total	220		96	
	Chi-Sqr df=13,X^2=12.67, p=0.47				
	*Chi-Sqr df=6, X^2=8.37, p=0.21				

Table 10: *Allele frequencies of the TLR2 MS in HIV resistant and HIV susceptible women. Allele frequencies show distribution on either chromosome (2n), therefore the study number is the total/2. Chi-square was used to analyze the entire table and repeated after grouping less frequent alleles(*).*

15a.



15b.

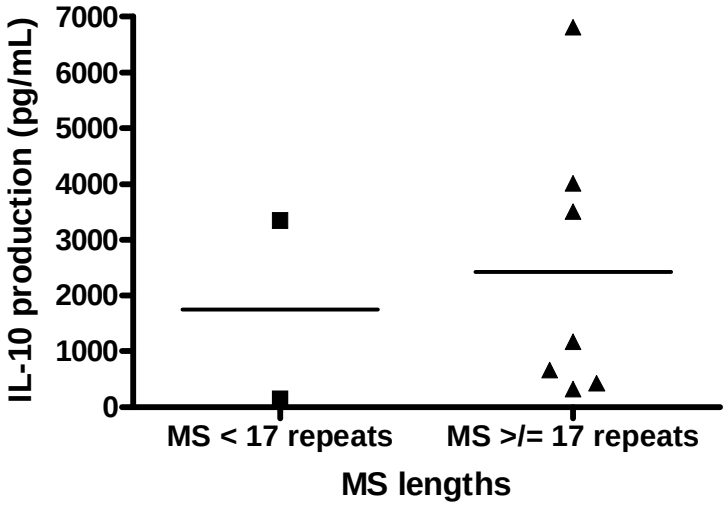


Figure 15a: TLR2 MS lengths and IFNγ production
Figure 15b: TLR2 MS lengths and IL-10 production

Figure 15a: *Mean IFNγ production after peptidoglycan stimulus in individuals with MS lengths less than 17 repeats and those with equal to or greater than 17 repeats.*
Figure15b: *Mean IL-10 production after peptidoglycan stimulus in individuals with MS lengths less than 17 repeats and those with equal to or greater than 17 repeats.*

This correction also failed to elicit a significant difference in IL-10 production between the two groups ($p=0.7777$) (Figure 15b). The analysis suggests that the TLR2 microsatellite is not the cause of the altered cytokine response to TLR ligands in this population.

Discussion

Within a cohort of commercial sex workers in Nairobi, Kenya, a small proportion of women appear to be resistant to infection by HIV-1. This epidemiologically defined definition of resistance gives hope to the existence of a natural protection mechanism against HIV. Research on this cohort has correlated resistance with a number of adaptive immune response elements, as well as genetic components. Ultimately, the reason why small groups of individuals are able to resist infection remains a critical question in HIV research.

Research has shown that host genetics not only play an important role in susceptibility to infections but also in the course of such infections. This is mirrored in HIV by various HLA genotypes and other genetic polymorphisms which have been associated with altered susceptibility. There has been a number of critical observations that led to the premise of the research contained within this thesis: many of the HIV resistant women within the Nairobi cohort are related to one another, HIV resistant women have altered adaptive immune responses and SNPs located within genes critical to adaptive immune responses appear to be associated with HIV resistance, and these women have altered

innate immune responses to certain stimulation. These observations led to the two hypotheses tested within this thesis. First, that polymorphisms associated with HIV resistance would also be present and associated with altered susceptibility to HIV in family members of HIV resistant women. Secondly, that mutations in critical innate immune genes are also a potential explanation for altered susceptibility to HIV through altered innate responses, and thus the generation of altered adaptive responses to HIV in these women.

Genetic studies on non-CSW relatives of HIV Resistant Women

To confirm a genetic association with HIV resistance, previously identified associations with genetic polymorphisms were sought out in non-CSW kindred of these women. As these kindred are a low risk population, the presence of these genotypes would provide further data that HIV resistance is genetically inherent as opposed to acquired through repeated exposure. Kindred of both HIV resistant and HIV susceptible women were therefore genotyped for a number of IRF-1 polymorphisms as well as a MS within the Th2/IL-4 gene cluster, previously associated with HIV resistance.

Total Allele Distribution of IRF-1 MS and D5S1984 MS is not Altered between HIV Resistant and HIV Susceptible Kin

The Kindred cohort was established in 1996 to fully investigate the suspected genetic component of HIV resistance. It consists of female and male relatives of both HIV

susceptible women and those women who meet our definition of HIV resistance. It was previously noticed that the kin of HIV resistant women were less likely to contract HIV than those related to HIV susceptible women (Ball, 2001). From this we theorized that genetic traits associated with HIV resistance would also be present in HIV resistant kin, and, in turn, associate with their HIV status.

Two microsatellites within the Th2/IL-4 gene cluster were investigated, D5S1984 and IRF-1. Once genotyped, allele frequencies for both microsatellites were compared between HIV resistant and HIV susceptible kindred (Table 3a and 3b). There appeared to be a significant difference in IRF-1 MS allele distribution between the two groups, however, when less frequent alleles were grouped together and Chi-square analysis repeated, the p value failed to remain significant. Chi-square analysis was also performed on the D5S1984 MS, showing no difference in the overall distribution of alleles between the two groups ($p=0.1211$).

No association was found between the distribution of IRF-1 and D5S1984 microsatellites and HIV resistant kindred as a whole, although this is not completely unexpected. A lack of association was also noted between the HIV resistant women and these microsatellites as a whole. The kindred also complicate this matter as they can not be labeled as HIV resistant due to lack of exposure and the association is based solely on whether they are related to an HIV resistant or HIV susceptible CSW. This finding neither negates nor proves an association between microsatellite alleles within the Th2 gene cluster and HIV

resistance, as an association was only previously noted between a specific MS length and HIV resistance, not MS distribution as a whole.

IRF-1 179 MS is Associated with HIV Resistant Kin

Due to its previously established association (Ball, 2001), further analysis was done on the distribution of the IRF-1 179 allele specifically. A comparison in allele frequency between HIV resistant kin (64/178 or 35.96%) and HIV susceptible kin (113/242 or 46.69%), showed a significantly higher proportion of HIV resistant kin possessing the 179 allele ($p=0.036$). This adds more weight to previous findings in which this particular allele may be associated with HIV resistance through some currently unknown mechanism. However, whether the increased 179 allele frequency in both HIV resistant women and HIV resistant kindred demonstrates altered susceptibility or simply relatedness is up for debate. IRF-1 179 is not likely the sole determinant of HIV resistance as that phenotype can not be determined in the Kindred cohort, suggesting other factors may be important. No other individual alleles were significantly differentially distributed between the two study groups.

As this microsatellite is contained within an intron, it is speculated that this is merely a marker for resistance and that the actual genotype responsible for, or involved in HIV resistance, is in LD with this MS. This particular allele length may affect the IRF-1 gene through a number of possible mechanisms. Intronic mutations can affect mRNA splicing, gene silencing or gene transcription/translation, which can affect protein function and

phenotypic changes, possibly leading to neuronal disease and cancers in humans (Li et al., 2004). Whether the association between HIV resistance is with the IRF-1 microsatellite itself or with another polymorphism in LD with the MS is yet to be discovered, although evidence would suggest the latter.

Neither D5S1984 MS nor IRF-1 MS is Associated with HIV Status in the Kindred Cohort

Previous analysis was carried out by separating the subjects based on whether they were related to an HIV resistant or HIV susceptible women. This analysis was carried out to assess the genetic correlates of HIV resistance within the group. Although this analysis is useful, it does not take into account the kindred's own HIV status. Due to this, subsequent analyses were conducted separating the kindred based on their own HIV status, resulting in two groups, HIV negative and HIV positive kindred. There is no HIV resistant group as non-CSW kindred are low risk and therefore do not meet our definition of resistance.

When allele frequencies were compared between the newly formulated study groups, there was no significant difference in distribution, suggesting no association between MS length and HIV status (Table 4a and 4b). Due to its previous variation, results were also compared for the IRF-1 179 allele alone. This comparison once again resulted in no significant associations signifying no association between IRF-1 179 and kindred HIV status.

Although there appears to be no association between these microsatellites and kindred HIV status, this does not contradict previous findings. As kindred can not be defined as resistant, it is hard to make correlations between resistance and polymorphisms within this study population. In addition, these samples are from a single time point in 1996 and therefore the HIV status of the individuals could have changed over the last decade. Additional studies on this cohort with more recent samples would provide additional and important information. Although this study yielded negative results and failed to show associations between HIV resistant kindred and the microsatellites as a whole, it was an important investigation into the genetics of HIV resistance.

IRF-1 179 MS Allele Instability Creates Uncertainty in HIV Resistance Association

Microsatellites are highly mutable and can expand or contract due to slippage during replication. Their abundance and high variability has led to their common use during genetic analyses (Ellegren, 2000). To use this dynamic marker in genetic studies, estimates of their mutation rates are usually required; therefore this type of analysis was attempted with the IRF-1 179 allele.

As the IRF-1 microsatellite occurs with an intron in the IRF-1 gene and therefore has no known functional role, it is most likely in linkage disequilibrium with another marker located at an alternate loci. By evaluating whether the kindred that possessed the 179 allele had relatives with that allele, we could examine both the stability of the

microsatellite and the likelihood of the kindred inheriting the identical allele and all loci in LD with the MS. Chi-square analysis was used to compare the HIV status of the 179+ kindred of 179+ HIV resistant women and the HIV status of 179+ kindred of 179- HIV resistant women (see Figure 2). If the kindred of the 179+ HIV resistant women were more likely to be HIV negative, it would suggest that the kindred most likely inherited the identical gene, and did not just have the 179+ allele due to MS mutation. Unfortunately, analysis did not show any difference between the two groups, and thus leaves one to believe that the IRF-1 MS is highly mutable and questions a genuine association between IRF-1 179 and HIV resistant kindred.

Making an association between microsatellites and any genetic condition is difficult without accurate information on the stability and mutation rate of the microsatellite. More participants in the kindred cohort, as well as more information on their relationships, would aid the investigation into the IRF-1 MS stability and thus give a more accurate view of any associations.

IRF-1 SNPs are Associated or Trending with HIV Resistant Kindred

Previous work had associated the 619A and 6516G alleles in IRF-1 with HIV resistance within the ML cohort. Chi-square analysis was carried out in an attempt to associate the same IRF-1 SNPs with the HIV resistant kindred. Kindred were once again separated into HIV resistant and HIV susceptible kindred and Chi-square analysis employed. This analysis failed to show an association for either SNP ($p=0.156$ and $p=0.229$ respectively).

However, concerns about this analysis were raised due to the composition of the Kindred cohort. Because Chi-square analysis works on the premise of observed and expected values, confounding factors that may affect expected values must be taken into account. The Kindred cohort is composed of varying degrees of relatives, and thus their genetic similarity to the respective ML cohort member varies depending on that degree. The degree of each relative, and thus genetic similarity was not taken into account in the original analysis and therefore Mantel-Haenszel Chi-square was used. This alternate statistical test takes into account the degree of relation of each kindred and thus accounts for the varied genetic similarity and expected values. When this test was employed, an association between HIV resistant kindred and the 6516G allele was shown ($p=0.042$). Although results for the 619 SNP were not statistically significant ($p=0.075$), the p value suggests a definite trend between 619A and HIV resistant kindred. Once again the elevated proportion of 619A alleles in HIV resistant kindred may be due to relatedness or due to an authentic association with HIV resistance. There was a small group of kindred, whose degree of relation was unknown. When these kindred were excluded, there was no longer a statistically significant association between HIV resistant kindred and either SNP, although this is most likely due to the decrease in sample size.

The association of the IRF SNPs with HIV resistance is an interesting and preliminary analysis. Additional work on the ML cohort has shown that the presence of the "protective" allele, (619A and 6516G), actually results in a lower basal expression of IRF-1 in PBMCs. This finding leads one to hypothesize that with a lower expression of IRF, the body may have a "window of opportunity" in which to control HIV infection

prior to initiation of replication by IRF-1. As studies of immunological parameters of HIV exposure have not been performed on members of the kindred cohort, it is harder to apply this hypothesis, yet it still serves as a plausible explanation for the decreased risk of HIV seroconversion. Further immunological and genetic studies on the kindred cohort are needed to further confirm these SNP associations.

IRF-1 SNPs are not Associated with HIV Status in the Kindred Cohort

As kindred can not be classified as resistant, as with the microsatellites, SNP analysis was repeated with kindred separated based on their own HIV status. When this analysis was done no association was found between HIV status and the presence of the "protective" 619A and 6516G alleles. Once again, as members of the kindred cohort can not be defined as resistant, any statistics are comparing HIV status and not resistance directly. A significant increase in sample size could overcome this lack of phenotype and provide more statistical power.

TLR Variants and Immunological Responses in HIV Resistant and HIV Susceptible CSWs

Previous work within the ML cohort has shown that HIV resistant women have altered cytokine responses to Toll-like receptor ligands. These altered responses include increased IL-10 response to TLR2 and a decreased IFNγ response to TLR4 when compared to new negatives and low risk control groups. This altered innate immune

response may help explain the altered adaptive immune response also noted within these women. It is hypothesized that the explanation for this altered TLR response is due to mutations within the receptors themselves. Two SNPs within TLR4, Asp299Gly and Thr399Ile, have been well described within the literature and result in amino acid changes in the cytoplasmic region of TLR4. These polymorphisms have been linked to an abundance of infectious diseases and may result in altered ligand binding. Specific lengths of a microsatellite within the 5'UTR region of TLR2 have also been associated with altered immune responses to exogenous stimuli and altered susceptibility to TB in Koreans. Genotyping of these polymorphisms was undertaken in the ML cohort in an attempt to elucidate the cause of the altered immune responses in HIV resistant women.

TLR4 Asp299Gly Polymorphism is not Associated with HIV Resistance

Genotyping was performed blind on members of the ML cohort by RFLP analysis. Sequencing was also performed on a portion of these samples to confirm the RFLP results. Both sequencing and RFLP failed to yield any heterozygotes for the Thr399Ile polymorphism so we excluded the Thr399Ile polymorphism from the study. Loci in linkage disequilibrium vary between populations and may explain the discordance found here.

ML cohort members were separated into those that met the definition of HIV resistance and those that did not. Chi-square analysis was then conducted to compare the polymorphism distribution between the two groups. This analysis failed to show an

association (p=0.3718), so the non-HIV resistant group was further divided into HIV negative and HIV positive and the analysis repeated (see Figure 9). This analysis again failed to show any correlation between the presence of the Asp299Gly polymorphism and HIV resistance.

No previous literature has shown a link between HIV and TLR polymorphisms, although associations with other infectious diseases have been noted, therefore such a hypothesis is not unwarranted. Discrepancies between our results and the literature regarding the linkage of the two polymorphisms prevent any assumptions of genotype regarding the Thr399Ile allele. However, evidence from the literature suggests that the Asp299Gly polymorphism has a greater functional impact and is actually the one responsible for the altered response to LPS (Arbour et al., 2000). The fact that there was no association with HIV resistance suggests that this gene may not be significantly involved in HIV susceptibility. In addition, the altered TLR responses in the HIV resistant women could be the result of altered TLR expression, as it is possible that the decreased response may be due to a lack of expression, rather than a default in the receptors themselves.

Asp299Gly Polymorphism is not Associated with Decreased IFNγ Production

As the investigation into TLR polymorphisms was established based on evidence of an altered innate immune response in HIV resistant women, it was necessary to attempt to correlate the two. Mean IFNγ production was calculated for both HIV resistant and HIV susceptible women, using the values measured in the prior innate studies. These values

were measured in pg/mL and obtained by ELISA following PBMC stimulation by the TLR4 ligand LPS. Initially, analysis was completed using only the HIV resistant group; where the mean IFNγ production for those who possess the polymorphic allele was compared to the mean production of those who do not. The results of this t test suggested no difference in IFNγ production between those who possessed the polymorphic allele and those who did not ($p=0.7698$). This analysis was also done on all samples with available cytokine data which included HIV resistant, HIV negative and HIV positive samples ($p=0.9584$). Although no correlation was discovered, this readout may not be dependent on the TLR2 SNP or it may not be functional in all populations. Furthermore, this analysis was conducted on a small number of the genotyped individuals due to a lack of cytokine data and more data could not be obtained due to a lack of appropriate biological samples.

A total of 44 SNPs have been identified within TLR4, therefore, a lack of association between 2 SNPs and HIV resistance does not completely eliminate the possibility of such an association. Complete gene sequencing could be carried out in an attempt to correlate the presence of any know SNPs within TLR4 and HIV resistance or more importantly, a correlation with IFNγ production. Furthermore, information on both cytokine production and genotypes on an adequate sample size would give more power to any observations that may be made.

Overall TLR2 MS Frequency is not Associated with HIV Resistance

Microsatellite typing was performed on the same sample set as the TLR4 polymorphisms. This was once again performed blind and HIV status revealed subsequent to the completion of genotyping. Individuals were separated into HIV resistant and HIV susceptible individuals and the microsatellite allele frequency compared between the two groups. When Chi-square analysis was completed on all alleles, no difference between the two groups was established ($p=0.47$). This analysis was repeated by grouping the less frequent alleles and reanalyzing the resulting contingency table. This once again however did not show a difference in allele distribution between HIV resistant and HIV susceptible women ($p=0.21$). The failure of the TLR2 polymorphism to associate with HIV resistance suggests that the polymorphism is not important to, or is not associated with TLR2 function. Once again altered TLR2 expression should also be evaluated in an attempt to elucidate the cause of the altered immune responses.

As research progresses into the mechanism by which this microsatellite causes altered susceptibility to TB and exogenous stimuli, more potential associations to HIV may be revealed. The relationship may also become complex through numerous co-infections of TB and HIV within patients.

TLR2 21 Repeat MS is Associated with HIV Susceptibility

Analysis was conducted individually for each microsatellite allele in an attempt to associate a specific allele with HIV resistance. Only one specific allele appeared to be differently distributed between the two study groups, allele 151, or 21 GT repeats. This

allele was present in only 1% of resistant women compared to 7.27% of susceptible women. This length of allele appears to be associated with a decreased probability of being HIV resistant.

Research on this particular microsatellite is in its infantry and thus not a lot is known about its functional implications. As with all microsatellites in non-coding regions, different alleles may have different downstream affects, such as splicing changes and may also be in LD with downstream loci. In addition, multiple statistical analyses with a 95% confidence interval leaves the possibility of an association due to chance rather than a genuine finding.

TLR2 MS Length is not Associated with IL-10 or IFNγ Production

As a measure of TLR2 function, we compared peptidoglycan driven production of IL-10 and IFNγ between various microsatellite lengths. Kindred were arbitrarily grouped into those with a MS allele of less than 17 repeats and those with a MS allele of 17 or greater repeats. Comparison of mean IFNγ production between these groups failed to yield an association with MS length. A similar comparison was conducted using mean IL-10 production, which also failed to show an association with MS length.

The altered cytokine response to TLR ligands within this population is not due to the microsatellite in TLR2. This microsatellite may not be involved in cytokine production through TLR2 or TLR2 function as a whole. Other polymorphisms within TLR2 and

TLR2 expression should be investigated in an attempt to elucidate the basis of this altered response.

Significance and future directions

The association between IRF-1 polymorphisms and HIV resistant kindred offers insight into possible protective immunological mechanisms into HIV resistance. IRF-1 is an important immunoregulatory gene and has an enormous impact on HIV replication. Additional research has shown that the 619A and 6516G alleles of these polymorphisms lead to a decreased basal expression of IRF-1, and perhaps provides a window in which the body can control HIV replication. The most important aspect of my contribution to these findings is the additional evidence of a genetic component to HIV resistance, rather than an acquired environmental factor. A longitudinal study of the members of the Kindred cohort would be an asset as HIV status is not static and may change the statistical analyses.

This research on TLRs has not shown any concrete associations with HIV resistance, but explored an important observation regarding these women. This research also attempts to bridge observations in both immunology and genetics, providing an explanation for correlations observed in both fields. Genomic scans and expression studies are needed to completely explain the association between TLR responses and HIV resistant women. Knowing that signaling through these TLRs varies does not shed light on the mechanism by which this occurs, and insufficient signaling through TLRs is not the only option.

Altered expression of specific TLRs, as well as expression variations on cell types are alternate explanations. In addition, an increase in sample size would provide the most power to any observations, and ensure that no associations are missed due to a lack of available subjects.

As not all resistant women have the "protective" alleles, and recent observations suggest an environmental impact on resistance, it is becoming clear that this is not an all or nothing phenomenon. Resistance is most likely multifactorial and has manifested itself as one, clearly evident phenotype. The amalgamation of many research projects will hopefully provide a blueprint for resistance and a natural model on which to base a desperately needed vaccine.

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Abbreviations used

AIDS	Acquired Immunodeficiency Syndrome
CSWs	Sex-Workers
d.d.H_20	Distilled, Deionized Water
DCs	Dendritic Cells
dNTPs	Deoxynucleotide Triphosphates
ELISA	Enzyme-Linked ImmunoSorbent Assay
EtBr	Ethidium Bromide
HIV resistant	HIV Resistant Women
HIV susceptible	HIV Susceptible Women
HIV	Human Immunodeficiency Virus
IRF-1	Interferon Regulatory Factor 1
IRF-3	Interferon Regulatory Factor 3
Kindred cohort	Family Members of ML Cohort
LD	Linkage Disequilibrium
LPS	Lipopolysaccharide
LRR	Leucine Rich Region (extracellular domain of TLRs)
LTNP	Long Term Non-Progressors
LTR	Long Terminal Repeat
ML cohort	Nairobi Sex-Worker Cohort
mM	Milimolar
MS	Microsatellite
MyD88	Myeloid Differentiation Factor 88
NFκB	Nuclear Factor kappa B
PAMP	Pathogen Associated Molecular Pattern
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PG	Peptidoglycan
PRR	Pattern Recognition Receptor
RFLP	Restriction Fragment Length Polymorphism
SNP	Single Nucleotide Polymorphism
Th1	T-helper 1 Immune Response
Th2	T-helper 2 Immune Response
TIR	Toll/IL-1R Region (cytoplasmic domain of TLRs)
TLR	Toll-like Receptor
TLR	Toll-like Receptor
µl	Microlitre
µM	Micromolar