A GENETIC BASIS FOR RESISTANCE

TO INFECTION BY HIV-1

BY

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A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

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Abstract:

The Human Immunodeficiency Virus 1(HIV-1) pandemic continues unabated despite increasing public health efforts and intense research. Considerable effort has gone into the development of HIV vaccines, and natural model of resistance to HIV-1 would be invaluable in this endeavor. Within a sex worker cohort in Nairobi, Kenya we have identified a group of women who are resistant to infection by HIV-1.

Resistance correlates with cellular and mucosal immune responses to HIV-1 suggesting that these woman have developed acquired immunity to HIV-1 thus providing a natural model of immunity. The question remains however as to why do these women appear to be able to mount an effective immune response to HIV-1 while the vast majority of individuals are unable to? It is our hypothesis that resistance to HIV-1 is mediated by genetic factors that are involved in the regulation of protective immune responses to HIV-1.

Using the tools of epidemiology, immunology, and genetics we provide data to show that there is a genetic basis for resistance to infection by HIV-1. We show that individuals related to a HIV resistant woman are less likely to become infected by the HIV-1 virus compared to individuals related to an HIV susceptible woman. This strongly suggests that there is a genetic component responsible for resistance to infection by HIV-1. To further investigate this finding we investigated polymorphisms in a number of genes involved in immune responses to HIV-1. These included microsatellite markers in a region important in regulating cellular and humoral immune responses. We identified an allele in the immunoregulatory molecule Interferon Regulatory Factor 1 (IRF-1) that was found at an increased frequency in HIV resistant women. This allele (IRF-1 179) was

associated with the resistance phenotype and was shown to protect against HIV infection. This is the first report of a polymorphism in a transcription factor that may account for differential susceptibility to HIV-1. We believe that individuals with the IRF-1 179 allele are better able to respond to HIV with what has been proposed to be protective cellular immune responses. This finding confirms our hypothesis that there are genetic factors responsible for resistance to infection by HIV-1.

Dedication:

More than any other, I thank my wife Monique for her love, support and understanding during the work on this project. She has given me the determination and confidence necessary to believe not only in this work, but also in myself. This has made me not only a better scientist, but also a better person.

Monique my love, I dedicate this thesis to you.

We Did It.

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Overview

The HIV pandemic continues unabated despite increasing public health efforts and intense research. Estimates by the World Health Organization suggest that over 33 million people are currently infected with HIV-1, with more than 15,000 new infections occurring per day (WHO, 1999). Despite advances in HIV research and therapy, the majority of those infected will develop AIDS (Advanced Immunodeficiency Syndrome) and die within 7-10 years (Hessol et al., 1994; Rutherford et al., 1990). This epidemic has put increased strain on health care systems in the developed world and is having a catastrophic effect on developing nations (Bloom, 1999). Over 90% of those infected by HIV-1 are from developing nations where access to quality health care and antiretroviral therapy is severely limited (Over, 1999). Some countries in sub-Saharan Africa are reporting HIV infection in up to 15-20% of the general population (Nicoll and Gill, 1999). Clearly, these countries will not be able to afford the current anti-retroviral therapies being developed and used in North America and Europe. In any event, since these therapies are not curative, they do not solve the problem. Prophylactic vaccines have proven to be the most effective control strategy for many infectious diseases.

Considerable effort has gone into the development of HIV vaccines, and a number of candidate vaccines are in various phases of development. Unfortunately, the likely efficacy of many of these vaccine candidates is not promising (Frey, 1999). A natural model of resistance to a pathogen is desirable in aiding the design and development of vaccines, and a natural model for resistance to HIV-1 would be invaluable in this

endeavor. A number of studies have reported groups of individuals who appear to be highly exposed to HIV-1 but do not become infected (Shearer and Clerici, 1996). Relative resistance to infection by HIV-1 in these individuals has been linked with primarily cellular immune responses against the virus (Haynes, Pantaleo and Fauci, 1996). This allows us to have a model of natural immunity to HIV-1, and an idea of what type of immune responses may protect against HIV-1 infection. However we do not have any idea about how and why these individuals are able to mount these immune responses. What is different about these individuals that allows them to resist infection by the virus, while the vast majority of individuals appear to be susceptible to infection? In this thesis we address the question of why some individuals are relatively resistant to HIV-1 infection by providing evidence for a familial, perhaps genetic basis for resistance to HIV-1 and investigate a number of genetic mechanisms that may be involved in conferring these protective immune responses.

Discovery of HIV-1 and its role in AIDS

The first reported cases of AIDS are generally considered to have occured in 1981 when a cluster of 5 otherwise healthy men with *Pneumocystis carinii* pneumonia (PCP) were identified by the Centers for Disease Control in (CDC, 1981b). By the following year numerous cases of PCP and other conditions associated with severe immunodeficiency were reported in otherwise healthy individuals (CDC, 1981a). AIDS, as this new condition was named, appeared to occur primarily in gay men, intravenous drug users, and hemophiliacs. However it became clear that AIDS was not restricted to these groups. Individuals who did not belong to these groups were identified with this

condition (Chamberland et al., 1984; Kreiss et al., 1985). Epidemiologic evidence suggested that an infectious agent was responsible for this disease, and that this agent was transmitted via blood, blood products or bodily fluids.

In 1983 a human retrovirus was isolated from the lymph node of a man with an AIDS related syndrome (Barre-Sinoussi et al., 1983). The isolated virus had characteristics similar to those described for the human T-cell leukemia virus (HTLV). A similar virus was concomitantly isolated from another individual with AIDS by a separate research group (Gallo et al., 1983). Further investigation revealed that these new viruses were not HTLV, but a similar virus with different *in vitro* growth characteristics. Initially called HTLV-III, LAV and ARV by different groups, this new virus was eventually identified as a new viral species, the Human Immunodeficiency Virus and classified into the lentivirus group (Coffin et al., 1986). HIV-1 has since been confirmed by numerous studies to be the etiologic agent for AIDS (O'Brien and Goedert, 1996). In 1986 a separate subtype, HIV-2 was identified in Western Africa (Clavel et al., 1986). Both subtypes can lead to AIDS although the pathology of the two viruses is quite different with HIV-2 having a longer and less severe pathogenic course (D'Aquila, 1996). There is now convincing evidence that both HIV-1 and HIV-2 have passed to humans by zoonosis from chimpanzees who harbor a related simian immunodeficiency virus (SIV) (Gao et al., 1999).

HIV-1 is transmitted horizontally through the exchange of blood, blood products, and other body fluids from infected individuals (Signorelli and Joseph, 1998). HIV can also be transmitted vertically from mother to child either *in utero*, during delivery, or through breast milk (Burns and Mofenson, 1999). Initially, the majority of HIV-1 transmission in North America was found to be through contaminated blood and blood

products, via exchange of contaminated needles by intravenous (IV) drug users, and through risky sexual practices. However, with the advent of blood screening protocols and safer sex practices in certain high-risk populations the focus of the epidemic has shifted. The majority of new infections occurring in both North America and the developing world appear to be transmitted primarily through heterosexual sex (Vernazza et al., 1999). Transmission associated with IV drug use still continues to be a concern primarily in Europe, North America and parts of Asia (Dehne et al., 1999; Schechter et al., 1999).

Heterosexual transmission of HIV-1 is the cause of over 90% of current HIV infections in adults (Bloom, 1998). A number of cofactors have been identified that influence an individuals susceptibility to HIV-1 including concurrent 'conventional' sexually transmitted infections (Simonsen et al., 1988), genital tract trauma (Cameron et al., 1989), contraceptive methods (Royce et al., 1997), and lack of circumcision in men (Moses, Nagelkerke and Blanchard, 1999). Transmission can also depend on the stage of disease in infected individuals (Simmonds, 1990), and the practice of risky sex habits (Ruiz, Facer and Sun, 1998). The exact role of many of these cofactors in HIV transmission is unclear, they may be mediated by inflammatory responses, damage to host epithelial surfaces and the recruitment of target cells, or by immune mechanisms responsible for upregulating viral expression (Simonsen et al., 1998). A number of host derived factors have been implicated in transmission and susceptibility to infection by HIV-1 and will be discussed in a later section.

HIV Structure and Replicative Cycle

HIV-1 belongs to the lentivirus subfamily of Retroviridae. Lentiviruses are characterized by causing disease with long incubation periods in a restricted host range. Lentivirus genomes have a typical retroviral genomic organization consisting of [*gag* (group specific antigen), *pol* (polymerase), and *env* (envelope) genes], with the addition of several regulatory and accessory proteins. HIV-1 has the typical retroviral structure with a cone shaped core formed of the p24 (*gag*) protein that encloses 2 positive sense RNA genomes of approximately 9.8 kb each, that are encapsidated by p7 and p9 nucleocapsid proteins (*gag*). The core also contains the *pol* gene products, the RNA dependent DNA polymerase, or reverse transcriptase (RT), integrase (IN) and protease (PR) molecules. The core is surrounded by another *gag* gene product, a myristoylated p17 protein that provides the matrix (MA) for the viral envelope. The envelope is host derived and contains a variety of host-derived proteins on its surface as well as approximately 72 knobs formed from trimers of the *env* glycoproteins gp41 and gp120. The transmembrane protein (gp41) is non-covalently linked to the cell attachment protein (gp120). The HIV-1 genome has the characteristic retrovirus structure of the *gag*, *pol* and *env* genes arranged in order 5' to 3', flanked by 2 long terminal repeat elements. HIV-1 also encodes the regulatory proteins Tat, Rev and Nef, as well as accessory proteins Vif, Vpr, Vpu, Vpx, and Tev (Levy, 1993; Wong-Staal, 1995).

HIV-1 replicates in CD4 positive cells, which are primarily lymphocytes, although CD4 positive macrophage and dendritic cells can also be infected (Gartner et al., 1986). HIV-1 utilizes CD4 as its primary cellular receptor, (Sattentau and Weiss, 1988) although alternative receptors have been identified, their use appears to be rare

(Bhat et al., 1991; Schneider-Schaulies et al., 1992). HIV-1 binds to CD4 via a high affinity interaction between CD4 and the fourth conserved region of the gp120 molecule near the carboxyl terminus (Deen et al., 1988). Recent data from crystallographic studies suggest that this interaction involves select amino acid side chains, with the majority of the contacts with CD4 involving interaction with the peptide backbone of the gp120 molecule (Kwong et al., 1998). This interaction is necessary but not sufficient for HIV-1 binding and attachment (Maddon et al., 1986). HIV-1 attachment and invasion also depends on interaction with co-receptor molecules. (Ashorn, Berger and Moss, 1990; Page, Landau and Littman, 1990) A number of seven transmembrane, G-protein coupled chemokine receptors (CCR2, CCR3, CXCR4, CCR5) have been identified as being capable of acting as a co-receptor for HIV-1 (Rowland-Jones, 1999). After binding to the CD4 molecule, gp120 is thought to undergo a conformational shift to allow for the interaction of regions underlying the V3 loop with chemokine receptors to facilitate entry of the cell (Kwong et al., 1998). The chemokine receptors most commonly utilized by HIV-1 are variably expressed on lymphocytes (CXCR4) and monocytes (CCR5) and are believed to account for the selection of cellular tropism of T cell tropic and macrophage tropic HIV-1 strains respectively (Berger, Murphy and Farber, 1999). The binding of gp120 with CD4 and the chemokine co-receptor molecule is believed to result in a further conformational change resulting in the exposure of a fusogenic site on gp41 (White, 1990). This site is responsible for mediating the fusion of viral and host membranes and the subsequent internalization of the viral core.

After the virus has gained entry to the cell the viral reverse transcriptase (RT) transcribes the viral RNA genome into a single strand cDNA. The RT has a relatively low fidelity due to lack of a proofreading function and has a misincorporation rate of roughly

1 nucleotide per 10^4 to 10^5 nucleotides transcribed (Coffin, 1990). This high mutation rate coupled with HIV's rapid replication account for the high degree of sequence diversity observed in this virus. After the reverse transcription step, the RT's RNase H activity digests RNA from the RNA/DNA hybrid, allowing the transcription of the complementary strand (Jacobo-Molina and Arnold, 1991). The double stranded DNA (dsDNA) is then translocated to the nucleus where the viral integrase inserts the dsDNA randomly into the host chromosome (Vaishnav and Wong-Staal, 1991). HIV-1 is now in its proviral form and can begin its replication upon host cell activation.

Gene expression by HIV-1 is a complex and tightly regulated process that involves both host and viral cofactors that may be expressed constituativly or upon activation by cellular or viral factors (Roebuck and Saifuddin, 1999). Generally, viral non-structural proteins with regulatory functions (vif, nef, tat) are expressed first from spliced messenger RNA transcripts. This is followed by the expression of structural proteins (*gag* and the *gag-pol* polyprotein) produced from unspliced viral transcripts. Finally, full length genomic mRNA is transcribed.

HIV-1 virions are assembled at the cell membrane. The *gag-pol* polyprotein containing the reverse transcriptase, protease and integrase enzymes associate with the cell membrane. During viral budding the *gag-pol* polyprotein undergoes autocatalysis to release the RT and IN gene products as well as the protease enzyme. The *gag* gene product also associates with the cell membrane and is proteolytically cleaved into its constitutive parts p17, p24, p7 and p9 by the protease gene during budding. The capsid protein (p9) specifically interacts with the 5' end of the RNA genome to incorporate the genome into the budding virion. The *env* gene encodes the gp120 and gp41 envelope glycoproteins, which are cleaved from a gp160 polyprotein in the golgi and assembled at

the cell membrane (Levy, 1993; Wong-Staal, 1995). All of these products self-assemble during budding to produce an intact HIV-1 virion capable of carrying on the infection cycle.

Dynamics of HIV-1 Replication and the Host Immune Response

The general course of HIV-1 infection in a human host can be roughly divided into three distinct phases, primary infection, so-called clinical latency, and end stage disease. The primary phase of viral infection is characterized by fever, headaches and lymphadenopathy. This lasts for approximately 6 weeks, during this period HIV-1 levels increase rapidly resulting in a heavy viremia, or viral load that peaks at 6 weeks (Pantaleo and Fauci, 1996). At this point both cellular and humoral immune responses are developing. It is believed that these immune responses are able to obtain some level of control over replicating virus at this point, as viral load declines to very low levels. This early drop in viral load is coincident with the appearance of HIV specific CTL. However, the appearance of HIV specific neutralizing antibodies is not detected until later in the course of infection (Walker et al., 1998). Viral load remains relatively low for a considerable time, averaging from 3 to 12 years depending on the clinical population in question (Anzala et al., 1995; Bacchetti and Moss, 1989) This period is classified as "clinical latency" and the infected host generally remains healthy. Evidence over the past few years has changed the concept of this clinical latency period. Originally, it was believed that HIV-1 replication was very low during this period. It has now been established that HIV replication during this time is intense with up to 10^9-10^{10} new virions being produced daily. The hosts CD4 positive T cells are also being replenished

rapidly having a turnover rate approaching 10^8-10^9 cells per day (Perelson et al., 1996). It appears that the high level of viral replication is being balanced by intense immune control. Eventually, the host is unable to maintain this immune control resulting in a gradual loss of CD4 lymphocytes and eventually severe immunodeficiency (Kitchen and Zack, 1998). The decline in CD4 positive lymphocytes results in the eventual loss of both cellular and humoral immune responses to HIV-1 as well as other pathogens. The mechanisms responsible for the loss of CD4 lymphocytes are still unclear. A number of models have been proposed to explain the loss of CD4 cells. These include: eventual immune exhaustion (the so-called tap and drain hypothesis) (Amadori, Zamarchi and Chieco-Bianchi, 1996), HIV induced apoptosis of CD4 cells (Goldberg and Stricker, 1999), and the potential toxic effects of certain HIV proteins such as tat (Magnuson et al., 1995).

The Host Immune Response to HIV-1

The function of the host immune response is due to two different and complementary mechanisms for recognizing an invading pathogen, the innate and the adaptive immune response. Innate responses depend on the discrimination of self and non-self by the recognition of specific molecular patterns like bacterial lipopolysaccharide, and viral double stranded RNA (Medzhitov and Janeway, 1997). Adaptive immune responses rely upon the clonal expansion of lymphocytes capable of recognizing and responding to specific amino acid peptide patterns or epitopes (Janeway, 1992). The two systems are tightly interconnected. Innate responses help control the induction of the adaptive immune response by regulating the expression of co-stimulatory

molecules on antigen presenting cells, and instructs the adaptive response to develop a particular effector response based upon the release of cytokines and other mediators (Fearon and Locksley, 1996). The effector responses generated can be broadly categorized into humoral responses, mediated primarily by antibody producing B cells and cellular responses mediated primarily by effector T cells. These effector responses are regulated by helper T cells that modulate these functions by the release of cytokines and other stimulatory molecules.

Humoral Immune Responses to HIV-1

The conventional humoral response of a host to viral infection is the production of antibodies to inactivate or neutralize the virus. HIV specific IgM and IgG can be detected in HIV-1 infected hosts 1-3 and 2-6 weeks post infection respectively (Tindall and Cooper, 1991). The HIV envelope proteins gp120 and gp41 appear to be the major target for this antibody response. Antibodies capable of neutralizing HIV-1 in *in vitro* infection assays have been mapped to gp120 and surface exposed regions of gp41 (Parren et al., 1997). Antibodies capable of recognizing other HIV-1 viral proteins have been identified, but their neutralization efficacy is unclear (Papsidero, Sheu and Ruscetti, 1989).

Most studies have identified 6 regions that could be involved in neutralization of HIV-1 infection, 5 in gp120, and one in gp41 (Levy, 1993). The region most recognized by neutralizing antibodies is believed to be the V3 loop at the amino terminus of gp120 (aa308-322) (Zwart et al., 1991). This region is responsible for the interaction with the chemokine co-receptor molecules. However, gp120/chemokine receptor interactions have been shown to involve cryptic sites underlying this region (Kwong et al., 1998). Thus

neutralizing antibodies that recognize this region must interfere with the conformational change necessary for revealing this site for binding to the chemokine receptor. The CD4 binding domain (aa413-447) has also been identified as a target for neutralizing antibodies, as have carbohydrate motifs on other parts of the gp120 molecule (Hioe et al., 1997). These antibodies appear to recognize both linear and conformational epitopes (Ho, Sarngadharan and Hirsch, 1987). Neutralization may be due to direct steric inhibition of HIV-1 binding and attachment to host cells, or cause conformational changes to gp120 that may interfere with binding and attachment.

Antibodies isolated from infected individuals have been shown (*in vitro*) to be capable of neutralizing laboratory-derived strains of HIV-1 (McKeating et al., 1989), and strains autologous to that host that had been isolated at earlier time points (von Gegerfelt, Albert and Morfeldt-Manson, 1991). However these antibodies were not capable of neutralizing autologous virus isolated at the same time as the neutralizing antibodies (Cheng-Mayer et al., 1988; Weiss et al., 1986). These findings suggest that *in vitro* neutralizing antibody responses are not effective in controlling HIV infection *in vivo*. This may be due to the inability of antibodies to effectively neutralize HIV-1 *in vivo*. Crystallography data suggests that due to gp120's extensive glycosylation, neutralizing antibodies may not be able to effectively target gp120 peptides (Kwong et al., 1998). Additionally, binding domains important for cell attachment and interaction with host molecules of the gp120 molecule appear to be cryptic, and become exposed only after conformational change (Rizzuto et al., 1998). These recognition sites appear to be able to undergo extensive amino acid variation (immune evasion) and still maintain binding ability (Wang et al., 1999). The high mutation rate of HIV-1's reverse transcriptase coupled with the high number of viral progeny produced per day allows for extensive

variability in the virus. This allows for the rapid evolution of virus capable of evading the host humoral immune response.

Non-neutralizing antibodies to HIV-1 have been identified as being important in controlling HIV infection (Wyatt et al., 1998). IgG specific for non-neutralizing epitopes on either gp41 or gp120 can be recognized by Fc receptors on natural killer (NK) cells or macrophage (Biron et al., 1999; Trinchieri, 1989). These cells are therefore capable of recognizing infected cells and lysing them via antibody dependent cellular cytotoxicity (ADCC) mechanisms (Skowron et al., 1997; Ullum et al., 1999). The role of ADCC in controlling HIV infection is still not known, although it has been shown to be important in other viral infections (Biron, 1997).

The clinical relevance of both neutralizing antibodies and ADCC mechanisms of suppression of viral replication are still unclear. Vaccines designed to elicit antibody responses to HIV-1 have been largely unsuccessful (Gotch, Hardy and Imami, 1999). No clear data exists to suggest that antibody responses to HIV-1 are associated with protection from infection or disease progression. Although humoral responses are likely to play some role in protective immune responses to HIV-1, these antibody responses by themselves do not appear to be sufficient. Like the immune response to most pathogens the immune response to HIV-1 likely needs to elicit both cellular and humoral responses.

Cellular Immune Responses to HIV-1

The outcome of many viral infections is immune containment of the infection due to the action of cellular immune responses mediated by CD8 positive, cytotoxic T lymphocytes (CTL). Numerous mouse and human studies have suggested that immune

control of viral infections by CTL are due to their capacity to lyse infected cells before the release of progeny virus (Zinkernagel and Althage, 1977). CTL recognize viral peptides produced by infected cells complexed with host major histocompatibility complex (MHC) class I molecules and β_2-microglobin, and specifically lyse target cells by the release of granzymes and perforins (Pamer and Cresswell, 1998).

CTL responses to HIV-1 were first described over 10 years ago (Plata et al., 1987; Walker et al., 1987). Specific CTL epitopes and their restricting human leukocyte antigen (HLA) alleles have been extensively characterized. This suggests that HIV-1 proteins contain a large variety of CTL targets, in structural as well as regulatory proteins (Haas et al., 1998; Harrer et al., 1998; Ikeda-Moore et al., 1997; Ogg et al., 1998a; Rowland-Jones et al., 1998). Unlike humoral immune responses, HIV-1 disease progression has been correlated with a loss of CTL activity (Brander and Walker, 1999). Compelling evidence for a protective role of CTL in HIV infection comes from quantitative assays indicating a strong association between CTL activity and HIV viral load (Ogg et al., 1998b). Further evidence has come from *in vivo* studies in which macaques were experimentally infected with SIV and in which CD8 positive cells (CTL) were depleted. With the loss of CTL came a drastic increase in SIV viral load, and as the CD8 cell numbers recovered, there was a concomitant decline in viral load that was shown to be mediated by virus specific CTL (Jin et al., 1999; Schmitz et al., 1999).

Recent evidence has suggested that the maintenance of effective CTL responses require virus specific T helper cells. Strong CTL activity in long-term non-progressors (LTNP, individuals who are infected with HIV-1 but do not progress to AIDS) was associated with the presence of strong virus specific T helper cell responses (Brander and Walker, 1999; Rosenberg et al., 1997).

Some have hypothesized that HIV specific CTL fail to control HIV replication for the same reason that humoral immune responses fail, the development of so-called immune escape mutants (Borrow et al., 1997; Goulder et al., 1997; Sewell et al., 1997). That is, viral strains that contain a mutation in a CTL targeted epitope due to viral sequence variation are able to "escape" immune surveillance by HIV specific CTL. Others disagree with the importance of CTL escape mutants as they suggest that the immune system fails to control HIV replication even in the absence of CTL escape mutants, (Brander et al., 1998; Safrit et al., 1994). Further evidence comes from a perinatal transmission study that showed that mothers who transmitted the virus to their children had a higher incidence of escape mutants than those who did not transmit the virus. However, the transmitted viruses were not the CTL escape mutants, but rather the CTL susceptible viruses (Wilson et al., 1999). This suggests that transmission of the virus was independent of the virus's evasion of the mother's CTL response.

An alternate hypothesis on why CTL responses are unable to effectively control HIV replication suggests that CTL responses are not fully effective without strong CD4 positive T cell help. Most laboratory tests for detection of CTL include at least one round of replication *in vitro* and require the addition of exogenous IL-2 or IL-7. This additional manipulation may conceal the unresponsiveness of CTL *in vivo* (Lalvani et al., 1997). This hypothesis is supported by studies of chronic viral infection in mice. High frequencies of viral specific CTL were found to persist *in vivo* with little or no antiviral effector functions (Brander et al., 1999). This unresponsiveness was more pronounced in CD4 deficient mice, highlighting the importance of both CD8 CTL and CD4 helper cells in the control of viral infection (Zajac et al., 1998). Further support that CTL alone are inefficient in the regulation of HIV-1 replication comes from adaptive transfer studies in

which autologous CTL clones are isolated from an infected patient, their numbers expanded *ex vivo*, and then transferred back into the patient. This led to a transient decrease in HIV infected CD4 cells, which rebounded rapidly even in the presence of high frequencies of CTL (Brodie et al., 1999). It was thought that the ineffectiveness of CTL in controlling HIV replication was due to the lack of HIV specific T cell help necessary to maintain functional CTL. Indeed high frequencies of HIV specific CTL clones have been identified for many years in infected adults without achieving immunologic control (Kalams et al., 1994; Moss et al., 1995). In several cases high CTL precursor levels were shown to be ineffective in control of viral infection, and this was found to be associated with deficiencies in CD4+ T helper cells (Brander et al., 1998).

Besides the MHC class I directed lytic activity, CD8+ T cells have been shown to repress HIV-1 replication without lysis of the infected cell. An as yet unidentified soluble factor from CD8+ T cells, cellular antiviral factor (CAF) has been suggested to be responsible for this suppression (Walker et al., 1986). Other factors besides CAF have been shown to inhibit HIV replication; these factors include IL-16 and certain chemokines (Maciaszek et al., 1997). The β-chemokines MIP-1α, MIP-1β and RANTES are all natural ligands for the CCR5 chemokine receptor and have been shown to inhibit cell infection by macrophage tropic HIV-1 strains (Cocchi et al., 1995). Increased β-chemokine expression has been associated with protection against infection in some human and animal studies (Gallo, Garzino-Demo and DeVico, 1999; Paxton et al., 1996). Studies suggest that both CD4+ T cells and CD8 cells can be sources for these inhibitory chemokines (Baggiolini, Dewald and Moser, 1997).

It is clear that cellular immune responses to HIV-1 are necessary for the control of HIV-1 replication. While humoral responses do appear to be important, perhaps even

necessary, their role in control of HIV-1 replication appears to be secondary to cellular responses. Regardless of the mechanism, HIV-1 is ultimately able to escape from immune control, either by the generation of immune escape mutants, or by the eventual loss of CD4 positive T helper cells. It is apparent that the loss of these responses result in increased HIV-1 replication and the eventual destruction of the host's immune system. It is likely that the regulation of cellular and humoral immune responses to HIV-1 is of utmost importance in HIV-1 pathogenesis.

Type-1 and Type-2 Immune Responses

CD4 positive lymphocytes can direct immune responses by the secretion of specific cytokines (Mosmann and Sad, 1996), and in some cases may act as effector cells themselves, however this appears to be relatively rare (Curiel et al., 1993; Orentas et al., 1990). Cytokines are an extremely important group of messenger molecules responsible for the regulation of the immune response, as well as having other biological effects. In mouse models it was observed that murine T cell clones could be divided into two groups based upon the cytokines that they produce. Th1 clones produced IFN-γ, IL-2, lymphotoxin and IL-3, while Th2 clones produced IL-4, IL-3, IL-5, IL-6, IL-10, and IL-13 (Mosmann et al., 1986). Generally Th1 cytokines were shown to enhance cellular immune responses, while Th2 cytokines enhanced humoral responses (Mosmann and Coffman, 1989). It was later demonstrated that human T cell clones could be similarly classified into Th1 and Th2 subsets (Peltz, 1991). Human T cells appeared to be much more complex and often a third subset of cells called Th0 that secreted both Th1 and Th2

cytokines could be identified (Romagnani, 1991). Although initial classification identified CD4+ T cells as the primary source of these cytokines, it became clear that a wide variety of cell types (monocytes, macrophage, NK cells and B cells) also contribute to the production of these cytokines (Mosmann and Sad, 1996). This eventually led to a new terminology termed type-1 and type-2 responses, that not only denote the type of cytokines being produced, but also the associated effector response (Abbas, Murphy and Sher, 1996; Bloom, Salgame and Diamond, 1992; Clerici and Shearer, 1994b).

Type-1 responses result primarily in cell mediated immune responses (DTH and CTL), while type-2 responses result primarily in antibody production. IL-4 and IL-12 in conjunction with other mediators, and through interaction with antigen presenting cells, appear to be the primary determinants in the type of T helper response (Fernandez-Botran et al., 1988; Reiser and Stadecker, 1996). Although not mutually exclusive, extensive cross-regulation results in reciprocal control such that the production of one type of response tends to suppress the other. For example, IL-4 (a type-2 cytokine) has been shown to inhibit the development of type-1 T helper cells, while increasing the development of type-2 cells (Seder et al., 1992; Swain et al., 1990).

Type-1 and type-2 responses appear to be more complex in humans and can serve as a useful model for T cell responsiveness, but may oversimplify the *in vivo* situation. Human T cells tend to exhibit a spectrum of cytokine secretion profiles with many cells having no clear Th1 or Th2 cytokine production patterns (Kelso, 1995). Thus cells that exhibit the prototypic type-1 and type-2 responses may represent the most extreme phenotypes in a broad set of T cell responses.

The type-1/type-2 model has become widely used by many investigators for description of a variety of biological systems, including resistance to infectious diseases,

and the effects of allergic responses. A predominant type-1 or type-2 response to a variety of infectious diseases has been reported (Lucey, Clerici and Shearer, 1996). In leishmaniasis a dominant type-1 or cellular response by C57BL/6 mice has been associated with the production of type-1 cytokines and protection from the disease. In contrast BALB/c mice are susceptible to disease and develop a primarily humoral response with the production of type-2 cytokines (Sadick et al., 1990; Scott, 1998). Polarization of the immune response has also been associated with resistance to Chlamydial infection as well (Yang, HayGlass and Brunham, 1996). In humans, the more severe lepromatous (disseminated) leprosy is associated with type-2 responses and cytokine production, while the less severe tuberculoid leprosy is associated with type-1 responses (Modlin and Rea, 1988; Sher, 1992). Resistance to schistosomiasis has also been identified as resulting from dichotomous type-1 and type-2 responses (Couissinier-Paris and Dessein, 1995). Further associations between disease state and dichotomous type-1/type-2 responses can be observed in human allergic diseases. Type-2 cytokine responses are associated with antigenic challenge by allergens in allergic individuals, while non-allergic individuals exhibit primarily type-1 responses to the same antigen (HayGlass, 1995; Imada et al., 1995).

Type-1/Type-2 Responses and HIV-1

It is well established that HIV disease progression is associated with increased B cell activity, increases in serum IgG levels (Clerici et al., 1993a; Pahwa et al., 1986) and decreased T cell proliferation, IL-2 production and CTL responses (Clerici, 1993). This has led to a hypothesis that a shift in type-1 to type-2 cytokine responses may play an

important role in disease progression (Clerici and Shearer, 1993; Clerici and Shearer, 1994b). Studies have suggested that as HIV disease progresses there is a gradual loss of response by helper T cells (measured by IL-2 and IFN-γ production) to recall antigens, mirrored by an increase in IL-4 production (Clerici et al., 1993a; Clerici et al., 1994b). These findings remain controversial. Critics disagree with some of the conclusions drawn due to the different stimuli used for the cytokine assays (IL-2 and IFN-γ responses were in response to recall antigens, while IL-4 responses were in response to polyclonal activators) (Romagnani et al., 1994; Romagnani, Maggi and Del Prete, 1994). Additional studies of cytokine profiles and HIV disease progression have failed to completely corroborate these findings (Graziosi et al., 1994). The consensus appears to be that as HIV disease progresses, type-1 cytokine levels do indeed decrease, but type-2 cytokine levels do as well, with possible increases in circulating Th0, or non-type-1/non-type-2 cells.

Further investigation regarding type-1 and type-2 cytokines have failed to entirely prove or disprove this hypothesis. This may be due to the lack of a standardized system to measure these responses. A variety of techniques have been used to measure cytokine production ranging from ELISA based systems (Clerici et al., 1993a), to cellular staining (Hagiwara et al., 1996), to measuring cytokine mRNA levels (Graziosi et al., 1994). The variety of cytokine assay systems used to measure cytokine production has been equally matched by the variety of experimental designs used to assay for T helper cell responses. Cytokine output has been studied from peripheral blood mononuclear cells (PBMC's) extracted directly *ex vivo*, or cultured for various periods of time (Fan, Bass and Fahey, 1993), from immortalized T cell clones (Maggi et al., 1994), and from plasma (Agarwal

and Marshall, 1998). Thus it has been difficult to come to a clear conclusion on HIV disease progression and the occurrence of a switch in cytokine production.

To add further complexity, there have been various reports that type-1 and type-2 cytokines can effect HIV replication rates within host cells (Fauci, 1993; Poli and Fauci, 1993). It has also been reported that HIV-1 preferentially infects and replicates in type-1 or type-2 cell lines (Maggi et al., 1994; Vyakarnam et al., 1995). The ability of cytokines to effect HIV replication, at least *in vitro* seems to be dependent on cell dependent effects of certain cytokines. For example IFN-γ down-regulates HIV replication in T cells and up-regulates its replication in monocytes and macrophage (Levy, 1993). It is still unclear whether cytokines effect HIV replication directly, or through an indirect manner. This may be due to their having an effect on cellular activation (Phair, 1999; Wells, Proudfoot and Power, 1999), or through the regulation of chemokine receptor expression (Gallo, Garzino-Demo and DeVico, 1999). The preferential replication of HIV in either Th1 or Th2 cells is though to be strain dependent. T cell tropic HIV strains appear to prefer Th2 cells rather than Th1 or Th0 cells (Wang et al., 1998), while macrophage tropic strains appear to replicate more efficiently in Th1 cells rather than Th2 or Th0 cells (Suzuki et al., 1999). It seems likely that this strain dependence on Th1 or Th2 cells is due to altered chemokine receptor expression on either T helper cell type. Recent evidence suggests that chemokine receptor expression may be dependent on the type of T helper cell being investigated. Th1 cells seem to be more likely to express CCR5, while Th2 cells are more likely to express CCR4 and CCR3 (Sallusto, Lanzavecchia and Mackay, 1998). It is still unclear however whether altered chemokine expression on Th1 or Th2 cell types is stable, or is suitable for use as a phenotypic marker for these cell types.

No clear consensus has been reached on the complex subject of a type-1 to type-2 switch in HIV disease. The switch of the immune system to a specific type of cytokine response will be the result of complex interactions between antigen presenting cells (APC's), T-helper cells, and effector cells such as CD8+ T cells and B cells. The emphasis for this model should be that as HIV disease progresses type-1 cytokines that *predominantly* direct the immune system towards protective cellular responses decrease. Type-2 cytokine levels on the other hand may increase, decrease or remain relatively stable. This will have less of an effect on HIV disease progression due to the apparent inefficiency of type-2 response in controlling HIV-1 replication. Humoral responses, mediated by type-2 cytokines do not appear to be protective and have not been associated with altered disease progression. Cellular responses mediated by type-1 responses appear to be able to provide some control of HIV-1 replication and loss of CTL has been strongly associated with increases in HIV-1 viral load, decline in CD4+ cells and other surrogate measures of disease progression.

The effect of various cytokines on HIV replication is still unclear and may act at many levels. Not only do they regulate the immune response to HIV-1; they may also be responsible for viral strain variation by the regulation of strain specific chemokine co-receptors. Thus associations of disease progression and type-2 responses, may not only be due to down-regulation of cellular responses, but may also be due to selection of viral strains that replicate and eliminate Th1 T helper cells.

Resistance to Infection by HIV-1

Over the past few years evidence has emerged that there is variability in susceptibility to HIV-1 and HIV disease progression. Individuals have been identified who appear to be able to resist infection, while others, once infected remain well for long periods of time, or progress rapidly to AIDS and death (Simonsen et al., 1998). Evidence for protection against HIV-1 infection has come from several sources. In Kenyan prostitutes the mean duration of prostitution was shorter in HIV infected women compared to non-infected women (Simonsen et al., 1990). In sex partners of HIV infected hemophiliacs the risk of infection was independent of sexual exposure (Ragni et al., 1989). HIV uninfected gay men and accidentally exposed health care workers have been identified with T cell responses to HIV-1 after several unprotected exposures (Barcellini et al., 1995; Beretta et al., 1996; Clerici et al., 1992; Clerici et al., 1994a; Clerici et al., 1993b; Paxton et al., 1996; Ranki et al., 1989). HIV specific CTL have been reported in uninfected children of infected mothers (Cheynier et al., 1992; Rowland-Jones et al., 1993) and uninfected sex partners of infected men and women (Pinto et al., 1995; Rowland-Jones et al., 1995).

This suggests that the immune system may be capable of eliminating infectious HIV-1 through protective immune responses in the absence of infection. Compelling evidence to support this has come from the identification of an HIV positive child infected *in utero*, who appeared to clear an active HIV-1 infection (Gompels, Spickett and Curtis, 1995). This observation remains quite controversial, and has been supported by some studies (Roques et al., 1995), but not by others (Bravo et al., 1996). These data are important and may provide evidence of clearance of HIV infection, but are limited in

that they do not provide convincing evidence of protection. Other than recipients of blood transfusions, most individuals do not become infected after one or a few exposures to the virus (Mastro and Kitayaporn, 1998). Only the Nairobi study and few others have demonstrated sufficient exposure to HIV-1 to suggest resistance to infection by HIV-1 (Fowke et al., 1996; Rowland-Jones et al., 1995).

With the recognition of the dichotomy of type-1 and type-2 T helper immune responses, the concept that cellular immune mechanisms may mediate protective immunity in the absence of systemic humoral responses has been forwarded (Salk et al., 1993). Evidence in support of this hypothesis has come from several sources. Animal models utilizing SIV have shown that low dose immunization with an SIV vaccine capable of eliciting CTL responses in the absence of humoral responses, protects against infection by further challenge (Clerici and Shearer, 1994a). Many of the exposed uninfected (EU) groups described above have evidence of CTL or T helper responses to HIV-1 in the absence of a humoral response (Barcelliniet al., 1995; Beretta et al., 1996; Clerici et al., 1992; Clerici et al., 1994a; Clerici et al., 1993b; Paxton et al., 1996; Ranki et al., 1989). Other cellular responses may also play a role in protective immunity to HIV-1. There is some evidence of suppression of HIV-1 replication by CAF and soluble antiviral factors produced by CD8+ cells and other cell types in some EU individuals (Ferbas, 1998). On the basis of this data it has been hypothesized that cellular immunity to HIV-1 rather than humoral immunity is important in protection against infection. This hypothesis is supported by some studies of Hepatitis C in which possibly protective cellular immune responses have been identified in the absence of humoral responses (Bronowicki et al., 1997).

A surprising finding in some EU women was that they appeared to have HIV-1 specific vaginal and cervical IgA antibodies (Mazzoli et al., 1997). These EU women do not have HIV-1 specific IgM and IgG responses, but have a localized mucosal antibody response that utilizes neutralizing antibodies with better neutralization activity than antibodies isolated from serum or plasma (Mazzoli et al., 1999). A type-1 cytokine profile is necessary for the induction of cellular immune responses, but it is unknown if these cytokines stimulate, or inhibit a localized, perhaps compartmentalized mucosal antibody response.

Considerable data has been gathered suggesting that a subset of women from the Nairobi sex worker (SW) cohort from the Pumwani district in Nairobi, Kenya have acquired immunity to HIV-1. These women are under extreme infection pressure (an estimated 60 or more unprotected exposures to HIV-1 per year) and are relatively resistant to infection by HIV-1 (Fowke et al., 1996). Both MHC class I and class II alleles have been identified that are associated with resistance to infection by HIV-1. The HLA class A2/6802 super allelic group has been associated with resistance (Incidence rate ratio (IRR)= 0.59, CI95% 0.39-0.87,$p<0.009$) as has the class II allele DR*0102 (IRR=0.21, CI95% 0.04-0.64, $p<0.001$) (MacDonald et al., 2000). These associations are consistent with MHC restriction of CTL (class I) and T helper (class II) responses to HIV-1. Resistant women have evidence of T helper cell responses to HIV-1 envelope peptides (Fowke et al., 2000). These women also have evidence of CTL responses to HIV-1 both measured by lysis of autologous B cell lines infected with vaccinia-HIV constructs, and by peptide based ELISPOT assays (Fowke et al., 2000; Rowland-Jones et al., 1998). As has been reported for other EU groups, they also have evidence of HIV specific IgA in their genital tracts (Kaul et al., 1999). Recent data from this group suggest that these

women have a global deficit in antigen driven IL-4 production (Trivedi et al., 2000). In light of the above findings of cellular responses to HIV-1 in the absence of serum humoral responses, we believe that these women have a measure of acquired immunity to HIV-1 mediated by strong type-1 responses to the virus.

Immunogenetics and Resistance to Infectious Diseases

In the majority of infectious diseases only a proportion of people exposed to particular pathogens become infected and go on to develop clinically significant diseases. There is evidence that host genetics are important in both innate resistance and acquired immunity to infection (Hill, 1998; Newport, 1997). The most exciting observations have been made in the study of immune mediated mechanisms of protection that support the development of acquired immunity to a pathogen. It has been observed that there appears to be familial clustering of many infectious diseases independent of exposure to the causative pathogen.

An example of the importance of the host's genetic component in infectious diseases has come from twin and adoptee studies. One of the seminal observations comes from a study of premature death due to infectious disease. It was observed that adoptees who had a biologic parent die from an infection before the age of 50 had a 5-6 fold increased risk of also dying of an infectious diseases before the age of 50. This was in contrast to the risk of death if the adoptive parent died of an infection before 50. No such increased risk was observed in the adopted children (Sorensen et al., 1988). Further evidence of the importance of the host genetic contribution is found from the study of twins and tuberculosis (TB). Monozygotic twins were found to be more likely concordant

for the disease than dizygotic twins (Comstock, 1978). Similar results were obtained from the study of Hepatitis B and *Heliobacter pylori*, in which monozygotic twins were again more likely to be concordant for disease state than dizygotic twins (Lin et al., 1989). Evidence that genetic factors effect the pathogenesis of a disease come from twin studies on malaria in The Gambia. Although there was no detectable effect on the risk of infection, there was significant concordance rates on the amount of fever caused by malaria in monozygotic twins, suggesting that genetic factors were influencing the immune response to the disease (Jepson et al., 1995). Further studies by this group showed a genetic basis for both humoral and cellular immune responses to malarial antigens (Jepson et al., 1997).

In addition to twin and adoptee studies there are considerable data on the role of specific host genes on the susceptibility to infectious diseases (Hill, 1998). The majority of these studies have examined the role of genetic factors on immune mechanisms in infectious diseases including studies on HLA and cytokine and chemokine genes. Interpretation of the literature is complicated by the differences between the populations studied in these analyses. Further difficulties arise from the complexities involved in analyzing polygenic processes such as the immune response, that potentially involve interactions with hundreds of genes. These studies tend to only identify genetic differences or polymorphisms that have large effects.

Certain HLA alleles have been associated with altered susceptibility to many infectious diseases including malaria, tuberculosis, leprosy, Hepatitis B and C persistence, and severity of disease caused by Chlamydia (Hill, 1997; Peano et al., 1994; Singh et al., 1983; Thursz et al., 1995; van Eden et al., 1980). Most assume that these associations result from the ability, or inability of certain HLA molecules to better

present immunogenic peptides to the host immune response. Evidence to support this hypothesis has come from many sources, including studies of malaria (Gilbert et al., 1998). It was shown that the differences in the ability of CTL clones to recognize malarial epitopes depended upon the presence of protective HLA alleles (Aidoo et al., 2000).

Polymorphisms in cytokine and chemokine genes have also been associated with resistance to many infections and altered disease progression in others. The driving force behind these investigations has come from studies on infections in gene knockout mice. Alterations in the IFN-γ receptor have been associated with an increased risk of mycobacterial infection (Newport et al., 1996). Studies of polymorphisms in the TNF-α gene found associations with an increased risk of cerebral malaria (McGuire et al., 1994), an increased risk of mucocutanious leishmaniasis (Cabrera et al., 1995), and increased risk of lepromatous leprosy (Roy et al., 1997). These were all associated with a point mutation in the TNF-α gene promoter that appears to result in increased transcription (Wilson et al., 1997). This polymorphism has also been associated with Hepatitis B persistence (Thursz and Thomas, 1997), and increased mortality from meningitis (Nadel et al., 1996) and asthma (Moffatt and Cookson, 1997).

Polymorphisms in other immunomodulatory genes have also been associated with altered susceptibility to various infectious diseases. Polymorphisms in the mannose binding lectin, an important molecule in innate immune responses, has been associated with some immunodeficiencies, and some infections (Mead et al., 1997). Polymorphisms in the receptor for Vitamin D, an important immunoregulatory hormone has been associated with resistance to tuberculosis and Hepatitis B (Bellamy and Hill, 1998) and

altered pathogenesis of leprosy (Roy et al., 1999). Several mutations have been identified in the human natural resistance associated macrophage protein (NRAMP) (Blackwell et al., 1995), a protein involved in macrophage activation (Govoni and Gros, 1998). In inbred mouse strains NRAMP-1 deficient mutants were susceptible to a wide variety of pathogens including Leishmania, Salmonella, and Mycobacterial infection (McLeod et al., 1995). In humans, mutations or polymorphisms in the human analog of this gene have been associated with altered pathogenesis of both tuberculosis and leprosy (Bellamy, 1999).

Interestingly, resistance to infection by Leishmania in mice also depends on the immunologic background of the mouse strain infected. As described, C57BL/6 mice raise a predominantly cellular (type-1) response and are protected from infection, while BALB/c mice are susceptible to disease and their response is primarily humoral (type-2) (Sadick et al., 1990; Scott, 1998). Because disease pathogenesis in humans is often related to the type of T cell response, it is likely that genetic factors that control this response will be important in human disease susceptibility (Lucey, Clerici and Shearer, 1996). Recently studies with schistosomiasis have shown that type-2 responses mediated by IL-4 and IL-5 play a role in protection against disease (Couissinier-Paris and Dessein, 1995). Segregation analysis and genetic studies have identified a single locus in the IL-4 gene cluster in chromosome 5 that appears to be at least in part responsible for protective immunity to this pathogen (Abel et al., 1991; Marquet et al., 1996). It is not unreasonable to assume that other diseases such as HIV-1 that appear to have differential pathogenesis based upon type-1 and type-2 responses may also be regulated by genetic factors. Pathogenesis of asthma and allergy has also been linked to differential type-1 and type-2

immune responses, and studies have identified the IL-4 cluster as being important in genetically mediated susceptibility to asthma and allergy (Marsh et al., 1994).

Genetic Mechanisms of Resistance to HIV-1

In the past several years considerable research has been conducted on the role of host genetic mechanisms and both resistance to HIV-1 infection and altered disease progression. Shortly after the identification of CCR5 and CXCR4 as the primary co-receptor molecules for HIV-1, three independent groups identified a mutant allele that contained a 32 base pair deletion in the open reading frame of CCR5 (Δ32 CCR5). This mutation resulted in the production of a truncated, non-functional CCR5 molecule (Dean et al., 1996; Deng et al., 1996; Samson et al., 1996). This polymorphism was associated with resistance to HIV-1 in individuals homozygous for the mutation (Paxton et al., 1996), although this resistance does not appear to be absolute (Biti et al., 1997; O'Brien et al., 1997). The role of this polymorphism in heterozygous individuals is unclear; some reports have identified a protective effect (Zimmerman et al., 1997), while others have been unable to replicate these results (Huang et al., 1996). The Δ32 CCR5 polymorphism is rare in Africans, Asians and persons of Latin American descent, but is fairly common in those individuals of European descent (Samson et al., 1996). This polymorphism has also been associated with decreased disease progression in a wide variety of studies, although the protective effect varies depending upon the group being studied (Eugen-Olsen et al., 1997; Meyer et al., 1997).

Additional polymorphisms in chemokine receptor genes have also been identified as having a role in disease progression. A conservative mutation resulting in the

substitution of a valine for isoleucine in the minor chemokine receptor CCR2b has been associated with decreased disease progression in some (Anzala et al., 1998; Kostrikis et al., 1998; Smith et al., 1997), but not all populations (Michael et al., 1997). Recently several independent groups have identified a number (at least 5 so far) of single nucleotide polymorphisms in the 5′ region of the CCR5 molecule (Easterbrook et al., 1999; Mummidi et al., 1998). These polymorphisms are in the promoter region of CCR5 and some appear to have an effect on CCR5 transcription (Alkhatib et al., 1997). Reports have suggested that the CCR2 polymorphism is in linkage disequilibrium with some of these promoter polymorphisms, and that the CCR5 promoter polymorphisms represent functional mutations (Kostrikis et al., 1998). Other groups argue that these protective effects are independent of each other (Easterbrook et al., 1999). A polymorphism in the 3′ untranslated region of the SDF-1 molecule has also been associated with decreased disease progression (Su et al., 1999; Winkler et al., 1998), but again other reports have failed to confirm this finding (Meyer et al., 1999). SDF-1 is the natural ligand for CXCR4 and the particular polymorphism is believed to result in increased mRNA stability, and presumably increased translation of the SDF-1 gene product, possibly inhibiting CXCR4 utilizing HIV strains (T cell tropic) replication (Marechal et al., 1999). This finding is also controversial (Arya et al., 1999). Similar reasoning may explain the effects of a recently identified polymorphism in the RANTES promoter region that has also been associated with disease progression (Liu et al., 1999). Since many of these polymorphisms are present in some populations but not others, it is difficult to ascertain the precise role that these polymorphisms play in HIV-1 disease progression. Of concern is that the population of the cohorts under investigation in each study are very different. For example, some study populations are North American and European cohorts

consisting primarily of gay men, hemophiliacs or IV drug users (Huang et al., 1996; Winkler et al., 1998). Other studies have been carried out on African cohorts that consist of both men and women (Kostrikis et al., 1998; Mummidi et al., 1998). Another issue is the failure to control for drug therapy in many of these studies. It is clear that further studies must be done to clarify what associations are population dependent, what polymorphisms are in linkage disequilibrium with one another, and whether the observed effects are due to increased gene expression, cross-regulatory mechanisms, or due to some other explanation.

In any case, resistance to infection by HIV-1 mediated by innate mechanisms is unlikely to account for resistance in the Pumwani cohort, or many of the other EU groups. In the Pumwani cohort resistance is not associated with Δ32 CCR5 polymorphisms, altered CCR5 expression levels, or increased β-chemokine expression. In fact studies have shown that HIV-1 is quite capable of replicating *in vitro* in cells obtained from these women (Fowke et al., 1998).

The most exciting associations described to date are polymorphisms in genes that may play a role in conferring protection against infection, or disease progression through mechanisms that may be responsible for conferring acquired immune responses to HIV-1. A large number of studies (>50) have identified a wide array of HLA Class I and Class II alleles that are associated with differential disease progression, disease manifestations, and susceptibility to infection and have been the subject of numerous reviews (Just, 1995; Michael, 1999; Kaslow and McNicholl, 1999). Obviously, it is difficult to draw any conclusions with the large number of associations identified, this may be due to population differences among the groups being studied, as well as the extreme variability of HIV-1. Polymorphisms in other immune response genes have also been identified that

may play a role in altered susceptibility. Mutations in TNF-α (Knuchel et al., 1998), Mannose binding protein (Amoroso et al., 1999), the Vitamin D receptor and a gene involved in secretor status (Ali, 1998), have all been associated with altered HIV susceptibility. However many of these findings have yet to be confirmed.

Hypothesis and Objectives

We have identified a group of women who appear to be relatively resistant to infection by HIV-1. These women have evidence of cellular, but not systemic humoral immune responses to the virus, indicative of a type-1 response. There is considerable evidence that genetic factors can influence host immune responses, including type-1 and type-2 responses. Anecdotal evidence from the Pumwani cohort suggests that several of the HIV resistant women may be related, suggesting a familial basis for resistance. We also know that there are no known innate mechanisms of resistance so far identified in this cohort. This line of reasoning leads us directly to our hypothesis:

Resistance to Infection by HIV-1 is Mediated by Genetic Factors that are Involved in Regulation of Protective Immune Responses to HIV-1.

In the studies done to test this hypothesis we had three main objectives:

1) To determine if there was altered susceptibility to HIV-1 in those related to a resistant woman in both the Nairobi sex worker cohort and in other family members.

2) To better identify the resistance phenotype by characterizing a number of immunologic parameters that may be involved in regulation of, or reflect type-1 immune responses.

3) To investigate the role of several candidate genes that have been previously associated with resistance to infection by HIV-1, or that may arise out of objective 2.

Materials and Methods:

I. Materials:

Source of Biologic Material:

Female sex workers attending the Majengo sexually transmitted infection (STI) clinic in the slum district of Pumwani in Nairobi, Kenya were enrolled into the ML study cohort and were one of the sources of biologic material for this study. This well described, open cohort has been followed since 1985 in a number of studies investigating a variety of STI's (Kreiss et al., 1986; Plummer and Ndinya-Achola, 1990; Ronald et al., 1991). The studies involving this cohort had been approved by University of Manitoba and University of Nairobi ethics review panels. After informed consent was obtained upon enrollment, women were assigned a unique study number (ML number) and identification card to ensure patient identity for further visits. Women were encouraged to return to the clinic twice yearly, or whenever they felt the need to return for medical treatment. At each visit the women underwent physical examination, and were interviewed using a standard questionnaire for basic demographic information, and for sexual, medical, obstetric and contraceptive histories. Biologic samples were obtained for the purpose of this study, as well as a variety of other laboratory tests, including culture for *Neisseria gonorrhoeae*, *Haemophilis ducreyi*, and *Chlamydia trachomatis* performed as previously described (D'Costa et al., 1985). Rapid plasma reagin and *Treponema pallidum* hemagglutination (TPHA) were used to test for syphilis. *Chlamydia trachomatis* was detected from endocervical secretions using a commercial enzyme

immunoassay (Chlamydiazyme). Women were also typed for HLA Class I alleles using standard serological methodology (Vartdal et al., 1986), and in some cases molecularly typed for certain alleles (Krausa and Browning, 1996). HIV serology and detection of proviral DNA were performed. The women attended the clinic on average 2-4 times yearly for the treatment of STDs and related concerns. To date over 1900 individuals have been enrolled in this study.

Families of women enrolled into the ML cohort were considered for recruitment into a separate study (ML Kindred) after informed consent was obtained from index ML women. Index cases were interviewed in detail about their family structure and were informed of the rationale and purpose of the kindred study. Relationships to other members of the ML cohort were established and confirmed via triangulation. Index ML women were encouraged to contact relatives who were not members of the cohort to inquire about their willingness to participate in this study. Those family members who agreed to participate were either visited in their rural home by a health care worker and their index ML relative, or were invited to visit the Pumwani clinic. The purpose of the study was explained to the individuals and informed consent was obtained.

Subjects agreeing to participate were given a unique study number (ML Kindred number) and were interviewed using a standard questionnaire on basic demographic information, and for sexual, medical, obstetric and contraceptive histories. Biologic samples were obtained for HIV-1 serology, RPR, and serologic HLA Class I typing, as well as certain immunologic and genetic assays described in this study.

HIV-1 seronegative individuals who were considered low-risk for acquiring HIV-1 (negative controls) were recruited from Winnipeg laboratory staff after informed consent was obtained. Low risk Kenyan seronegative individuals were recruited (after

informed consent was obtained) from the well-described mother-to-child transmission study (MCH) in Nairobi (Datta et al., 1994; Embree et al., 1992). The biologic samples obtained were used for the immunologic and genetic assays described in this study.

General Chemicals:

Unless otherwise noted general laboratory chemicals were obtained either from Sigma Chemical Company or Fisher Scientific Canada.

Molecular Biology Solutions:

10X Tris-Borate EDTA Buffer

121.1 grams Tris Base, 61.8 grams Boric Acid, 3.72 grams Na_2EDTA, Adjust volume to 1 litre, final pH should be 8.3.

10X SSC

175.3 grams NaCl, 88.2 grams of Sodium Citrate, Adjust pH to 7.0 with 5M NaOH, bring up volume to 1 litre.

10X Gel Loading Dye

25 milligrams of bromophenol blue (0.25%), 1.5 grams Ficoll (Type 400 Pharmacia), bring up to 10 ml final volume.

10 mM Tris 1 mM EDTA (TE)

10 ml of 1M Tris*HCl (pH 8.0), 2 ml of 0.5 M EDTA (pH 8.0), bring up volume to 1 litre.

20X SSPE

210 grams NaCl, 28.6 grams $Na_2H_2PO_4$, 7.4 grams EDTA, bring up volume to 1 litre.

Cell Culture Reagents

Set-up Media (RPMI)

RPMI 1640 plus 25 mM HEPES and L-glutamine pH 7.2 (Gibco), 2 grams $NaHCO_3$, 100 units/ml Penicillin G Sodium, 0.1 mg/ml Streptomycin Sulfate, 0.25 ug/ml amphotericin B.

Culture Media (RPMI-10%)

900 ml set-up media as above, plus 100 ml Foetal Bovine Serum Modified (Gibco/BRL) heat inactivated 40 minutes at 56°C.

ABS Media (RPMI 2%ABS)

980 ml set-up media as above, plus 20 ml pooled human AB serum (Sigma).

Freezing Media (10% DMSO)

90 ml Foetal Bovine Serum (inactivated) + 10 ml DMSO (Sigma)

Phosphate Buffered Saline (PBS)

137mM NaCl, 2.7 mM KCl, 10.1 mM Na2HPO4, 1.76 mM KH_2PO_4, pH 7.0, heat sterilized.

Cell lines Utilized

K-562 (American Type Culture Collection (ATCC) 45507)

This cell line is derived from a myelogenous leukemia cell line that has been shown to be sensitive to lysis by natural killer cells due to its lack of surface expressed HLA antigens. The cell line was a generous gift from Dr. Anna Gomez, INR Research Ltd. (Vancouver, BC).

CEM-NK^R (ATCC CRL-1991)

This cell line is derived from a myelogenous leukemia cell line that has been shown to be resistant to lysis by natural killer cells due to its surface expression of HLA antigens. The cell line was a generous gift from Dr. Anna Gomez, INR Research Ltd. (Vancouver, BC).

B95-8 (ATCC CRL-1612)

This cell line is a transformed lymphoblastoid cell line from marmosets that produces high titre infectious Epstein-Barr Virus, which is used to immortalize human B lymphocytes. This cell line was a generous gift from Dr. Richard Warrington, Rheumatic Disease Laboratory (University of Manitoba).

II. Methods:

General Methods:

Data Entry and Statistical Methods of Analysis:

Epidemiological and laboratory data were computer coded and entered into a SPSS (SPSS 9.0, SPSS Science, Chicago, Ill) database. Data was analyzed using SPSS statistical packages. Data was tested for normal distribution by Kolmogorov-Smirnov tests. Standard parametric tests (Students t-test and analysis of variance (ANOVA)) and non-parametric statistical tests (Mann-Whitney U and Kruskal-Wallis) were used, with 95% confidence intervals. Standard survival analysis (Kaplain-Meier survival and log-rank tests) was used for univariate analysis of time dependent data. Multivariate analysis was carried out by Cox linear regression.

HIV-1 Serology and Polymerase Chain Reaction (PCR)

HIV-1 Serology was performed on all subjects using a synthetic peptide immunoassay (Detect HIV: Biochem Immunosystems Inc.). Positive tests were confirmed using recombinant antigen enzyme immunoassay (Recombigen HIV-1/2 EIA; Cambridge Biotech Corporation).

HIV-1 PCR was performed on DNA isolated from peripheral blood mononuclear cells (PBMC's) (see Methods: DNA Isolation and Source of Genetic Material) using primers and probes developed for African isolates. (Dawood et al., 1992) Briefly, PCR was carried out for *vif*, *nef* and *env* genes (described in Table 1) on samples that were positive for an internal control PCR reaction (HLA-DQα1, Table 1). Conditions for 50 ul reactions were as follows: Buffer (1mM Tris-HCl, 50 mM KCl), 1.5 mM $MgCl_2$, 0.001% gelatin, 200 uM of the 4 deoxynucleotide triphosphates (dNTP's) (Pharmacia), 0.5 uM of each specific primer pair, and 2.5 units AmpliTaq DNA polymerase (Perkin Elmer).

Table 1: HLA/HIV Primers and Probes.

Name	Oligonucleotide Sequence	Notes
HLA-5'	GTGCTGCAGGTGTAAACTTTGTACCAG	HLA- DQα Conserved 5'
HLA-3'	CACGGATCCGGTAGCAGCGGTAGAGTTG	HLA- DQα Conserved 3'
Vif-5'	ATTGTGTGGCAAGTAGACAGGATGA	HIV-1_{HXB2} Posn. 5064-5088
Vif-3'	CTAGTGGGATGTGTACTTCTGAACT	HIV-1_{HXB2} Posn. 5217-5193
Vif Probe	AGTTTAGTAAAACACCATATGTATGTTTCA (Tm = 68°)	HIV-1_{HXB2} Posn. 5106-5135
Env-5	AGCAGCAGGAAGCACIATGG	HIV-1_{HXB2} Posn. 7795-7814
Env-3	CCAGACIGTGAGTTGCAACAG	HIV-1_{HXB2} Posn. 7936-7916
Env Probe	ACGGTACAGGCCAGACAITTATTGTCTGGTATAGT (Tm = 73°)	HIV-1_{HXB2} Posn. 7835-7869
Nef-5	ACCTCAGGTACCTTTAAGACCAATG	HIV-1_{HXB2} Posn. 9008-9032
Nef-3	TGTGTAGTTCTGCCAATCAGGGAA	HIV-1_{HXB2} Posn. 9179-9156
Nef-Probe	GATCTCAGCCACTTTTTAAAAGAAAAGGGGGGACTG (Tm = 68°)	HIV-1_{HXB2} Posn. 9051-9086

Amplification was carried out on a PE 9700 thermocycler (PE applied Biosystems) using the following protocol: 35 cycles of (1 min @ 94°C, 1 min @ 56°C, 1 min @72°C) followed by 7 minutes at 72°C. Standard PCR techniques were followed throughout the experiment; including the use of positive and negative controls, aerosol resistant tips, and the prevention of PCR carry-over contamination, by using "clean" and "dirty" rooms for preparation and analysis respectively.

Twenty ul of the reaction was resolved on a 3.5% ethidium bromide (EtBr) stained agarose gel in 0.5X TBE running buffer, molecular weight standards (DNA Molecular Weight Standards V, Roche Biochemicals) were included to determine product size. The gel was placed on a transilluminator and photographed. The confirmation of whether a sample was positive or negative was then determined by Southern blot using the probes described in Table 1.

For Southern blots, DNA was transferred to a nylon membrane (Genescreen Plus, NEN Dupont) using the capillary transfer method as described by the manufacturer. DNA contained within the gel was denatured for 30 minutes in 0.4 N NaOH/0.6 M NaCl at room temperature with gentle agitation. The gel was neutralized by incubating in 1.5 M NaCl/0.5 M Tris-HCl, pH 7.5 for 30 minutes at room temp with gentle agitation. The transfer was performed in 10X SSC as follows: A glass plate was suspended over a glass dish containing 10X SSC. A filter paper wick was draped over the plate into the 10X SSC. The agarose gel was placed (upside down) on top of the filter paper layer. The hybridization membrane was placed directly over the gel, with three layers of filter paper laid on top of the transfer membrane. The whole overlay was then topped with a stack of absorbent towels with a weight placed on top of them. Capillary transfer was allowed to

take place for 16-72 hours. The nylon membrane was immersed in 0.4 N NaOH for 1 minute and then neutralized in 0.2 M Tris-HCl, pH7.5/ 2XSSC.

Membranes were then hybridized with the appropriate radiolabelled probe prepared as follows. Ten pmol (1pmol/ul) of probe was labeled with 2 ul of 10X polynucleotide kinase buffer (Pharmacia Biotech), 7 ul of γ-^{32}P Adenosine 5' triphosphate (ATP) (3000 ci/mmol, 10 mCi/ml, NEN Dupont) and 1 ul T4 polynucleotide kinase (5.3 U/ul) for 45 minutes at 37°C. Free γ-^{32}P-ATP was removed by passing through a 1 ml syringe spin column of Sephadex G-50 (Pharmacia). Specific activity of the purified oligonucleotide was determined by counting an aliquot of the oligonucleotide in 1-5 ml liquid scintillation cocktail (Ecolume, ICN) in a scintillation counter (LS 500 CE, Beckman). From 5-6 x 10^8 counts per minute/ug of radio-labeled oligonucleotides were then incubated in a sealed plastic bag with the membrane for 16-18 hours at between 48-56°C depending on the melting temperature (Tm) of the specific probe (generally Tm-5°C). After hybridization, the membrane was washed twice for 30 minutes in a 1% SDS/1X SSC solution at the hybridization temperature to remove unbound probe. The membrane was then exposed to X-ray film (Kodak X-O-Mat AR5) for between 6-48 hours. The films were developed using an automated film processor (MiniMed/90 X-ray Film Processor, AFP Imaging Corp.).

Immunologic Methods.

Identification of Study Groups for Immunologic Studies

During the efforts to identify a marker for the resistance phenotype we studied a number of defined groups in an effort to properly control for these assays. These groups and the reasons that they were chosen are as follows:

HIV resistant women from the ML cohort: These individuals are defined as resistant based upon epidemiologic parameters. If there is a genetically mediated immunologic phenotypic marker for resistance than individuals should respond differently in the immunologic studies chosen than susceptible individuals, and presumably differently than uninfected, or control groups, the vast majority of whom would be equally susceptible to HIV-1.

HIV susceptible women from the ML cohort: These individuals are infected with HIV-1 and therefore susceptible to infection. A differential immune response between these individuals and resistant individuals can not be readily used as a marker for resistance as numerous studies have shown (Graziosi et al., 1998) that even recently infected individuals can have seriously perturbed immune responses. Thus any differences observed between these two groups may be the result of general immune dysfunction due to HIV-1 infection and not due to underlying genetic differences in immune responses.

Kin of Resistant Sex Workers: These subjects from the kindred cohort are HIV uninfected relatives of resistant SWs from the ML cohort. If there is an underlying genetic mechanism responsible for an altered immune response, then these individuals may also share this response. One could expect that up to 50% of this group would

behave similarly to the resistant woman, while the remainder would behave similar to susceptible individuals. The kindred cohort also contained HIV infected relatives of the resistant women. These were excluded from the analysis for reasons described for the HIV infected sex workers (i.e. any alteration in the immune response is more likely due to the effects of HIV infection).

Kin of Susceptible SW's: These subjects from the kindred cohort are HIV uninfected relatives of susceptible SW's from the ML cohort. If there is an underlying genetic basis for resistance responsible for an altered immune response, then these individuals would best represent "normals" as they would be least likely to have a genetic factor responsible for resistance and would be expected to have the most normal immune responses in our tests. Again, we also had HIV infected relatives of susceptible SW's who were also excluded from analysis.

Finally, we used one (or both) of the following control groups. HIV uninfected North American controls recruited from Winnipeg laboratory staff. These were used as controls (if possible) for most of the assays performed as there are little data from African populations for a number of these experiments. The second control group consisted of low-risk Kenyan controls attending an antenatal clinic from the well-characterized MCH transmission study. These would represent normal Kenyan controls. The results obtained from this group should closely mirror that of the kin of susceptible study group, as neither group would be likely to have the genetic factor responsible for resistance.

Isolation of Peripheral Blood Mononuclear Cells (PBMC's)

Blood was drawn by venipuncture into sodium heparin vacutainers (Becton Dickinson) and mixed to prevent coagulation. Blood was layered onto one-third volume

of Ficoll-Hypaque (Sigma) as per the manufacturers instructions, and centrifuged at 500 x g (2000 rpm) for 30 minutes in a table top centrifuge (Beckman GPR) at room temperature. After centrifugation the plasma layer was removed aseptically in a laminar flow hood for long term cryopreservation and used for HIV-1 testing as well as other serological tests. The PBMC layer was similarly removed and transferred to a second sterile tube. The cells were then washed twice with 50 ml PBS at 400 x g (1800 rpm) for 20 minutes at room temperature. After the final wash the cells were stained with trypan blue staining and quantified using a heamocytometer and then resuspended at 3 x 10^6 cells per ml in setup media. The cells were then used as described in the following procedures.

Natural Killer Cell (NK)/ Lymphokine Activated Killer (LAK) Cell Assays

Analysis of NK and LAK activity were carried out as previously described (Grimm et al., 1982) with the following modifications. Nine to twelve x 10^6 PMBC's were set to 1 x 10^6 cells per ml in cell culture media (RPMI-10%) and incubated overnight in a 25 cm^3 flask (Corning, CoStar) at 37°C in a water-jacketed CO_2 incubator. The cells were then counted again using a heamocytometer and an aliquot of cells was resuspended to 5 x 10^5 cells per ml in RPMI-10%. These cells were used as the effector cells for the NK assay.

A two to three ml aliquot of mid-log phase CEM-NK^R and K-562 target cell lines were labeled with 50 ul of ^{51}Cr-sodium dichromate (2000 cu/mmol, 20 mCi/ml, NEN Dupont) for 90 minutes at 37°C. The cells were washed 4 times with 10 ml of warm PBS, counted, and resuspended at 1 x 10^5 per ml in RPMI-10%. These cells were used as the two target cell lines for the NK assay.

Fifty microlitres of target cells were plated out in a 96 well round bottom plate (Corning, CoStar) (in triplicate) for each of the two target cell lines. Experimental wells were set up at various effector to target cells ratios (50:1, 25:1 and 12.5:1), which was 50 ul, 25 ul and 12.5 ul of effectors to 50 ul of targets respectively. Spontaneous release wells of target cells alone (50 ul targets plus media), plus maximal release wells containing fully lysed target cells (50 ul of target cells plus 20 ul of 20% SDS) were also set up. All wells were brought up to 200 ul total volume in RPMI-10%.

The plate was then centrafuged at 400 x g (1500 rpm) for 5 minutes to maximize cell to cell contact and then incubated for 4 hours at 37°C. At the end of the incubation 100 ul of supernatant was removed from each well and combined with 1 ml of scintillation cocktail (Ecolume, ICN). ^{51}Cr release was detected using a liquid scintillation counter (LS 5000 CE, Beckman). Percent specific lysis for each cell ratio was calculated by {(Experimental – Spontaneous) / (Maximum – Spontaneous)}x 100%.

Remaining effector cells were set to 1 x 10^6 cells/ml and cultured a further 3-4 days in RPMI-10% at 37°C in the presence of recombinant IL-2 at 5 units per ml. The cells were again counted and resuspended to 5 x 10^5 per ml before being assayed as described above with freshly labeled target cell lines. This assay measures the lytic activity of lymphokine activated killer cells (LAK).

Lymphocyte Proliferation Assays

The proliferative response of PBMC's to a panel of recall antigens and mitogens were carried out similar to previous experiments. (Clerici et al., 1992) Briefly, 100 ul of isolated PBMC's in setup media set at 3 x 10^6 cells per ml (3 x10^5 cells total) were added in triplicate to 6 separate antigen/mitogen solutions in 96 well round bottom plates

(Corning, Costar). The solutions were as follows; 2% ABS Media alone (negative control), phytohemagglutinin M (1.25 ug/ml final concentration, Sigma), influenza antigen (FLU) (1:800 final concentration, a generous gift from Dr. Fred Aoki, University of Manitoba), tetanus toxoid (3.25 T units/ml Connaught Laboratories), candida (CAND) (10 ug/ml final concentration, Greer Laboratories), purified protein derivative of *Mycobacterium tuberculosis* (PPD) (5 ug/ml, Connaught Laboratories). The media contained 25 ul of a 1:100 dilution of anti-Tac monoclonal antibody (specific for the alpha chain of the IL-2 receptor) (a generous gift from Dr. Gene Shearer, NCI, NIH, Bethesda, Md) to prevent endogenous uptake of IL-2. The test solutions were then brought to 250 ul total volume in 2% ABS media. The cells were incubated with their respective antigen / mitogen for 4 days at 37°C in a water-jacketed CO_2 incubator. On the fourth day 200 ul of supernatant was harvested and stored frozen at -70°C until assayed for IL-2 production by a commercially available IL-2 Enzyme-linked Immunosorbant Assay (ELISA) kit (R&D Biosystems, Minneapolis, MN).

Monocyte / Macrophage Cytokine Assays

The cytokine response of specifically activated monocytes was determined as previously described, (Chougnet et al., 1996) with the following modifications. Basically, 350 ul of PBMC's in setup media (1.05×10^6 cells total, as described above) were added in duplicate to an F24 flat bottom tissue culture plate (Corning, Costar). One well (negative control) was brought up 700 ul total volume and 2% final ABS serum concentration. The experimental well was prepared as above, but contained 70 ul of a 1:100 dilution of a formalin inactivated, heat killed Cohen strain of *Staphylococcus*

aureus (SAC, Pansorbin, Calbiochem). The cells were then incubated overnight at 37°C in a water-jacketed CO_2 incubator. The next day 2 samples of 200 ul each of supernatant were harvested after centrafuging the plate at 400 x g (1500 rpm) for 5 minutes to pellet whole cells. The supernatant samples were frozen at -70°C until assayed for IL-10 and IL-12 levels by commercially available ELISA kits (R&D Biosystems, Minneapolis, MN). Assays were kindly performed at the laboratory of Dr Gene Shearer, NCI, NIH, Bethesda, Maryland.

B-cell Transformation and Cryopreservation of Viable Cells

Cells remaining after the previous experiments were used to establish immortalized B-lymphoblastoid (B-lcl) cell lines as a continuous source of genetic material, and were frozen as viable cells for further immunologic assays. Both of these procedures have been previously described, (Grant, 1976), (Sugden and Mark, 1977) and are briefly described below.

For B-lcl transformation, 5-10 x 10^6 PBMC's were centrifuged at 400 x g (1500 rpm) for 5 minutes and the supernatent removed. The cells were then resuspended in 1-2 ml of B-958 supernatant that was prepared by filtration through a 0.45 micron filter to remove any B-958 cells. The PBMC's and B-958 supernatant were then transferred to a F24 flat bottom culture plate. After 4-6 hours the media was doubled with fresh RPMI-10% and incubated for 2-3 days at 37°C. Roughly one-half of the spent media was then removed and the cells resupended in RPMI-10%. The cells were eventually split to 2 separate wells within the same plate, as cell numbers permitted. The cells were then split at roughly bi-weekly intervals for 3-5 weeks until being transferred to 25 cm^3 flasks. At

this point the cell lines were either maintained for a period of time, or were frozen as described below.

For the cryopreservation of viable cells, a known amount of cells was centrifuged at 400 x g (1500 rpm) for five minutes. The supernatant was then discarded and the cell pellet resuspended in ice cold freezing media to a final concentration of 5 x 10^6 cells per ml. Resuspended cells were transferred to pre-chilled cryovials (Nalgene) in 1 ml aliquots. The cryovial was then transferred to a pre-chilled cell freezing device (Stratacooler, Stratagene) and stored overnight at -80°C. The cell freezing device allowed a rate-specific drop in temperature to the cryovials. The next day, cryovials were transferred to liquid nitrogen canisters.

Candidate Gene Analysis Methods:

DNA Isolation and Source of Genetic Material

DNA used for genetic analysis was obtained from one of 2 main sources; either from PBMC's (isolated as described above) or from B-lymphoblastoid cell lines from the individuals to be analyzed. In either case DNA was extracted from between 5-6 x 10^6 cells using the PUREGENE^{TM} system and using the manufacturer's recommended procedures (Gentra Systems). Isolated DNA was finally resuspended in 100 ul of distilled deionized H_2O. The quality and quantity of isolated DNA was estimated by carrying out a test PCR reaction amplifying a single copy gene, a portion of the second exon of the HLA DQ-α1 gene and analyzing the resulting products on an agarose gel. Briefly, 1-5 ul of isolated DNA was used as template in the previously described HLA-DQα reaction mixture (See Methods: HIV-1 Serology and PCR) and resolved on an ethidium bromide (EtBr) stained 3.5% agarose gel in 0.5X TBE. The intensity of the PCR products was then compared to a known, standardized amount of control DNA, and the approximate DNA concentration was estimated. Alternatively, more precise calculations of DNA concentration was obtained on some samples by staining with a fluorescent dye (PICO Green, Molecular Probes). Briefly, a 1:100 dilution of DNA was incubated with PICO Green, which fluoresces when bound to double stranded DNA. Fluorescent intensity of the sample is then compared to a standard curve of known concentration, and the concentration of the unknown sample was derived.

Dot-Blot Analysis of Vitamin D Receptor and Fucosyl Transferase Polymorphisms

Single nucleotide polymorphisms in the Vitamin D Receptor (VDR) and Fucosyl transferase (Secretor) were detected by oligonucleotide specific hybridization to dot-blotted PCR fragments that spanned the polymorphic loci. The specific PCR primers and sequence specific oligonucleotide probes for these reactions are described in Table 2. PCR conditions for each of the 15 ul PCR reactions were as follows: approximately 50 ng of template DNA, PCR buffer 10 mM Tris-HCl pH 8.3, 25 mM KCl, 200 uM of each of the 4 deoxynucleotide triphosphates, 1 mM $MgCl_2$, 0.4 U of Amplitaq Gold Polymerase (Perkin-Elmer) and 1.5 pmol of each PCR primer, the mixture was then overlaid with 20 ul of mineral oil. PCR was carried out in a thermocycler (MJ Research) as follows: 14 minutes @ 94°C, followed by 35 cycles of 20s @ 94°C, 30s @ 58°C, 20s @ 72°C.

Two microlitres of PCR products were resolved on a EtBr stained 2% agarose gel in 0.5X TBE to confirm the presence of size specific amplicons, before dot-blotting the remaining product to a nylon membrane (Boehringer Mannheim) using a 96-well dot-blot device (Bio-Rad) following the manufacturer's recommended instructions. The blotted PCR fragments were then exposed to UV light for 2 minutes (Stratalinker UV Crosslinker, Stratagene) to crosslink the PCR fragments to the nylon membrane.

Oligonucleotide probes were end-labeled with Digoxigen (DIG) (Boehringer Mannheim) using the following reaction conditions. One hundred picomoles of probe were incubated in a terminal transferase buffer containing 200 mM Potassium Cacoadynylate, 25 mM Tris-HCL, 250 mg/ml BSA and supplemented with 5 mM Cobalt Chloride, 250 mM of DIG labeled ddUTP, and 5 units of terminal transferase.

Table 2: PCR Primers and Oligonucleotide Probes used in Dot-Blot Analysis of VDR and Secretor Polymorphisms.

Oligo Name	Oligonucleotide Sequence	Notes
VDR-Forward	CAGAGCATGGACAGGGAGCAAG	
VDR-Reverse	GGTGGCGGCAGCGGATGTA	
VDR-Wt-probe	GCGCTGATcGAGGC	Lowercase NT denotes polymorphic loci
VDR-Mut-Probe	GCGCTGATtGAGGC	Lowercase NT denotes polymorphic loci
Secretor-Forward	TACCGCCACATCCCGGGGG	
Secretor-Reverse	CCGGCTCCCGTTCACCTGC	
Secretor-Wt-probe	CCTGCTCCTgGACCTTCTA	Lowercase NT denotes polymorphic loci
Secretor-Mut-Probe	CCTGCTCCTaGACCTTCTA	Lowercase NT denotes polymorphic loci

The reaction tubes were incubated at 37°C for 25 minutes before being stopped by the addition of 0.005% glycogen/ 0.2 M EDTA, and brought up to 100 ul volume with ddH_20.

The membranes were pre-blocked for 30 minutes at room temperature with 10 ml blocking solution (4 x SSPE, 1% Blocking reagent (Boehringer), 0.1% laurylsarcosine). Blocking solution was then removed, and the membrane pre-hybridized with a Tetramethyl Ammonium Chloride (TMAC) hybridization solution (3M TMAC, 50 mM Tris-pH 8.0, 0.1% SDS, 2 mM EDTA) for 45 minutes at 50°C, before 25 ul of the labeled, diluted probe was added and incubated a further hour at 50°C. After the probe was hybridized, 2 non-specific 10 minute wash steps were carried out with 25 ml wash solution (2x SSPE, 0.1% SDS) before the probe-specific stringency wash was carried out. Stringency washes were then carried out at probe specific temperatures (VDR-wt 61°C, VDR-mut 58°C, Secretor-wt and Secretor-mut 58°C) for 25 minutes in TMAC hybridization solution (as above).

Detection was carried out by alkaline phosphatase reactions catalyzed by an alkaline phosphatase labeled anti-DIG monoclonal antibody. Membranes were first briefly washed in 0.1 M maleic acid, 0.15 M NaCl (pH 7.5), before being blocked by the addition of 0.3% Tween 20 to the wash buffer for 30 minutes at RT. Two ul of anti-DIG-alkaline phosphatase (1:5000) was then added to the buffer and allowed to incubate for a further 30 minutes. Excess antibody was removed by 2 x 15 minute washes using the wash buffer. The membranes were then briefly rinsed with reaction buffer, (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$ (pH 9.5)), before the addition of the AP substrate CSPD (1:100 dilution in reaction buffer) (Roche Biochemicals). Membranes

were then wrapped in Saran-wrap to maintain moisture, while the CSPD was activated by incubation at 37°C for 15 minutes. After this time the membranes were exposed to X-ray file (XAR5, Kodak Eastman) for 15-20 minutes before the X-ray film was developed in an automated film processor (Minimed/90, AFP Imaging Corp.). Samples were scored as positive for a specific genotype if the blot intensity of the sample was greater than that of an included positive control.

After analysis of the autoradiograph, the corresponding membrane was stripped for 30 minutes at 85°C in 2X SSC, 20 mM EDTA, followed by a wash at room temperature for 10 minutes in 2X SSC, 0.1% SDS, a wash for 30 minutes at 37°C in 0.2 M NaOH, 0.1% SDS and a final rinse in 2X SSC. Membranes could then be wrapped in Saran-wrap and stored short term at 4°C (< 1 month), or immediately re-probed with a second oligonucleotide probe (prepared as described above).

Complete genotypes for each sample were then determined by comparing the corresponding autoradiograph from each sample pair hybridized with the genotype specific probe. A sample positive for the VDR-mut probe alone, and negative for the VDR-wt probe is a homozygous mutant, a sample positive for both VDR-mut and VDR-wt probes would be considered heterozygous, while a sample positive for the VDR-wt probe alone would be a homozygous wild-type. Genotyping done in this manner was done blinded to sample identity and confirmed by a second reading done by another individual. For an example of dot-blot hybridization and genotyping by this method see Figure 1.

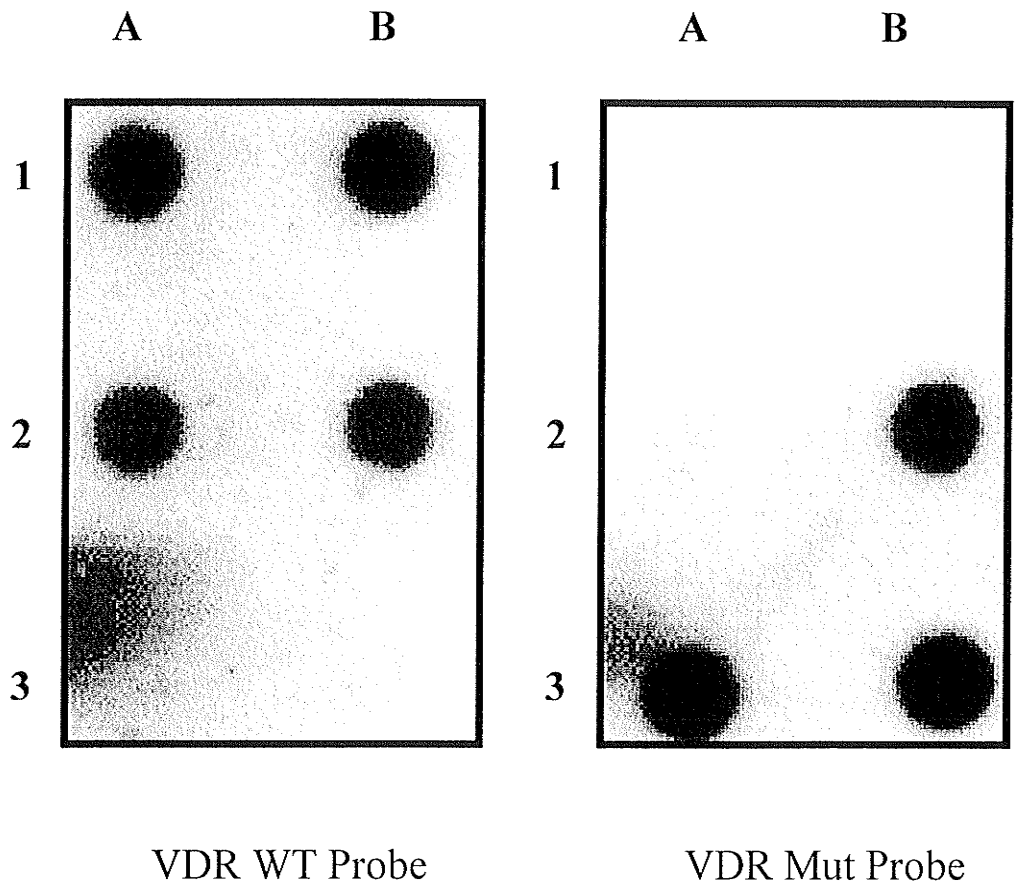

Figure 1. Genotyping of VDR Polymorphism by Dot-Blot Analysis.

Figure 1: Genotyping of VDR Polymorphism by Dot-Blot Analysis. Duplicate images of chemiluminescent autoradiographs of dot-blotted PCR amplicons containing the polymorphic loci from 6 individuals were hybridized with either the VDR wt probe (left panel), or stripped and re-probed with the VDR mut probe (right panel). Individuals A1, A2 and B1 are homozygous for the wild type allele, while A3 and B3 are homozygous for the mutant allele. The B2 individual's sample hybridizes with both probes and is heterozygous for this polymorphism.

CCR2-64I Polymorphism (RFLP Analysis)

A single nucleotide polymorphism in the CCR2b gene was detected by PCR amplification of a region spanning this polymorphism, and restriction fragment length polymorphism analysis (RFLP) was carried out by restriction endonuclease digestion specific to the polymorphic loci. PCR of the specific region of the CCR2b gene was carried in 50 ul reactions as follows: Buffer (1mM Tris-HCl, 50 mM KCl), 1.5 mM $MgCl_2$, 0.001% gelatin, 200 uM of the 4 deoxynucleotide triphosphates (dNTP's) (Pharmacia), 0.5 uM of each specific primer (CCR2-forward TTGTGGGCAACATGATGG, and CCR2-reverse GAGCCCACAATGGGAGAGTA) and 2.5 units AmpliTaq DNA polymerase (Perkin Elmer). Amplification was carried out on a PE 9700 Thermocycler (PE applied Biosystems) using the following protocol: 35 cycles of (45 seconds @ 94°C, 45 seconds @ 50°C, 45 seconds @72°C) followed by 7 minutes at 72°C. This PCR generated a 128 bp amplicon that contained a *Bsa* B1 restriction endonuclease site if it contained the mutant allele, or lacked this site if the allele was wild-type.

A 17.5 ul aliquot of the PCR reaction was then mixed with 2 ul of 10x NEBuffer2 (New England Biolabs), and 0.5 ul (10 units) of the restriction endonuclease (RE) *Bsa* B1 and incubated at 60°C for 1 hour. The resulting products were then resolved on an EtBr stained 3.5% agarose gel in 0.5X TBE.

Genotyping was performed by comparing the banding patterns of the digested PCR fragments. Wild-type genotype was distinguished by the fact that the wild-type sequence did not contain the *Bsa* B1 recognition site and was not digested, resulting in a single PCR product band (128bp). Conversely the mutant sequence contains the *Bsa* B1

RE site and was digested to completion, resulting in 2 distinct bands in the agarose gel (one band at 18 bp, and one at 110 bp). DNA from a heterozygous individual would therefore display both banding patterns (i.e. all three sizes of bands). Excess restriction enzyme was used to insure complete digestion of the PCR products occurred. An example of RFLP analysis and genotyping of the CCR2b polymorphism is shown in Figure 2.

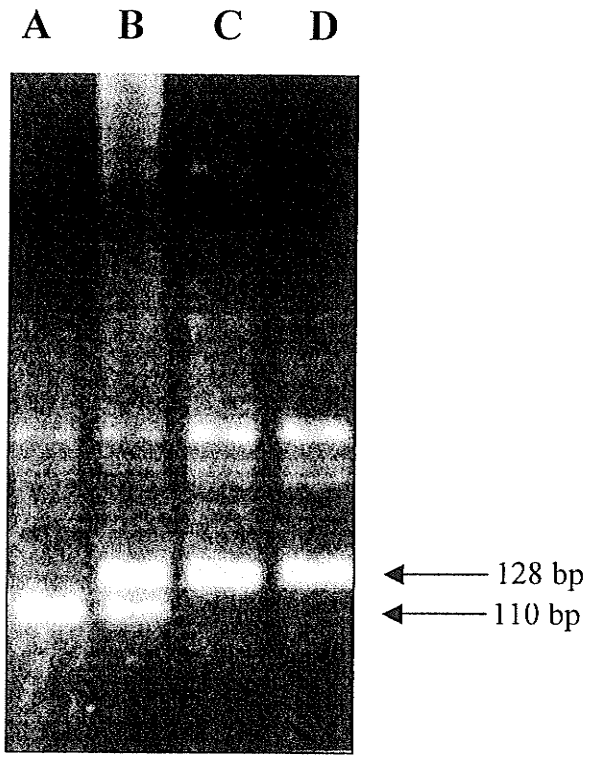

Figure 2. Genotyping of CCR2b Polymorphism by RFLP Analysis

Figure 2: Genotyping of CCR2b Polymorphism by RFLP Analysis. The image shows an EtBr stained agarose gel used to resolve a portion of the CCR2b gene from 4 individuals after digestion of the PCR product with *Bsa* B1. Sample A is homozygous for the mutant allele (product completely digested), sample B is heterozygous for this polymorphism (2 bands of the appropriate size), while samples C and D are considered homozygous wild type as their PCR amplicons do not contain the *Bsa* B1 site. Not shown on this gel is the 18 bp cleavage product that would be visible in lanes A and B.

IL-4 -590 Polymorphism (RFLP Analysis)

A single nucleotide polymorphism in the -590 position of the IL-4 promoter was detected by PCR amplification of a region containing this polymorphism, and RFLP analysis carried out by restriction endonuclease digestion of the polymorphic loci. PCR of the specific region of the IL-4 gene was carried out using the following reaction conditions. The 20 ul reactions were as follows: Buffer (1mM Tris-HCl, 50 mM KCl), 1.5 mM $MgCl_2$, 0.001% gelatin, 200uM of the 4 deoxynucleotide triphosphates (dNTP's) (Pharmacia), 0.5 uM of each primer (IL4 –590for ACTAGGCCTCACCTGATACG, and IL4 –590reverse GTTGTAATGCAGTCCTCCTG) and 2.5 units AmpliTaq DNA polymerase (Perkin Elmer). Amplification was carried out on a PE 9700 thermocycler (PE applied Biosystems) using the following protocol: 32 cycles of (30 seconds @ 94°C, 30 seconds @ 57°C, 30 seconds @72°C) followed by 7 minutes at 72°C. This PCR reaction produced a 226 bp amplicon that contained a *Bsm* F1 site if the wild type allele was present.

A 5 ul aliquot of the PCR reaction was then mixed with 2 ul of 10x NEBuffer 4 (New England Biolabs), 1 ul (2 units) of the restriction endonuclease *Bsm* F1, and 5 ul of H_20 and incubated at 37°C for 1 hour. The resulting products were then resolved on an EtBr stained 3% agarose gel in 0.5X TBE.

Genotyping was performed by comparing the banding patterns of the digested PCR fragments. Wild-type genotype was distinguished by the fact that the wild-type allele contains the *Bsm* F1 recognition site and was digested to completion, resulting in 2 distinct bands in the agarose gel (one 192 bp, and one 34 bp in length). Conversely the mutant sequence lacks this restriction enzyme site and is not digested resulting in a single

band (226 bp). A heterozygous individual would therefore display both banding patterns (and all three bands would be present). Excess restriction enzyme was used to insure complete digestion of the PCR products occurred. An example of RFLP analysis and genotyping of the IL-4 -590 polymorphism is shown in Figure 3.

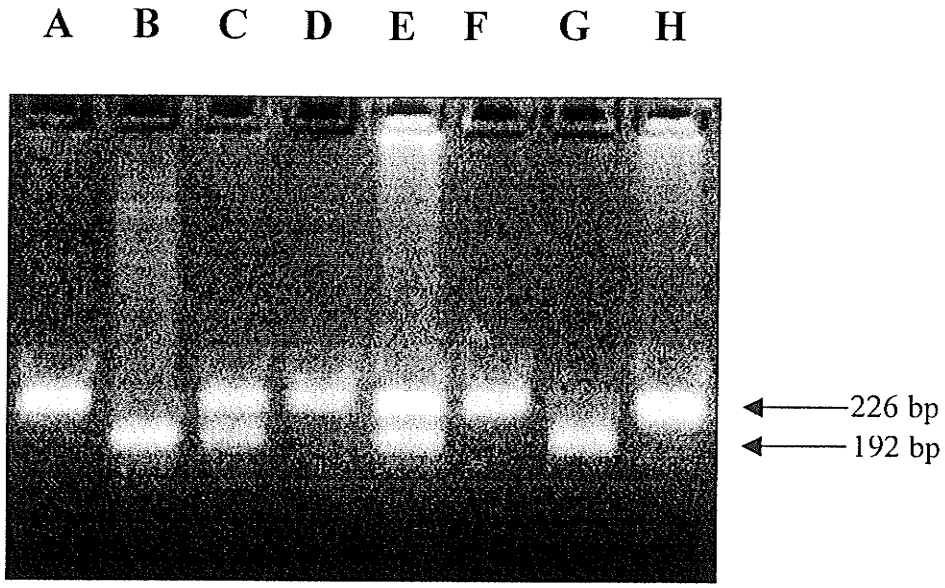

Figure 3. Genotyping of IL-4 –590 Promoter Polymorphism by RFLP Analysis.

Figure 3: Genotyping of IL-4 -590 Polymorphism by RFLP Analysis. The image shows an EtBr stained agarose gel used to resolve a portion of the IL-4 gene from 8 individuals after digestion of the PCR product with *Bsm* F1. Samples A, D, F and H are homozygous for the mutant allele (product undigested), samples C and D are heterozygous for this polymorphism (2 bands of the appropriate size), while samples B and G are considered homozygous wild type as their PCR amplicons contain the *Bsm* F1 site and are completely digested. Not shown on this gel is the 34 bp cleavage product that would be visible in lanes B, C, E and G.

IL-4 Promoter Polymorphism (SSCP/Sequence Analysis)

Potential polymorphisms in the IL-4 immediate upstream promoter were investigated by a combination of sequence analysis and single stranded conformational polymorphism analysis (SSCP). Primers were designed that would generate a PCR product corresponding to the first 452 bp of the IL-4 gene's immediate upstream promoter region and the first 15 bp of the IL-4 transcript. This PCR product was used for both SSCP and sequence analysis. PCR of this region of the IL-4 gene was carried out using the following reaction conditions. The 50 ul reactions were as follows: Buffer (1mM Tris-HCl, 50 mM KCl), 1.5 mM $MgCl_2$, 0.001% gelatin, 200 uM of the 4 deoxynucleotide triphosphates (dNTP's) (Pharmacia), 0.5 uM of each specific primer (IL-4prom-forward GAAGCTAACGATGCAATGCTG, and IL-4prom-reverse TTCCATGACAGGACAGTTTCC) and 2.5 units AmpliTaq DNA polymerase (Perkin Elmer). For the SSCP reactions 0.5 ul of α-32P deoxyadenosine 5' triphosphate (ATP) (3000ci/mmol, 10 mci/ml, NEN DuPont) was added so that amplicons could be detected by autoradiography during SSCP polyacrylamide gel electrophoresis. Amplification was carried out on a PE 9700 thermocycler (PE applied Biosystems) using the following protocol: 35 cycles of (30 seconds @ 94°C, 45 seconds @ 58°C, 45 seconds @72°C) followed by 7 minutes at 72°C. The presence of resulting products was confirmed by resolution on a 2.5% EtBr stained agarose gel.

The 467 PCR amplicon contained a single *Hae* III restriction endonuclease site. The amplicons were digested with *Hae* III in order to produce two smaller fragments for analysis using the SSCP protocol. Briefly 10 ul of PCR product was digested with 20

units of *Hae* III, and 2 ul of 10X NEBuffer2 (New England Biolabs) in a 20 ul reaction, for 2 hours at 37°C. The digested products were diluted 1:10 with ddH_2O and 2 ul of this dilution was mixed with 2 ul sequencing stop solution (95% formamide, 0.1% bromophenol blue, and 0.1% xylene cyanol). Samples were denatured by heating at 95°C for 3-5 minutes immediately before loading the sample on to a 5% acrylamide/10% glycerol polyacrylamide gel in 1X TBE. A non-denatured control sample was also included for each sample. The gel was run at 12 mA overnight, before drying and exposure to X-ray film for 18-24 hours and then developed in an automated film processor (Minimed/90, AFP Imaging Corp.). Autoradiographs were analyzed by comparing differences in the gel migration pattern between the samples.

Sequencing of the IL-4 promoter region was done by University of Calgary DNA sequencing services on PCR product generated as previously described. The PCR product was size separated on a 2.5% agarose gel, before being excised from the gel and purified using Agarose Gel DNA Extraction Kit (Boehringer, Mannheim).

Microsatellite Analysis of IL-4 / TH2 Gene Cluster

DNA microsatellite analysis of the IL-4/Th2 gene cluster was done essentially as previously described (Hughes, 1993), with the following modifications. PCR primer pairs specific for the indicated microsatellite markers were developed and are indicated in Table 3. Primers were ordered from PE-Applied Biosystems (Chesire, UK) with one primer from each pair being end-labeled with either FAM, TET or HEX fluorescent markers.

Conditions for the 15 ul PCR reactions were as follows for each of the microsatellite markers. Approximately 50 ng of template DNA was amplified in the following reaction mix. The PCR buffer was10 mM Tris-HCl pH 8.3, with 25 mM KCl, 200 uM of each of the 4 deoxynucleotide triphosphates, 1 mM $MgCl_2$, 0.4 U of Amplitaq Gold Polymerase (Perkin-Elmer) and 1.5 pmol of each PCR primer, the mixture was then overlaid with 20 ul of mineral oil. PCR was carried out in a thermocycler (MJ Research) as follows: 14 minutes @ 94°C, followed by 35 cycles of 15s @ 94°C, 30s @ the specified annealing temperature (55° or 58°C), 30s @ 72°C.

Table 3: PCR Primers for IL-4 Cluster Microsatellite Markers.

Microsatellite Marker	Oligonucleotide Primers	Annealing Temperature
D5S666	(f) AGCTGCATTCTCATGGTTTATCTTG	55°C
	(r) GTGCCTGGCTTATTTCACTTAACA	
D5S1984	(f) CCAGCCCGCTTAGTGT	58°C
	(r) TAGGAGGCTTCCCACATCT	
IL-4 ms	(f) TGCACCTGGGCAACAGTTTA	58°C
	(r) GTTGGATGGACTTGGAGATT	
IRF-1 ms	(f) ATGGCAGATAGGTCCACCGG	55°C
	(r) TCATCCTCATCTGTTGTAGC	
D5S2115	(f) GGCACTCATGCTGCACT	55°C
	(r) GTAAGCCCCTGGCTCCT	
D5S399	(f) GAGTGTATCAGTCAGGGTGC	58°C
	(r) GGCCTCAACTTCATAATCAA	

Resulting PCR products from the same individual were then pooled together in 96 well PCR plates (Corning, Costar) in set combinations so that differentially labeled products (i.e. similar sized amplicons, one labeled with FAM, and one with TET) could be resolved on the same sample run. Pooled products were then ethanol precipitated by the addition of 2.5 volumes 100% EtOH and centrifugation at 600 x g (2000 rpm) for 15 minutes. Ethanol was then removed and the samples allowed to air-dry at 55°C for 10 minutes. The precipitated samples were then resuspended in 4 ul of loading dye (PE Applied Biosystems) that contained a ROX 500 labeled size standard, and loaded onto an ABI Prism 373 automated DNA sequencer (PE-Applied Biosystems) and resolved for 6 hours at 1800 volts in 0.5X TBE. After the run, genotypes were determined for each microsatellite marker using the provided Genescan analysis software and the data recorded in an SPSS data file.

Results:

Overview:

The Nairobi prostitute cohort contains a sub-group of women who appear to be relatively resistant to infection by HIV-1. Previous studies have suggested that these women have acquired immunity to HIV-1 infection (Fowke et al., 2000). There also appears to be a familial basis for this resistance to infection. We attempted to determine if genetic factors were associated with resistance. To do this we first showed that being related to an HIV resistant woman led to a decreased likelihood of being infected with HIV-1. We then attempted to define a resistance phenotype, or marker for resistance that we could apply to non-prostitutes to better use the tools of genetic epidemiology to investigate these genetic factors. Candidate gene analysis of selected polymorphisms and microsatellite markers were used to ascertain these genetic factors.

Determination of HIV Susceptibility in Two Cohorts

HIV Resistant Phenotype in ML Prostitute Cohort

Currently over 1900 women have enrolled into the ML cohort, these women are all active sex workers and have been the subject of numerous studies. Within this overall group 97 women meet our epidemiologic definition of resistance to infection by HIV-1 (Fowke et al., 1996). To strictly meet our definition of resistance the woman must have been enrolled within our cohort for over 3 years, be persistently seronegative by HIV-1/2

EIA, and have no evidence of integrated proviral DNA as detected by our HIV-PCR methods.

Of these 97 women 7 have either been lost to follow-up (n=4), or we lack the biologic materials necessary to confirm their resistant status by PCR (n=3). For the purpose of this study we shall define the resistance phenotype based upon our epidemiological definition (active in sex work, >3 years follow up in our cohort, HIV EIA negative and HIV-PCR negative). The HIV-1 resistant women who have been used as subjects in the present study are detailed in Table 4. For this study, women in the ML cohort were divided into one of three phenotypes; *Resistant* (as above), *Susceptible* (HIV infected) or *Unknown* (HIV negative, but have not been enrolled into our cohort long enough to be considered resistant). For the majority of the immunologic and genetic studies carried out we compared resistant and susceptible individuals only.

It was established that a number of the resistant women appeared to be relatives of one another. Interviews with individual cohort members indicated that some of them had relatives (mothers, daughters, sisters, cousins and nieces) who were also members of the ML cohort. We identified as many relationships within the cohort as possible, as described in Materials and Methods. We then set up a simple case-control study within the ML cohort to determine if being biologically related to a resistant woman in any manner had an effect on HIV infection. The results are presented in Table 5A. To summarize 15/33 (45.5%) of those related to a resistant woman were HIV positive compared to 93/103 (90.3%) of those related to a susceptible woman. Individuals related to an HIV resistant women were significantly less likely to be HIV infected (OR 0.1, 95% CI .07-.26, $p<0.00001$).

Table 4: Characteristics of HIV Resistant Women.

ML #	Enrollment	Time	EIA	PCR	ML #	Enrollment	Time	EIA	PCR
187	4/16/1986	4.4	(-)	(-)	1515	6/23/1992	4.5	(-)	(-)
320	3/14/1985	12.9	(-)	(-)	1524	7/14/1992	5.4	(-)	(-)
326	3/14/1985	5.8	(-)	(-)	1529	9/21/1992	4.8	(-)	(-)
459	3/22/1985	12.4	(-)	(-)	1536	7/28/1992	6.5	(-)	(-)
556	6/6/1985	13.8	(-)	(-)	1544	8/25/1992	6.1	(-)	(-)
630	11/11/1986	10.3	(-)	(-)	1552	9/8/1992	4.8	(-)	(-)
767	1/13/1987	12.1	(-)	(-)	1562	9/15/1992	3	(-)	(-)
832	3/3/1987	3.6	(-)	(-)	1573	9/29/1992	6.3	(-)	(-)
851	4/14/1987	11.9	(-)	(-)	1589	3/11/1992	5.8	(-)	(-)
858	4/28/1987	9.2	(-)	(-)	1593	11/10/1992	6.1	(-)	(-)
881	4/7/1987	11.8	(-)	(-)	1601	12/1/1992	5.8	(-)	(-)
889	6/23/1987	11.7	(-)	(-)	1603	12/1/1992	3	(-)	(-)
893	6/20/1987	9.7	(-)	(-)	1604	12/2/1992	3.9	(-)	(-)
896	7/7/1987	11.7	(-)	(-)	1607	12/15/1992	5.5	(-)	(-)
923	8/25/1987	6.4	(-)	(-)	1608	12/15/1992	5	(-)	(-)
935	9/8/1987	11.4	(-)	(-)	1622	2/2/1993	5.9	(-)	(-)
948	10/6/1987	11.3	(-)	(-)	1626	2/2/1993	5.4	(-)	(-)
1025	6/21/1988	9.6	(-)	(-)	1635	2/23/1993	4.3	(-)	(-)
1070	1/24/1989	5.9	(-)	(-)	1643	2/23/1993	5.8	(-)	(-)
1072	2/7/1989	10	(-)	(-)	1655	3/9/1993	4.9	(-)	(-)
1192	11/14/1989	9.3	(-)	(-)	1663	3/30/1993	5.3	(-)	(-)
1250	3/30/1990	8.8	(-)	(-)	1668	4/29/1993	4.7	(-)	(-)
1260	8/21/1990	8.5	(-)	(-)	1671	4/20/1993	5.7	(-)	(-)
1266	8/28/1990	8.4	(-)	(-)	1681	5/11/1993	3.7	(-)	(-)
1275	11/13/1990	8.2	(-)	(-)	1700	6/30/1993	5.4	(-)	(-)
1286	9/11/1990	8.3	(-)	(-)	1702	7/13/1993	3.3	(-)	(-)
1293	7/3/1990	5.1	(-)	(-)	1705	7/20/1993	5.5	(-)	(-)
1327	10/16/1990	8.3	(-)	(-)	1726	10/13/1993	5.2	(-)	(-)
1358	3/6/1991	6.8	(-)	(-)	1730	10/19/1993	3.2	(-)	(-)
1362	3/19/1991	7.6	(-)	(-)	1732	10/26/1993	5.2	(-)	(-)
1371	5/7/1991	7.6	(-)	(-)	1742	1/18/1994	4.3	(-)	(-)
1376	5/22/1991	7.6	(-)	(-)	1747	2/1/1994	4.9	(-)	(-)
1378	1/11/1991	7	(-)	(-)	1749	2/8/1994	4.8	(-)	(-)
1394	7/30/1991	5.4	(-)	(-)	1763	7/26/1994	3.4	(-)	(-)
1402	9/17/1991	6.3	(-)	(-)	1764	7/26/1994	3.9	(-)	(-)
1430	12/10/1991	7.1	(-)	(-)	1766	8/11/1994	4.4	(-)	(-)
1431	12/10/1991	4.6	(-)	(-)	1769	8/23/1994	3.4	(-)	(-)
1434	1/14/1992	7	(-)	(-)	1775	9/20/1994	4.2	(-)	(-)
1437	1/14/1992	6.9	(-)	(-)	1776	9/21/1994	3.4	(-)	(-)
1441	1/21/1992	5.7	(-)	(-)	1782	1/18/1995	3.3	(-)	(-)
1458	3/3/1992	5.8	(-)	(-)	1792	4/26/1995	3.6	(-)	(-)
1488	5/12/1992	6.6	(-)	(-)	1796	5/10/1995	3.7	(-)	(-)
1490	5/12/1992	6.7	(-)	(-)	1797	5/10/1995	3.7	(-)	(-)
1498	5/26/1992	5.6	(-)	(-)	1803	8/8/1995	3.3	(-)	(-)
1513	6/16/1992	3.6	(-)	(-)	1807	8/24/1995	3.2	(-)	(-)

Table 4: Characteristics of HIV Resistant women. Listed are the characteristics of the ML cohort who we defined as resistant for the purposes of this study. Each woman has a unique identifying code number (ML #), followed by the date of enrollment into the cohort (enrollment), the amount of time (in years) she has remained persistently seronegative by EIA. This value is calculated as date of last negative visit minus her initial visit date. EIA is the results of the last known serologic test for HIV antibodies, while PCR is the results of the HIV-PCR diagnostic test described in Materials and Methods.

Table 5A: Clustering of HIV Negative Phenotype in Sex Worker Family Members.

	Index	Seronegative	Seropositive	HIV Prevalence
Resistant	55	18	15	(15/33) = 45.5%
Susceptible	89	10	93	(93/103) = 90.3%

(OR 0.1, 95%CI 0.07-0.26, p<0.00001)

Table 5B: Clustering of HIV Negative Phenotype in non-Sex Worker Family Members. Data from Kimani et al. (Kimani, 1999).

	Index	Seronegative	Seropositive	HIV Prevalence
Resistant	58	239	37	(37/276) = 13%
Susceptible	108	202	101	(101/303) = 33%

(OR 0.31, 95%CI 0.199-0.48, p<0.00001)

Table 5A: Clustering of HIV Negative Phenotype in Sex Worker Family Members. Listed is the prevalence of HIV in 33 women from the ML cohort who are related to one of 55 index resistant women, and in 103 women from the cohort who are related to one of 89 HIV susceptible women. Chi-square analysis shows the women related to an HIV resistant woman were 10 fold less likely to be HIV positive compared to those related to a susceptible woman.

Table 5B: Clustering of HIV Negative Phenotype in non-Sex Worker Family Members. Data from Kimani et al. (Kimani, 1999). Listed is the prevalence of HIV in 276 individuals from the kindred cohort who are related to one of 58 index resistant women, and in 303 individuals from the kindred cohort who are related to one of 108 HIV susceptible women. Chi-square analysis indicates that women related to an HIV resistant woman were three fold less likely to be HIV positive compared to those related to a susceptible woman.

HIV Status in Kindred Cohort

As a direct result of this research project, a new research cohort, ML Kindred was established in 1996. Based upon our observations of decreased prevalence of HIV-1 in SW family member's (Table 5A) we investigated what effect being related to an HIV resistant woman had on HIV prevalence in relatives who were not members of the ML SW cohort. As described in Materials and Methods non-SW family members were recruited into this new cohort. Members of this cohort were classified as being *Resistant Kin*, if any of their relations within the ML cohort met our definition of resistance. They were classified as being *Susceptible Kin* if they were related to an HIV susceptible woman within the ML cohort, and had no relatives who met our definition of resistance.

HIV serology was used to determine infection status for all family members and they were classified as either HIV infected or HIV uninfected. HIV negative individuals could not be classified as resistant as they were neither SW's followed in our study, nor have they been under follow-up for the 3 years necessary to be classified as resistant. Initial results from this cohort gathered for the purpose of this study showed a trend towards a less likely chance of infection if related to an HIV resistant woman. However, these numbers were small, and may be confounded by differences in sex habits, or behavior that this study (due to its small size) was unable to answer. For this reason, these initial results were used as the foundation for a much larger study further discussed by Kimani et al. (Kimani, 1999). The results of this study supported the validity of the earlier findings. A summary of the larger data set is shown in Table 5B and showed a reduced likelihood of infection. To summarize, 37/276 (13%) were HIV infected in those

related to a resistant woman compared to 101/303 (33%) in those related to a susceptible woman (OR 0.31, 95%CI 0.199-0.48, $p<0.00001$).

The individuals used in the preliminary study were the study subjects in a number of the following studies. HIV uninfected kindred of both resistant and susceptible individuals were used as subjects and controls for the immunologic tests described below.

Immunologic Determination of Resistance Phenotype

Natural Killer Cell /Lymphokine Activated Killer Cell Assays

Natural killer cells are an important part of the innate immune response to pathogens. They are responsible for the lytic destruction of cells that exhibit aberrant expression of MHC Class I molecules and play an important role in the immune response to pathogens including viruses and other intracellular pathogens (Biron et al., 1999). NK cells appear to be upregulated by a number of Type-1 cytokines (Raulet, 1999). If resistant women do have increased type-1 responses this may be reflected in higher NK activity in these women. We decided to investigate if natural killer cell activity was associated with resistance to infection by HIV-1. We determined NK activity of PBMC's from 25 resistant and 19 susceptible members of the ML cohort, 24 uninfected resistant kin and 11 uninfected susceptible kin from the Kindred cohort, as well as in 8 North American laboratory staff as controls. NK cell activity was determined by specific lysis of a Chromium 51 (^{51}Cr) labeled NK sensitive cell line (K-562) relative to a ^{51}Cr labeled cell line resistant to NK cell lysis (CEM-NK^R). Effector cells were PBMC isolated from the above individuals.

A representative example of NK results from a single ML patient is shown in Figure 4A. The NK activity at the various effector to target ratio's appears to be titrateable and corresponds to the decreasing E:T ratios. All NK data used in the following analyses showed this titration effect. Any NK data that did not appear to be titratable was discarded from the analysis.

The NK activity at 50:1 was compared among all the study groups and is shown in Figure 4B. The mean NK levels at 50:1 are indicated for all groups, with standard error of the mean indicated by the bars. The data appeared to be normally distributed (Kolmogorov-Smirnov test (KS test), therefore parametric analysis were used. Both Analysis of Variance (ANOVA) and student t-test were used to test for statistically significant differences in the overall data set (ANOVA) and to test for significant differences between individual study groups (t-test). As is evident from Figure 4B there did not appear to be any significant differences in NK activity in the groups studied, except that the mean NK activity of the North American controls was significantly higher than all the African study groups ($p<0.05$). Similar results were obtained at the 25:1 and 12.5:1 E:T ratios (data not shown).

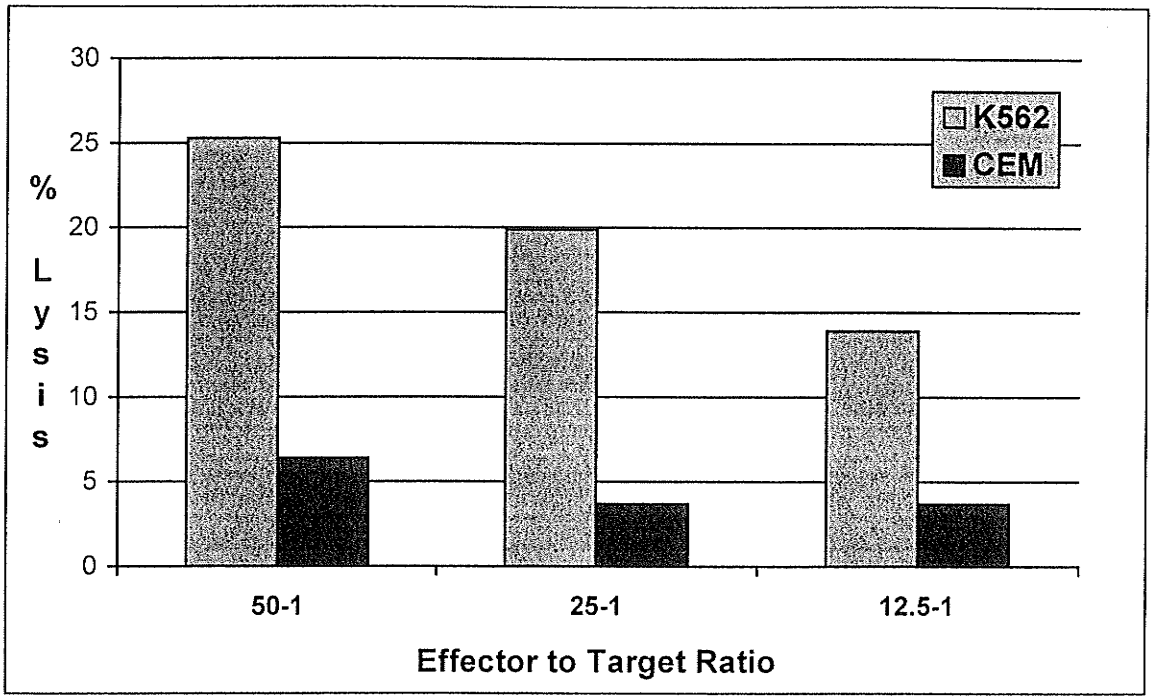

Figure 4A: Natural Killer Cell activity of ML 887 against Target Cell Lines.

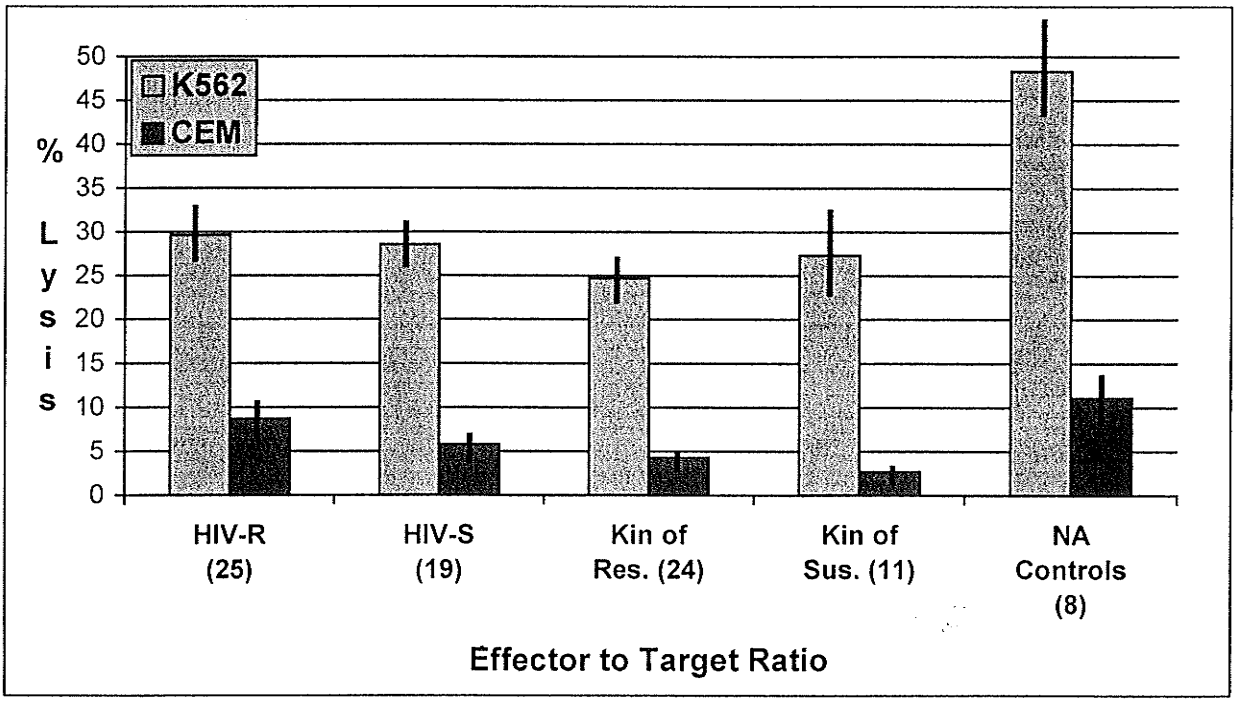

Figure 4B: Mean NK Activity at 50:1 E:T Ratio for 5 Study Groups.

Figure 4A: Natural Killer Cell Activity of ML 887 against Target Cell Lines. Percent specific lysis as measured by Cr^{51} release assay (as described in methods) of two target cell lines; K-562 (NK-sensitive) and CEM-NK^R (NK resistant) by PBMC's from ML 887. The lytic activity at the three effector to target ratios is shown. The values shown are the mean values of triplicate wells.

Figure 4B: Mean NK Activity at 50:1 E:T Ratio for 5 Study Groups. The mean percent specific lysis of the two target cell lines as measured by Cr^{51} release assay is shown for the 5 study groups; Resistant SW's, Susceptible SW's, Kindred of Resistant SW's, Kindred of Susceptible SW's and North American Controls (the numbers in each group indicated in brackets). The error bars indicate standard error of the mean.

Concurrent with the investigation of NK activity, the lytic activity of lymphokine activated killer cells was investigated in a subset of the individuals assayed for their NK activity. LAK cells are a functionally and phenotypically diverse set, and include all cells that exhibit lytic activity, including NK cells, non-specific CD8+ T lymphocytes as well as others (Frederick et al., 1997). They are upregulated by specific lymphokines (like IL-2) and in this case by the addition of recombinant IL-2 to the culture media. This assay is less specific than the NK assay as it measures a marker of an individuals non-specific (total) lytic activity. As can be seen from Figure 5A the lytic activity of ML 1785K5's PBMC against the NK sensitive cell line increased, although not substantially with the addition of IL-2 to the media and a further few days of culture. The LAK activity was again titrateable, and proportional to the amount of effector cells used. A more dramatic increase of NK-non-specific lysis is observable in the CEM-NK^R cell line. This is most likely the result of the IL-2 upregulation of a number of lytic cell types other than NK cells. If we again compare the mean NK and LAK levels of the five study groups (Figure 5B), we see that there does not appear to be any significant differences in LAK activity against the NK sensitive cell line nor against the NK resistant cell line among the study groups. This was in contrast to the NK results where we observed statistically significant differences between the NA controls and the African study groups.

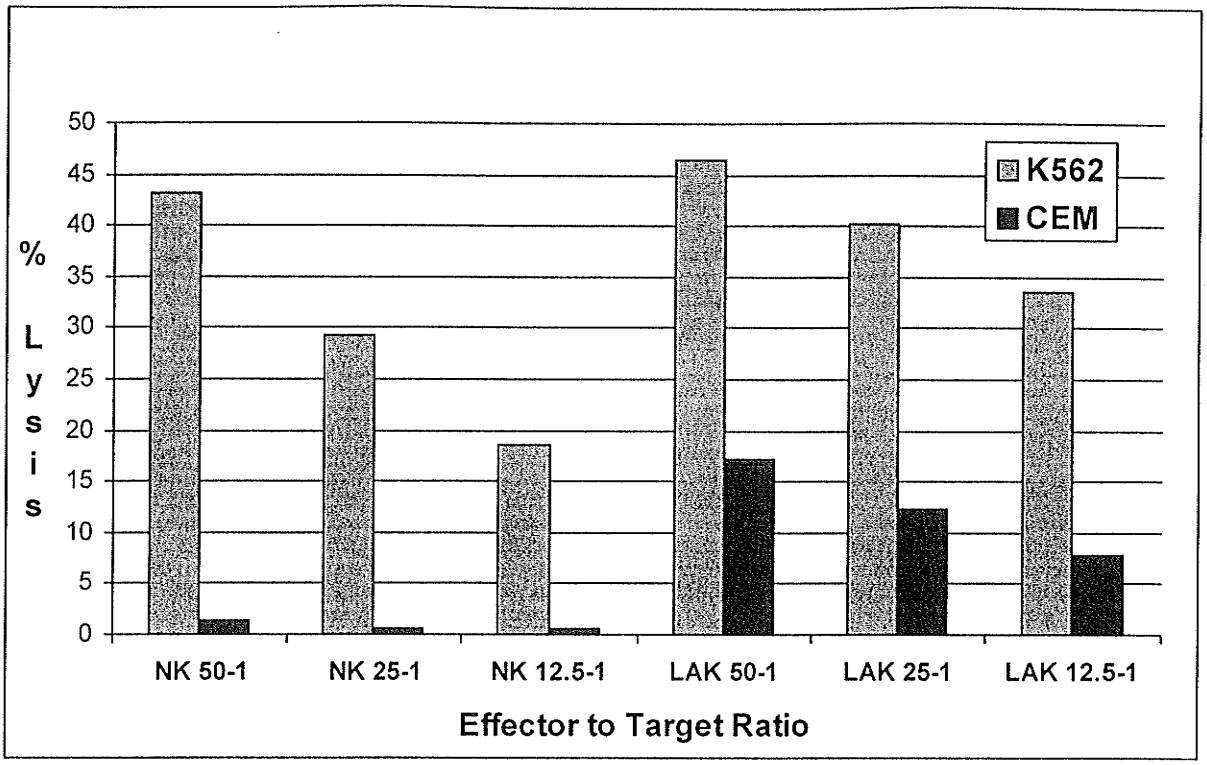

Figure 5A: Natural Killer and Lymphokine Activated Killer Cell Activity of Kindred 1787k5 Against Target Cell lines.

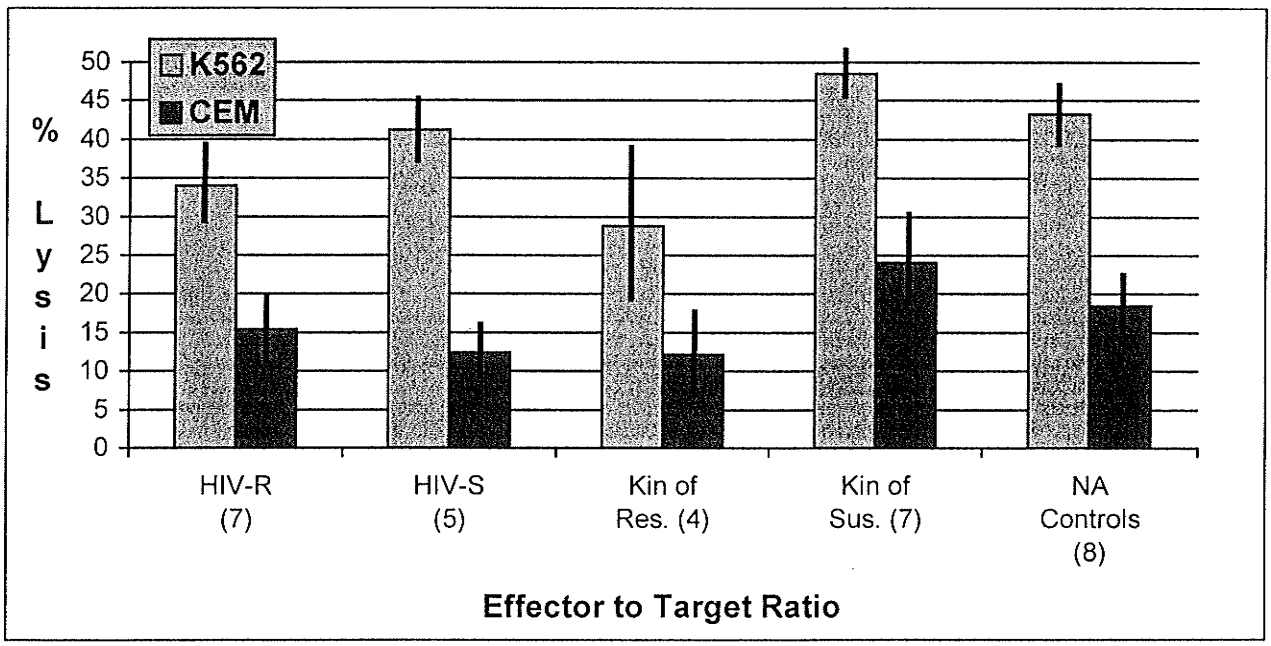

Figure 5B: Mean LAK Activity at 50:1 E:T Ratio for 5 Study Groups.

Figure 5A: Natural Killer and Lymphokine Activated Killer Cell Activity of Kindred 1787k5 Against Target Cell lines. Percent specific lysis as measured by Cr^{51} release assay (as described in methods) of two target cell lines; K-562 (NK-sensitive) and CEM-NK^R (NK resistant) by PBMC's of Kindred 1787K5 is shown for both NK and LAK assays. The lytic activity at the three effector to target ratios is shown. The values shown are the mean values of triplicate wells.

Figure 5B: Mean LAK Activity at 50:1 E:T Ratio for 5 Study Groups. The mean percent specific lysis of the two target cell lines as measured by Cr^{51} release assay is shown for the 5 study groups; Resistant SW's, Susceptible SW's, Kindred of Resistant SW's, Kindred of Susceptible SW's and North American Controls (the number in each group are indicated in brackets). The error bars indicate standard error of the mean.

Lymphocyte Proliferation Assays.

In a secondary immune response, T-lymphocytes will proliferate in response to stimulation by recognizable cognate antigens (Abbas, Murphy and Sher, 1996). HIV resistant women appear to have an altered immune response in that they have cellular, but not humoral immune responses to HIV (Fowke et al., 2000). This is in contrast to the vast majority of HIV infected individuals who exhibit both humoral and cellular responses to the virus. It is possible that these resistant women may show an altered secondary immune response to other pathogens, or commensal organisms. In addition, preliminary evidence from this cohort had suggested that PBMC's from HIV resistant women showed an increased production of IL-2, both unstimulated, and in response to polyclonal activation by Phytohemagglutinin (PHA) (Fowke et al., 2000). For these reasons we decided to investigate the proliferative response (as measured by IL-2 production) of T-lymphocytes and other cell types to a panel of common environmental antigens and pathogens.

As described in Materials and Methods, PBMC from 23 resistant women, 9 susceptible women, 15 kindred of resistant women, 11 kindred of susceptible women and 5 low-risk HIV negative controls were assayed for their proliferative response to media (negative control), PHA (polyclonal activator), Influenza antigens (viral recall), Tetanus toxoid antigens (vaccine recall), *Candida albicans* antigens (commensal recall), and Purified protein derivative (PPD) of *Mycobacterium tuberculosis* (vaccine recall).

Figures 6-11 show the responses to the stimulating antigen in each of the 5 study groups. Stimulation is expressed as the amount of IL-2 produced (pg/ml) in response to

the mitogen as well as being expressed as the stimulation index (SI) which calculates the specific increase in IL-2 production relative to the media alone.

The cut off values for these assays were 7 pg/ml, samples that scored below this cut off value were scored for data analysis as 7 pg/ml, as this would be the most conservative estimate of IL-2 production. SI's are derived from taking the total amount of IL-2 produced, and dividing it by the amount produced in the media alone. SI's of greater than or equal to 2 are considered to be a positive immune response to that mitogen. The majority of the data did not appear to follow normal distribution (K-S test); therefore non-parametric analyses were used to analyze this data set.

As can be seen in Figure 6, there did not appear to be much difference in the amount of IL-2 produced by unstimulated or "resting" PBMC's. A non-parametric comparison of all groups showed that there was no significant difference in IL-2 production among the 5 groups (Kruskal-Wallis). Although HIV susceptible women, appeared to have a larger "resting" response to media alone than the other groups, This difference was not statistically significant even when comparing HIV susceptibles to each group individually (2 independent samples, Mann-Whitney test).

Analysis of the IL-2 response to antigen and mitogen stimulation (Figures 7-11) did not appear to show any significant differences in the various study groups response to these antigens by the Kruskal-Wallis test. However, some antigens and mitogens such as PHA (Figure 7), FLU (Figure 8) and CAND (Figure 10) appeared to have a variable effect on the responses of some of the different study groups.

Both the mean IL-2 response and the SI response to PHA appeared to be depressed in the HIV susceptible (or infected) group. As described, this difference was not significant by the K-W test, nor when comparing each group individually (Mann-

Whitney). The response to FLU also appeared to be decreased in both susceptibles, and in the low-risk control group. Again, this was not significant when compared overall (KW test), but when each group was compared individually (Mann-Whitney) to groups that had high IL-2 responses a significant difference was observed (Susceptibles and Control compared to Resistants, $p<0.05$, Susceptibles and Control compared to Kin of resistants, $p<0.05$). Similar results were also observed in the CAND responses, The Susceptible group, and the Control group were significantly depressed only when compared individually to high responder groups. (Susceptible vs Resistant $p<0.05$, Control vs Resistant $p<0.05$)

The only observable pattern to these differences was that the HIV susceptible group appeared to have consistently depressed responses to most of the antigens (PHA, FLU and CAND). These "depressed" responses were duplicated in the low-risk control group. Once again, the differences between the groups were not statistically significant as measured by Kruskal-Wallis but when compared individually (2 independent sample, Mann-Whitney) to resistants, kin of resistants and kin of susceptibles, some significant differences were observed.

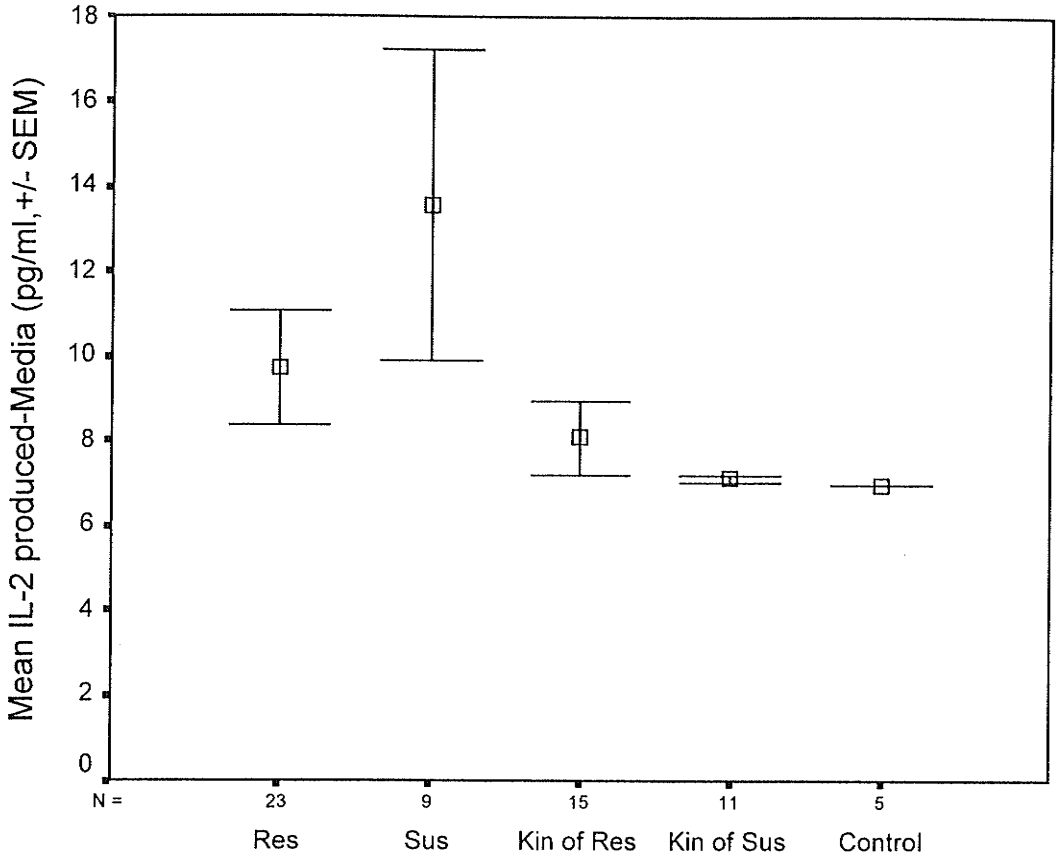

Figure 6: Mean IL-2 Produced in Response to Media in the 5 Study Groups.

Figure 6: Mean IL-2 Produced in Response to Media in the 5 Study Groups. The mean response of PBMC after stimulation with media as measured by the release of IL-2 into the culture supernatant. The number of individuals in each of the five groups tested (Resistant SW's, Susceptible SW's, Kin of Resistant SW's, Kin of Susceptible SW's, and low risk Kenyan Controls) is shown on the X axis. The square box is the mean IL-2 produced by each specific group, while the error bars represent the standard error of the mean.

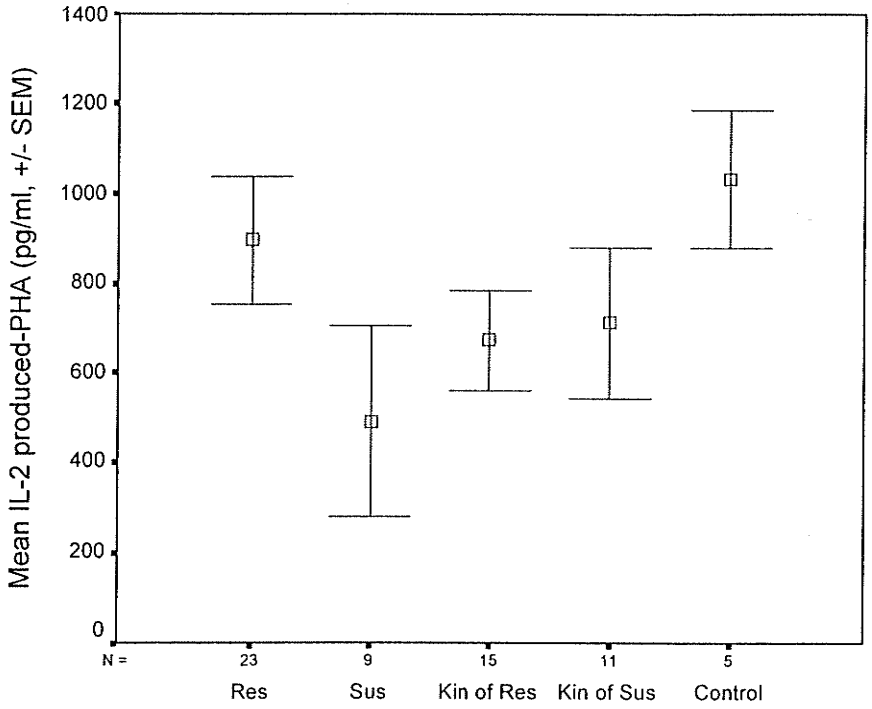

Figure 7A: Mean IL-2 Produced in Response to PHA in the 5 Study Groups.

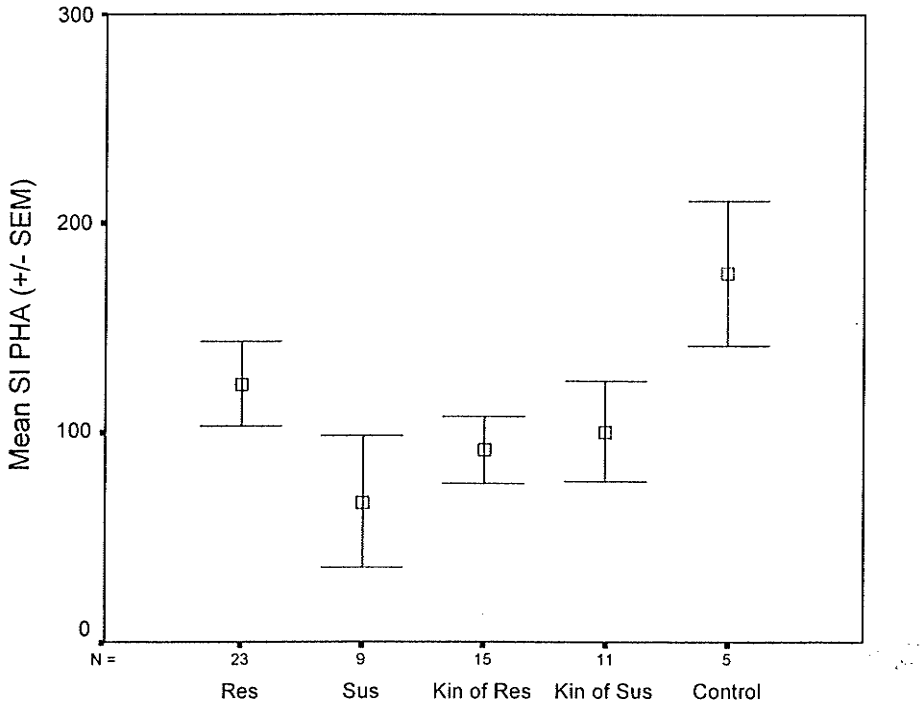

Figure 7B: Mean Stimulation Indexes in Response to PHA in the 5 Study Groups.

Figure 7A: Mean IL-2 Produced in Response to PHA in the 5 Study Groups. The mean response of PBMC after stimulation with PHA as measured by the release of IL-2 into the culture supernatant. The number of individuals in each of the five groups tested (Resistant SW's, Susceptible SW's, Kin of Resistant SW's, Kin of Susceptible SW's, and low risk Kenyan Controls) is shown on the X axis. The square box is the mean IL-2 produced by each specific group, while the error bars represent the standard error of the mean.

Figure 7B: Mean Stimulation Indexes in Response to PHA in the 5 Study Groups. The mean response of PBMC after stimulation with PHA as measured by the stimulation index (SI) in response to antigen/mitogen (SI = response to antigen (pg/ml)/ response to media (pg/ml)). The number of individuals in each of the five groups tested (Resistant SW's, Susceptible SW's, Kin of Resistant SW's, Kin of Susceptible SW's, and low risk Kenyan Controls) is shown on the X axis. The square box is the mean SI of each specific group, while the error bars represent the standard error of the mean.

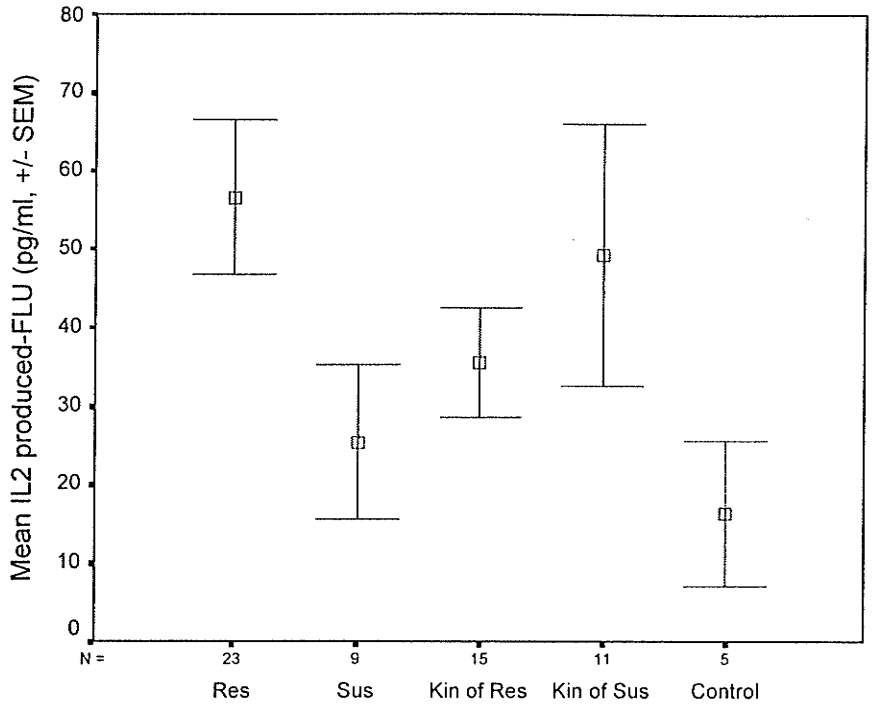

Figure 8A: Mean IL-2 Produced in Response to FLU in the 5 Study Groups.

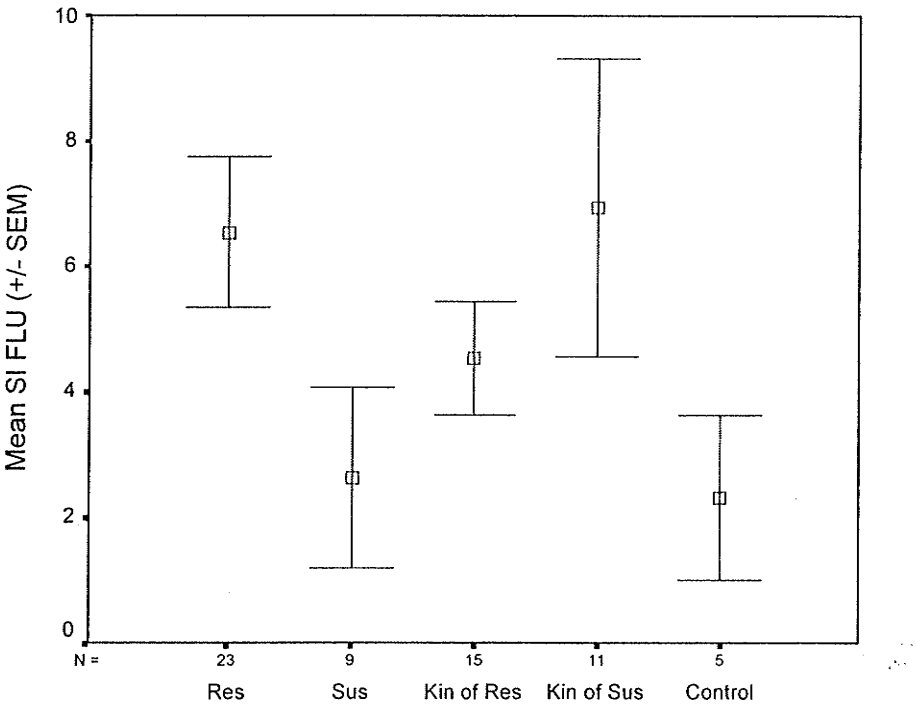

Figure 8B: Mean Stimulation Indexes in Response to FLU in the 5 Study Groups.

Figure 8A: Mean IL-2 Produced in Response to FLU in the 5 Study Groups. The mean response of PBMC after stimulation with FLU as measured by the release of IL-2 into the culture supernatant. The number of individuals in each of the five groups tested (Resistant SW's, Susceptible SW's, Kin of Resistant SW's, Kin of Susceptible SW's, and low risk Kenyan Controls) is shown on the X axis. The square box is the mean IL-2 produced by each specific group, while the error bars represent the standard error of the mean.

Figure 8B: Mean Stimulation Indexes in Response to FLU in the 5 Study Groups. The mean response of PBMC after stimulation with FLU as measured by the stimulation index (SI) in response to antigen/mitogen (SI = response to antigen (pg/ml)/ response to media (pg/ml)). The number of individuals in each of the five groups tested (Resistant SW's, Susceptible SW's, Kin of Resistant SW's, Kin of Susceptible SW's, and low risk Kenyan Controls) is shown on the X axis. The square box is the mean SI of each specific group, while the error bars represent the standard error of the mean.

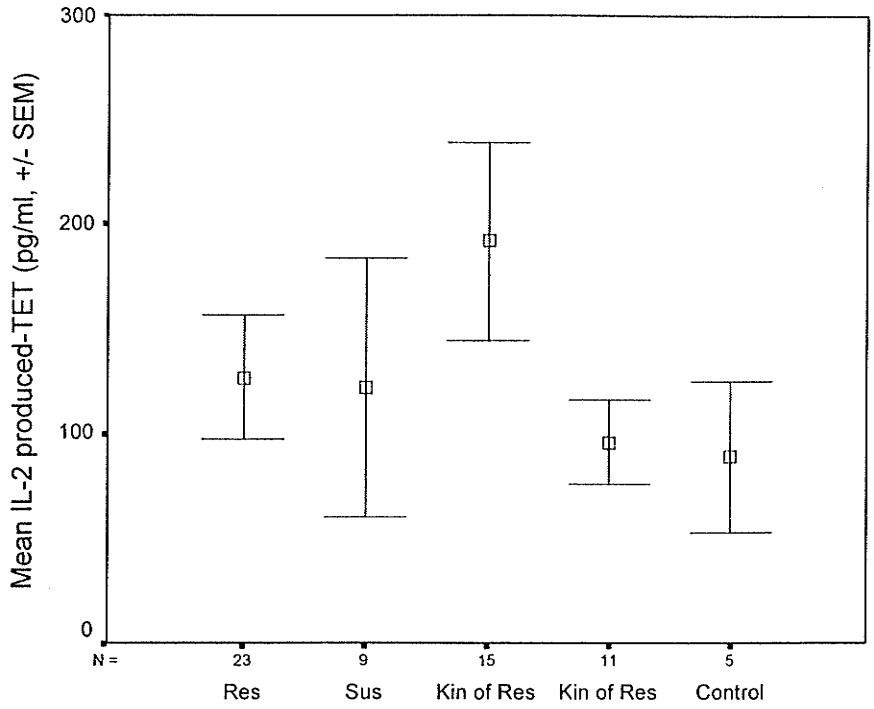

Figure 9A: Mean IL-2 Produced in Response to TET in the 5 Study Groups.

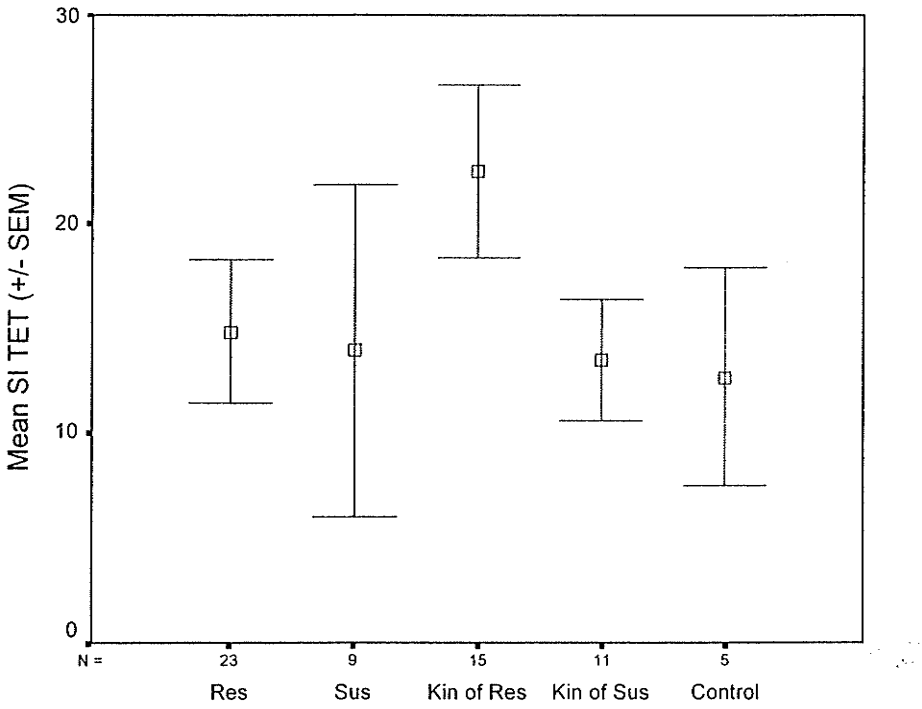

Figure 9B: Mean Stimulation Indexes in Response to TET in the 5 Study Groups.

Figure 9A: Mean IL-2 Produced in Response to TET in the 5 Study Groups. The mean response of PBMC after stimulation with TET as measured by the release of IL-2 into the culture supernatant. The number of individuals in each of the five groups tested (Resistant SW's, Susceptible SW's, Kin of Resistant SW's, Kin of Susceptible SW's, and low risk Kenyan Controls) is shown on the X axis. The square box is the mean IL-2 produced by each specific group, while the error bars represent the standard error of the mean.

Figure 9B: Mean Stimulation Indexes in Response to TET in the 5 Study Groups. The mean response of PBMC after stimulation with TET as measured by the stimulation index (SI) in response to antigen/mitogen (SI = response to antigen (pg/ml)/ response to media (pg/ml)). The number of individuals in each of the five groups tested (Resistant SW's, Susceptible SW's, Kin of Resistant SW's, Kin of Susceptible SW's, and low risk Kenyan Controls) is shown on the X axis. The square box is the mean SI of each specific group, while the error bars represent the standard error of the mean.

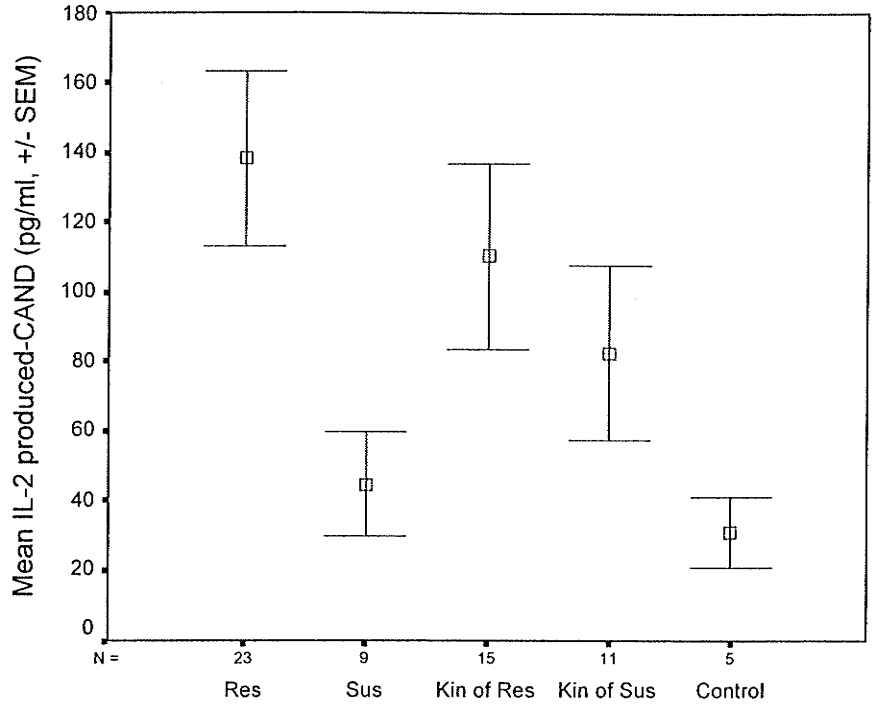

Figure 10A: Mean IL-2 Produced in Response to CAND in the 5 Study Groups.

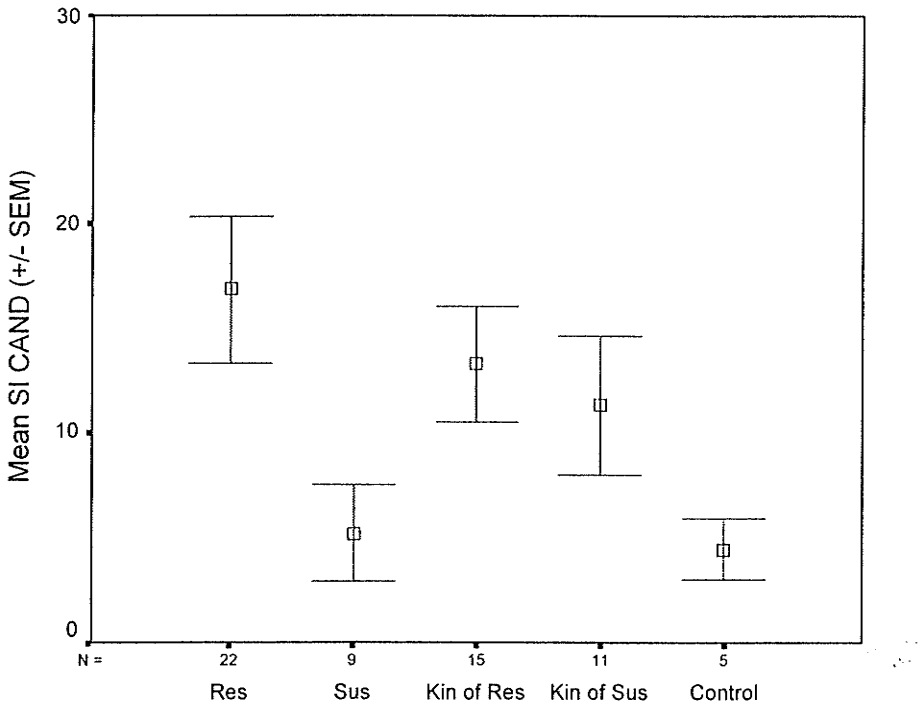

Figure 10B: Mean Stimulation Indexes in response to CAND in the 5 study groups.

Figure 10A: Mean IL-2 Produced in Response to CAND in the 5 Study Groups. The mean response of PBMC after stimulation with CAND as measured by the release of IL-2 into the culture supernatant. The number of individuals in each of the five groups tested (Resistant SW's, Susceptible SW's, Kin of Resistant SW's, Kin of Susceptible SW's, and low risk Kenyan Controls) is shown on the X axis. The square box is the mean IL-2 produced by each specific group, while the error bars represent the standard error of the mean.

Figure 10B: Mean Stimulation Indexes in Response to CAND in the 5 Study Groups. The mean response of PBMC after stimulation with CAND as measured by the stimulation index (SI) in response to antigen/mitogen (SI = response to antigen (pg/ml)/ response to media (pg/ml)). The number of individuals in each of the five groups tested (Resistant SW's, Susceptible SW's, Kin of Resistant SW's, Kin of Susceptible SW's, and low risk Kenyan Controls) is shown on the X axis. The square box is the mean SI of each specific group, while the error bars represent the standard error of the mean.

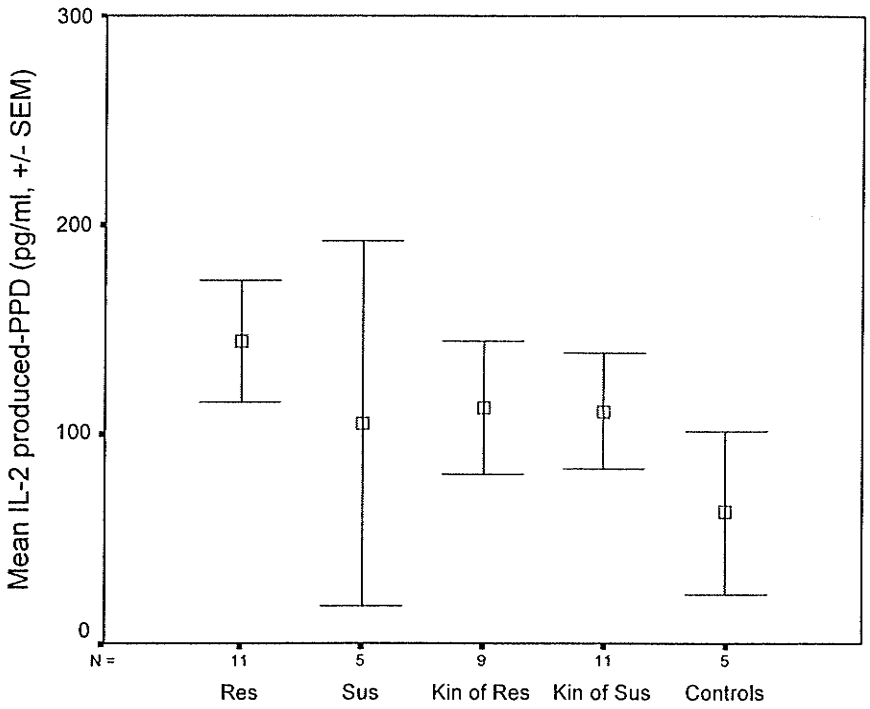

Figure 11A: Mean IL-2 Produced in Response to PPD in the 5 Study Groups.

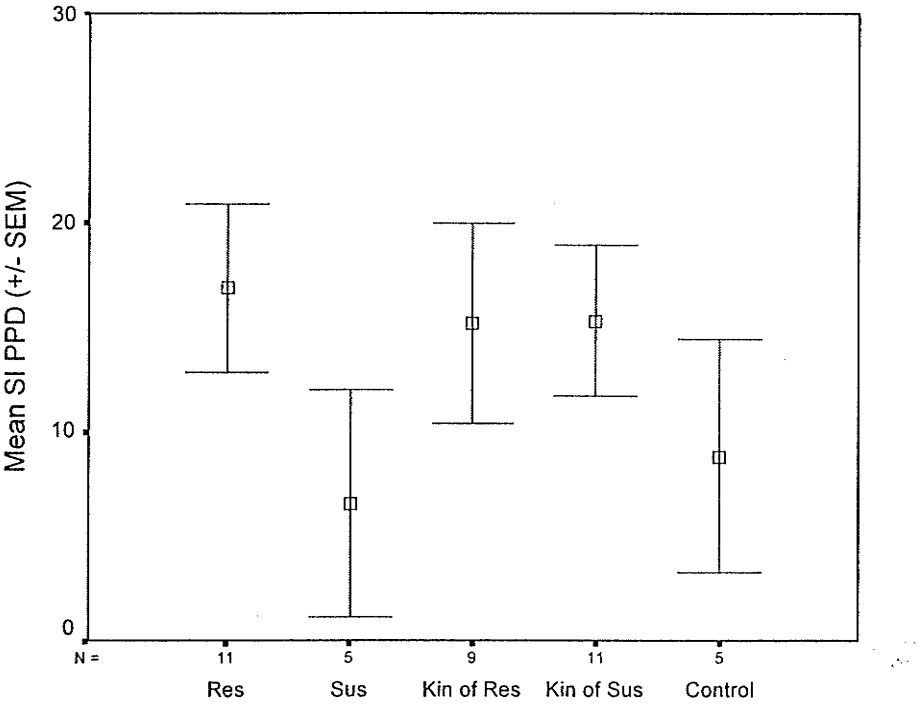

Figure 11B: Mean Stimulation Indexes in Response to PPD in the 5 Study Groups.

Figure 11A: Mean IL-2 Produced in Response to PPD in the 5 Study Groups. The mean response of PBMC after stimulation with PPD as measured by the release of IL-2 into the culture supernatant. The number of individuals in each of the five groups tested (Resistant SW's, Susceptible SW's, Kin of Resistant SW's, Kin of Susceptible SW's, and low risk Kenyan Controls) is shown on the X axis. The square box is the mean IL-2 produced by each specific group, while the error bars represent the standard error of the mean.

Figure 11B: Mean Stimulation Indexes in Response to PPD in the 5 Study Groups. The mean response of PBMC after stimulation with PPD as measured by the stimulation index (SI) in response to antigen/mitogen (SI = response to antigen (pg/ml)/ response to media (pg/ml)). The number of individuals in each of the five groups tested (Resistant SW's, Susceptible SW's, Kin of Resistant SW's, Kin of Susceptible SW's, and low risk Kenyan Controls) is shown on the X axis. The square box is the mean SI of each specific group, while the error bars represent the standard error of the mean.

Monocyte/Macrophage Cytokine Assays

Cells of the monocyte lineage are likely to be one of the first host cell types encountered by HIV-1 during natural infection (Pope, 1999). It is also likely that the initial monokine response by monocytes or macrophages may play a role in the development of the immune response to that pathogen. We have hypothesized that Type-1 or cell mediated immune responses may be protective against infection by HIV-1. Two interleukins produced by monocytes and macrophage in response to activation are IL-10 and IL-12, both of which are highly important in the development of a Type-2 and Type-1 immune responses respectively. If HIV resistant women do preferentially develop a Type-1 response to HIV-1, it is conceivable that this may be regulated by specific cytokines, such as IL-10 or IL-12 released by monocytes or macrophage. For this reason we tested the production of both IL-10 and IL-12 in monocytes specifically activated by a preparation of formalin inactivated, heat killed Cohen strain of *Staphylococcus aureus* (SAC).

As described in Materials and Methods, PBMC from 22 resistant, 19 susceptible, 21 kin of resistants, 14 kin of susceptibles 5 low risk Kenyan controls and 8 North American (NA) controls were stimulated with SAC and the quantity of IL-10 and IL-12 released into the supernatant were analyzed by ELISA. The data did not appear to have a normal distribution (K-W test); therefore non-parametric analyses were used. Figure 12 shows the mean release of IL-10 into the culture supernatant from unstimulated cells (media) or upon stimulation by SAC. As is evident from Figure 12, there were no significant differences in the IL-10 levels released by unstimulated macrophage and monocytes. Upon SAC activation however, there did appear to be some differences among the study groups in that HIV susceptible individuals produced less IL-10 upon

stimulation than some of the other groups, specifically compared to HIV resistant, kin of resistant, and kin of susceptible groups ($p<0.05$, Mann-Whitney).

Figure 13 shows the mean level of IL-12 produced in response to media and SAC in the various study groups as detected by an ELISA using a monoclonal antibody specific to the IL-12 p70 heterodimer. This heterodimer consists of a p40 and a p35 subunit, which together make up the intact, functional IL-12 molecule. IL-12 (p70) levels both stimulated by media and SAC among the study groups were similar in most groups. The Kruskal-Wallis test indicated a significant difference in the IL-12 production among the study groups. The HIV resistant individuals appeared to produce overall higher levels of IL-12 both unstimulated, and upon activation with SAC compared to HIV susceptible individuals, kin of susceptibles and kin of resistants. However this was only significant when compared to the kin of susceptibles group (Mann-Whitney, $p<0.05$) for unstimulated IL-12 p70 production, and compared to the kin of resistant group for SAC stimulated p70 production (Mann-Whitney, $p<0.05$)

There was some concern regarding this experiment in that the IL-12 response by the cells did not appear to respond to SAC stimulation to the same magnitude that was evident by the IL-10 response. For this reason we also assayed for the production of one of the IL-12 subunits, specifically p40. Figure 14 shows the mean release of IL-12 (p40) by both resting cells and upon SAC activation. There was definitely a large increase in p40 production upon stimulation by SAC. Again, the KW test suggested that there were significant differences in the levels of IL-12 p40 produced among the groups ($p<0.05$). The p40 production did not entirely confirm the observation made about the p70 increase observed in the resistant group. Unstimulated IL-12 p40 levels did appear to be increased in the resistant group compared to the kin of susceptible group (Mann-Whitney, $p<0.05$).

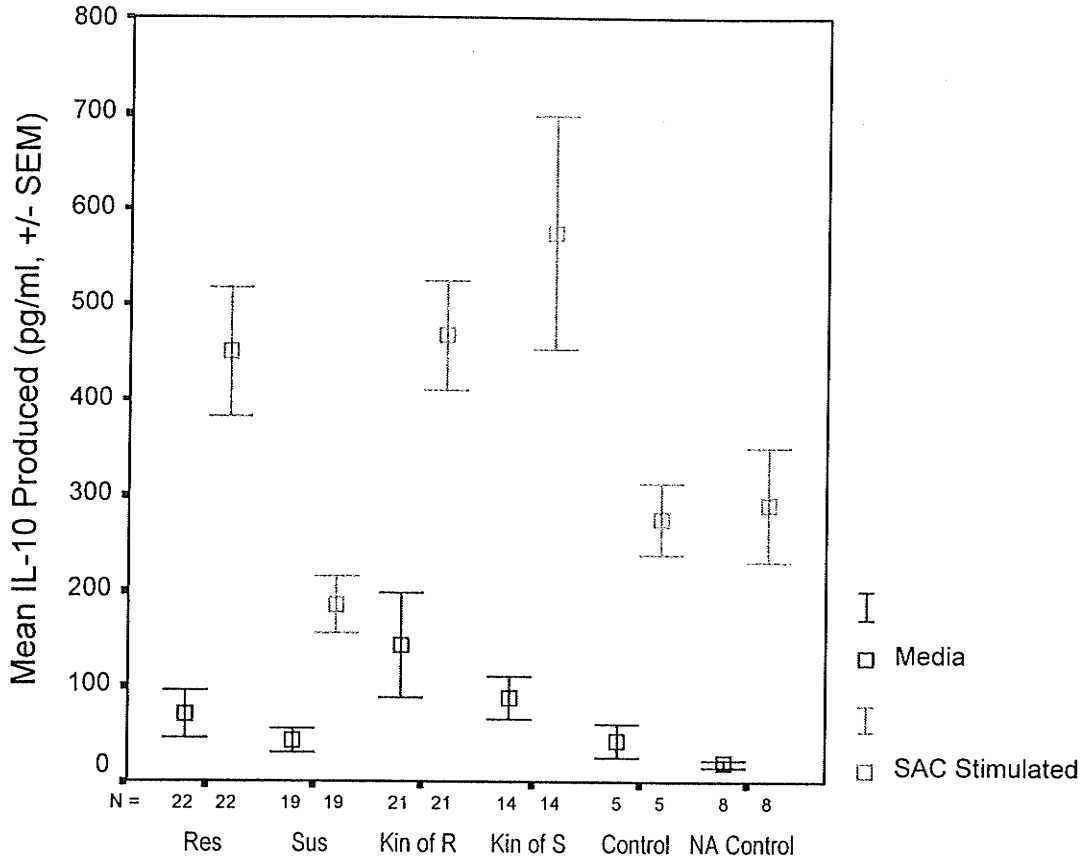

Figure 12: Mean IL-10 Production by PBMC's Stimulated with Media and SAC.

Figure 12: Mean IL-10 Production by PBMC's Stimulated with Media and SAC. The mean response of PBMC after stimulation with Media alone (black bars in each group) and with *Staphylococcus aureus* Cohen Strain Antigen (SAC) (grey bars in each group) as measured by the release of IL-10 into the culture supernatant. The number of individuals in each of the six groups tested (Resistant SW's, Susceptible SW's, Kin of Resistant SW's, Kin of Susceptible SW's, low risk Kenyan Controls, and North American Controls) is shown. The square box is the mean IL-10 produced by each specific group, while the error bars represent the standard error of the mean.

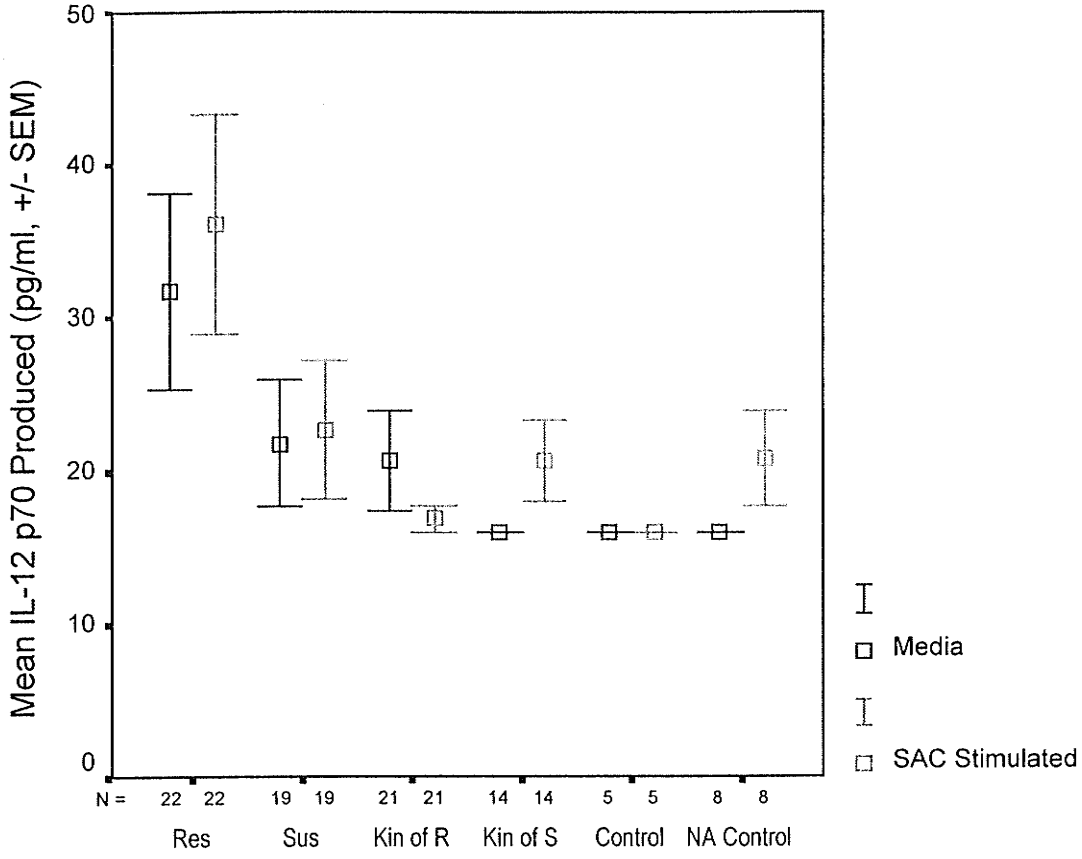

Figure 13: Mean IL-12 (p70 heterodimer) Production by PBMC's Stimulated with Media and SAC.

Figure 13: Mean IL-12 (p70 heterodimer) Production by PBMC's Stimulated with Media and SAC. The mean response of PBMC after stimulation with Media alone (black bars in each group) and with *Staphylococcus aureus* Cohen Strain Antigen (SAC) (grey bars in each group) as measured by the release of the IL-12 heterodimer into the culture supernatant. The number of individuals in each of the six groups tested (Resistant SW's, Susceptible SW's, Kin of Resistant SW's, Kin of Susceptible SW's, low risk Kenyan Controls, and North American Controls) is shown. The square box is the mean IL-12 (p70) produced by each specific group, while the error bars represent the standard error of the mean.

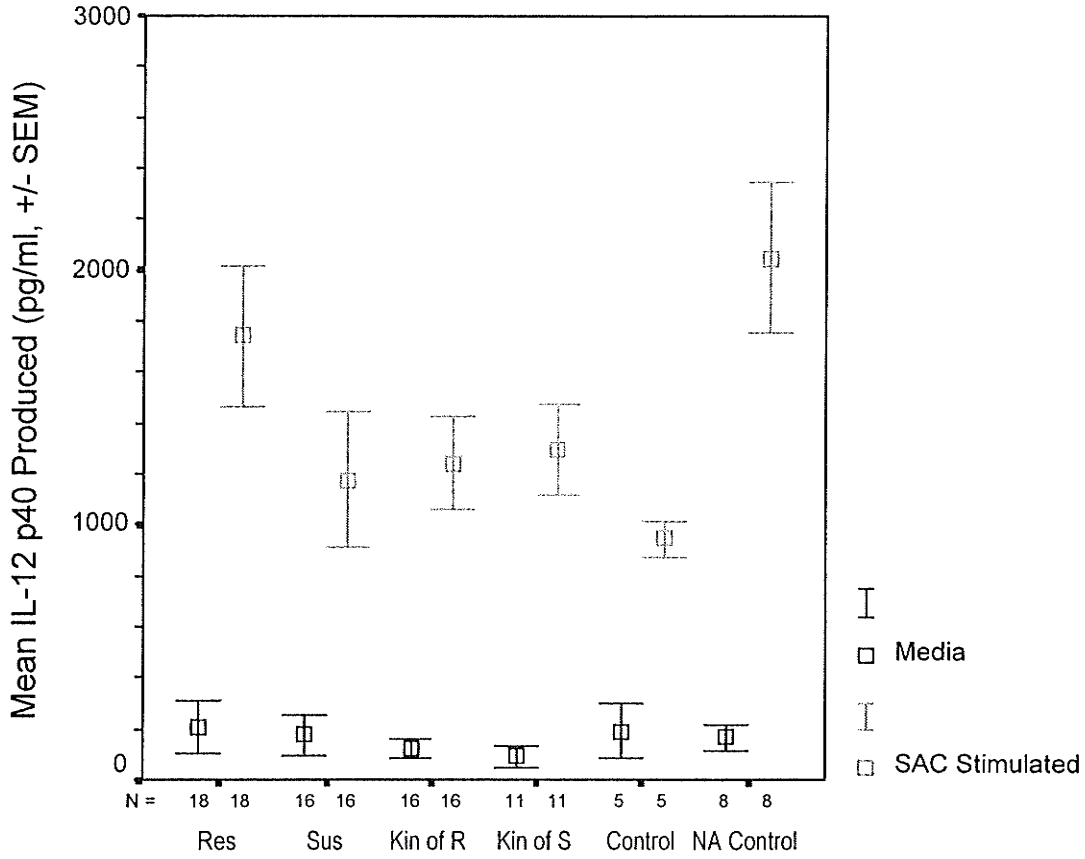

Figure 14: Mean IL-12 (p40 subunit) Production by PBMC's Stimulated with Media and SAC.

Figure 14: Mean IL-12 (p40 subunit) Production by PBMC's Stimulated with Media and SAC. The mean response of PBMC after stimulation with Media alone (black bars in each group) and with *Staphylococcus aureus* Cohen Strain Antigen (SAC) (grey bars in each group) as measured by the release of IL-12 p40 subunit into the culture supernatant. The number of individuals in each of the six groups tested (Resistant SW's, Susceptible SW's, Kin of Resistant SW's, Kin of Susceptible SW's, low risk Kenyan Controls, and North American Controls) is shown. The square box is the mean IL-12 (p40) produced by each specific group, while the error bars represent the standard error of the mean.

Candidate Gene Analysis

We decided to investigate a number of candidate genes that have been shown to be involved in altered susceptibility to HIV and other STI's, as well as genes that have been implicated in other Type-1 or Type-2 diseases or conditions. We are studying a group who can be epidemiologically defined as being resistant to infection by HIV-1, and this protection may be mediated by a predominantly cellular immune response to HIV-1. Clearly, if a genetic basis for resistance does exist, it may be the result of polymorphisms in any of the genes involved in the cellular or humoral immune response to HIV-1 or in genes that may effect HIV's replicative cycle.

Vitamin D Receptor

The Vitamin D receptor (VDR) is responsible for the uptake of the active metabolite of Vitamin D (1,25 dihydroxyvitamin D3) which is an important immunoregulatory hormone (Lemire, 1995). VDR is expressed on a number of cell types including monocytes and activated T and B lymphocytes (Manolagas et al., 1994). A number of polymorphic markers within the VDR have been associated with genetic variation in bone density, presumably due to Vitamin D's important role in calcium metabolism (Dusso and Brown, 1998). Recently however, a specific "mutant" genotype in a *Taq*-1 RFLP polymorphism in codon 352 in intron 8 of the VDR has been associated with altered susceptibility to Hepatitis B, Tuberculosis and with altered pathogenesis by *Mycobacterium leprae* (Bellamy and Hill, 1998; Roy et al., 1999). We investigated this polymorphism in our SW cohort by comparing the prevalence of the mutant genotype in

HIV resistant and HIV susceptible individuals. Genotyping for this polymorphism was carried out as described in Materials and Methods on 66 resistant women, and 322 susceptible women. As can be seen in Table 6A the prevalence of the 3 VDR genotypes for this polymorphism does not appear to be significantly different in the two study groups.

An alternate way of looking at this data is shown in Table 6B, which indicates the gene frequency of the mutant allele in each of the two populations. This analysis looks at the presence of each chromosomal copy of the allele independently; thus the denominator is twice that of the first analysis. Since all humans are diploid each individual would contribute either zero, one or two copies of the allele, to each study group. Again no significant differences were observed between the two populations.

Another analysis commonly used to identify associations between genetic polymorphism and altered disease susceptibility is to compare the rate at which different groups or genotypes become infected by HIV-1. In this case we compared seroconversion rates in HIV-1 susceptible individuals among the 3 different genotypes by looking at HIV free survival, or time until HIV-1 infected (Kaplain-Meier analysis, data not shown) and found no appreciable differences among the genotypes.

Table 6A: VDR Genotypes and Lack of Association with Resistance.

VDR Genotype		HIV Resistant		HIV Susceptible
0 (mut/mut)		12 (18.2%)		37 (11.5%)
1 (mut/wt)		20 (30.3%)		104 (32.3%)
2 (wt/wt)		34 (51.5%)		181 (56.2%)
	Total	66		322

X^2=2.22, p = 0.33

Table 6B: VDR *Taq I* Mutation Gene Frequencies and Lack of Association with Resistance.

VDR *Taq I* Mutant Allele		HIV Resistant		HIV Susceptible
Present		44 (33.3%)		178 (27.6%)
Absent		88 (66.7%)		466 (72.4%)
	Total	132		644

X^2=1.47, p = 0.23

Table 6A: VDR Genotypes and Lack of Association with Resistance. The Table shows the subjects genotyped for the VDR *Taq I* polymorphism. The number of individuals with a specific genotype in the two study groups is shown, along with the percentage of each group that has that genotype. VDR 0 is homozygous for the mutation; VDR 1 individuals are heterozygous, while VDR 2 are homozygous wild type.

Table 6B: VDR *Taq I* Mutation Gene Frequencies and Lack of Association with Resistance. The Table shows the frequency of the mutant allele within the HIV resistant and HIV susceptible populations. The values for each group were determined from Table 6A, looking at the prevalence of each allele independently, therefore n = twice the number of individuals typed above.

Since many of the known genetic polymorphisms so far associated with altered susceptibility to HIV-1 also appear to play a role in altered disease progression we further analyzed VDR genotype and mutant allele frequencies in HIV infected individuals. They were classified into one of 3 groups; Long-term non-progressors (LTNP), individuals who are infected, but do not progress to AIDS for >10 years; so-called "normal" progressors, whom in our cohort succumb to disease in 6-8 years; and rapid progressors (RP) who progress very rapidly to disease and death (<4 years). Again, VDR genotype and gene frequencies were not significantly different among the 3 groups (data not shown).

Fucosyl Transferase (Fut-2)

Fucosyl Transferase or Secretor is an enzyme involved in the secretion of Lewis and ABO blood group antigens into bodily fluids (Henry, Oriol and Samuelsson, 1995). An inactivating mutation in this gene, leads to non-secretor status in homozygous individuals, and leads to modified carbohydrate moieties on epithelial cell surfaces (Kelly et al., 1995). Non-secretor status has been associated with altered susceptibility to a number of infectious diseases including HIV and other STI's (Blackwell, 1989). Preliminary evidence suggested that secretor status may play a role in altered susceptibility to HIV-1 in a large cohort in Uganda (Ali, 1998). For this reason we genotyped 55 resistant, and 267 susceptible SWs from our cohort. As can be seen in Table 7A and 7B, secretor genotype or allele frequencies were not significantly different between the two groups. Secretor status did not appear to have any effect on seroconversion rates (data not shown), and its distribution was not associated with groups exhibiting altered disease progression (data not shown).

Table 7A: Secretor (Fut-2) Genotypes and Lack of Association with Resistance.

Secretor Genotype		HIV Resistant		HIV Susceptible
0 (mut/mut) non secretors		17 (30.9%)		50 (18.7%)
1 (mut/wt)		24 (43.6%)		152 (56.9%)
2 (wt/wt)		14 (25.5%)		65 (24.3%)
	Total	55		267

X^2=4.75, p = 0.09

Table 7B: Secretor Mutation Gene Frequencies and Lack of Association with Resistance.

Secretor Mutant Allele		HIV Resistant		HIV Susceptible
Present		58 (52.7%)		252 (47.2%)
Absent		52 (47.3%)		282 (52.8%)
	Total	110		534

X^2=0.91, p = 0.34

Table 7A: Secretor (Fut-2) Genotypes and Lack of Association with Resistance. The Table shows the subjects genotyped for the Secretor polymorphism. The number of individuals with a specific genotype in the two study groups is shown, along with the percentage of each group that has the specific genotype. Genotype 0 is homozygous for the mutation, genotype 1 individuals are heterozygous, while genotype 2 are homozygous wild type.

Table 7B: Secretor Mutation Gene Frequencies and Lack of Association with Resistance. The Table shows the frequency of the mutant allele within the HIV resistant and HIV susceptible populations. The values for each group were determined from Table 7A, looking at the prevalence of each allele independently, therefore n = twice the numbers of individuals typed above.

CCR2b

Polymorphisms in chemokine receptors were the first described genetic mechanisms linked with differences in susceptibility and pathogenesis of HIV-1. So far, an inactivating 32 base pair deletion in CCR5 (the co-receptor molecule for M-tropic HIV strains) has been associated in a number of studies with resistance to infection by HIV-1 and decreased disease progression and is the subject of a recent review (Rowland-Jones, 1999). A single nucleotide substitution in the CCR2b gene (Val>Ile 64) has been shown to be associated with decreased disease progression in some populations (Michael et al., 1997). The 32 base pair CCR5 deletion is not generally found in African populations, and none of the resistant women from our cohort so far examined display this mutation (Fowke et al., 1998). We were interested in determining if the CCR2b polymorphism was in any way associated with resistance to HIV-1 our cohort. As described in Materials and Methods we genotyped 51 resistant, and 425 susceptible members of our SW cohort for this mutation. The results are described in Table 8A. The CCR2b 64I mutation is not associated with resistance to infection by HIV-1. In fact, the mutation appears to be less prevalent in the resistant population when analyzed at the gene frequency level (Table 8B, $p<0.05$). The mutation appeared to have no effect on seroconversion rates (data not shown), but was significantly increased in the LTNP group of susceptible individuals, both at the genotypic ($p<0.03$), and the gene frequency level ($p<0.009$, Table 9A and 9B).

Table 8A: CCR2-64I Genotypes and Lack of Association with Resistance.

CCR2-64I Genotype		HIV Resistant		HIV Susceptible
0 (mut/mut)		1 (2%)		22 (5.2%)
1 (mut/wt)		12 (23.5%)		149 (35.1%)
2 (wt/wt)		38 (74.5%)		254 (59.8%)
	Total	51		425

X^2=4.38, p = 0.11

Table 8B: CCR2-64I Gene Frequencies and Inverse Association with Resistance.

CCR2-64I Mutant Allele		HIV Resistant		HIV Susceptible
Present		14 (13.7%)		193 (22.7%)
Absent		88 (86.3%)		657 (77.3%)
	Total	102		850

X^2=3.81, p = 0.05
(FET=.022), OR 0.54, 95%CI (.29-1.00)

Table 8A: CCR2-64I Genotypes and Lack of Association with Resistance. The Table shows the subjects genotyped for the CCR2-64I polymorphism. The number of individuals with a specific genotype in the two study groups is shown, along with the percentage of each group that has the specific genotype. Genotype 0 is homozygous for the mutation, genotype 1 individuals are heterozygous, while genotype 2 are homozygous wild type.

Table 8B: CCR2-64I Gene Frequencies and Inverse Association with Resistance. The Table shows the frequency of the mutant allele within the HIV resistant and HIV susceptible populations. The values for each group were determined from the Table 8A, looking at the prevalence of each allele independently, therefore n = twice the numbers of individuals typed above. The CCR2-64I allele is significantly under-represented in the HIV resistant group (FET=.022 OR .54 95%CI (0.29-1.00)).

Table 9A: CCR2-64I Genotypes and Association with Groups Exhibiting Altered Disease Progression.

CCR2-64I Genotype		HIV Infected LTNP		HIV Infected "normal" Prog.		HIV Infected Rapid Prog.
0 (mut/mut)		6 (11.8%)		15 (4.2%)		1 (6.7%)
1 (mut/wt)		23 (45.1%)		125 (35.5%)		2 (13.3%)
2 (wt/wt)		22 (43.1%)		212 (60.2%)		12 (80%)
	Total	51		352		15

X^2=11.6, 4df p = 0.021

Table 9B: CCR2-64I Gene Frequencies and Association with Groups Exhibiting Altered Disease Progression.

CCR2-64I Mutant Allele		HIV Infected LTNP		HIV Infected "normal" Prog.		HIV Infected Rapid Prog.
Allele Present		35 (34.3%)		155 (22%)		4 (13.3%)
Allele Absent		67 (65.7%)		549 (78%)		26 (86.7%)
	Total	102		704		30

X^2=9.3, 2df p = 0.01

Table 9A: CCR2-64I Genotypes and Association with Groups Exhibiting Altered Disease Progression. The Table shows the subjects genotyped for the CCR2-64I polymorphism. The number of individuals with a specific genotype in the three study groups is shown, along with the percentage of each group that has the specific genotype. The three study groups are long-term non-progressors (LTNP), "normal" progressors, and rapid progressors (RP). Genotype 0 is homozygous for the mutation, genotype 1 individuals are heterozygous, while genotype 2 are homozygous wild type. Significant differences were observed in the distribution of the CCR2-64I allele (X^2=11.6, 4df p = 0.021).

Table 9B: CCR2-64I Gene Frequencies and Association with Groups Exhibiting Altered Disease Progression. The Table shows the frequency of the mutant allele within three study populations. The values for each group were determined from the table 9A, looking at the prevalence of each allele independently, therefore n = twice the numbers of individuals typed above. The CCR2-64I allele is significantly over-represented in the long-term non-progressor group compared to both groups overall (X^2=9.3, 2df p = 0.01), and compared to each group individually. LTNP vs "normal" progressors (FET p = 0.009) OR 1.85 95%CI (1.16-2.97)) and LTNP vs RP (FET p = 0.05 OR 3.36 95%CI (1.01-12.52)).

IL-4 -590 Promoter

Recently, data from the Nairobi cohort suggest that HIV resistant women are deficient in IL-4 production compared with control groups (Trivedi et al., 2000). This is in agreement with our overall hypothesis that resistant women may be protected due to predominant Type-1 responses to HIV-1. A decreased IL-4 response to HIV-1 in particular and other antigens in general would tend to favor a Type-1 or cellular response. A single nucleotide polymorphism (-590 C > T) in the IL-4 upstream promoter region has been shown to be associated with increased allergic responses to house dust mite (Walley and Cookson, 1996), and has shown to be weakly associated with asthma and atopy (Noguchi et al., 1998). Since allergy and atopic reactions tend to be strongly influenced by IL-4, it seemed that this polymorphism may be a marker for IL-4 responsiveness in general and may play a role in the regulation of IL-4 synthesis. For this reason we investigated this mutation in our SW population. We genotyped 72 resistant and 464 susceptible women from the cohort for the IL-4 -590 mutation as described in Material and Methods. The results are described in Table 10. As seen in Table 10A and 10B this mutation was not associated with resistance in any way, nor was it associated with decreased seroconversion, altered disease progression or duration of survival (data not shown).

Table 10A: IL-4 -590 Genotypes and Lack of Association with Resistance.

IL4 (-590) Genotype		HIV Resistant		HIV Susceptible
0 (mut/mut)		32 (44.4%)		227 (48.9%)
1 (mut/wt)		35 (48.6%)		198 (42.7%)
2 (wt/wt)		5 (6.9%)		39 (8.4%)
	Total	72		464

X^2=0.93, 2df p = 0.66

Table 10B: IL-4 -590 Mutation Gene Frequencies and Lack of Association with Resistance.

IL4 (-590) Mutant Allele		HIV Resistant		HIV Susceptible
Present		99 (68.8%)		652 (70.3%)
Absent		45 (31.2%)		276 (29.7%)
	Total	144		928

X^2=0.08, 1df p = 0.79

Table 10A: IL-4 -590 Genotypes and Lack of Association with Resistance. The Table shows the subjects genotyped for the IL4 -590 polymorphism. The number of individuals with a specific genotype in the two study groups is shown, along with the percentage of each group that has the specific genotype. Genotype 0 is homozygous for the mutation, genotype 1 individuals are heterozygous, while genotype 2 are homozygous wild type.

Table 10B: IL-4 -590 Mutation Gene Frequencies and Lack of Association with Resistance. The Table shows the frequency of the mutant allele within the HIV resistant and HIV susceptible populations. The values for each group were determined from table 10A, looking at the prevalence of each allele independently, therefore n = twice the numbers of individuals typed above.

SSCP/Sequence Analysis of IL-4 Promoter

The lack of an association with HIV resistance and the IL-4 (-590) promoter polymorphism does not preclude other, currently unknown mutations or polymorphism's within this gene that may be associated with resistance to infection by HIV-1. We began an investigation into the immediate, upstream region of the IL-4 gene. This region of the IL-4 gene contains binding sites for the majority of the transcriptional regulatory molecules believed to be important in regulation of IL-4 production (Todd et al., 1993). Transcription regulation assays have suggested that over 90% of IL-4's transcriptional regulation can be accounted for in this region (Brown and Hural, 1997). For this reason we hypothesized that the HIV-1 resistant women may have sequence polymorphisms within this region, and this may explain their apparently altered IL-4 production.

Single stranded conformational polymorphism (SSCP) analysis is an established procedure designed to detect single base pair changes in short PCR amplicons based upon mobility differences in non-denaturing acrylamide gels due to conformation differences based upon nucleotide sequence. We carried out this analysis as described in Materials and Methods on 32 HIV resistant, 6 HIV Susceptible and 9 HIV negative low risk controls. SSCP analysis on 12 resistant women and 6 low risk controls are shown in Figure 15. As is evident from analysis of this gel, there appears to be no discernable differences among the fragments analyzed. In total, there did not appear to be any significant differences in SSCP banding patterns in any of the individuals tested.

To insure that the SSCP analysis accurately reflects the nucleotide sequences of these regions, we decided to carry out sequence analysis on 5 samples. Three HIV resistant individuals that had identical SSCP banding patterns (ML 887, ML 1293 and

ML 1358) and two low risk controls that exhibited SSCP banding identical to the HIV resistant individuals (MCH 3621 and MCH 5451

Analysis of the nucleotide sequence (Shown in Figure 16) of these samples showed that all of the samples were identical to each other, and to the published sequence of this region. Thus SSCP and sequence analysis of the IL-4 promoter showed no differences in the groups analyzed.

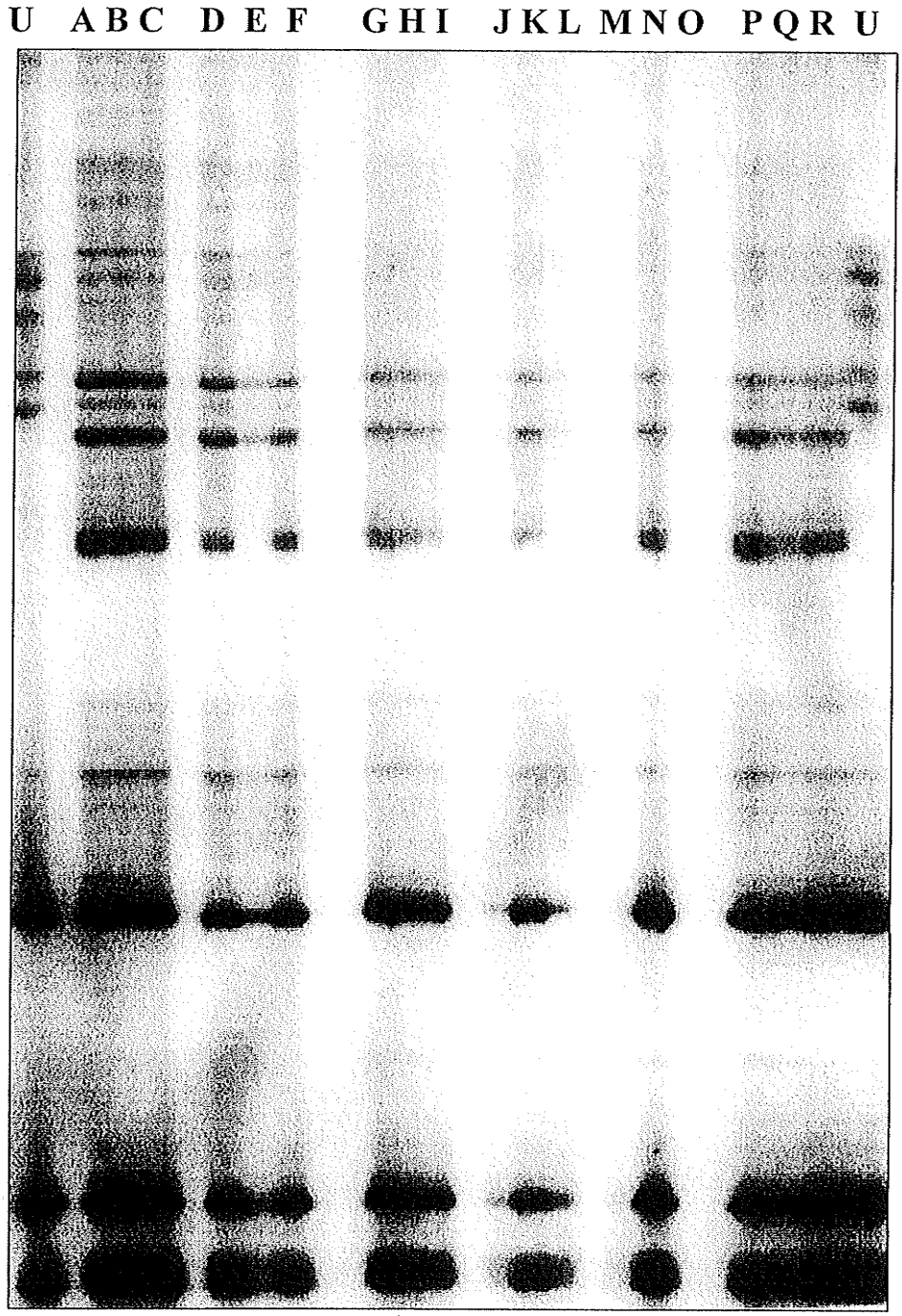

Figure 15: SSCP Analysis of IL-4 Promoter Region in HIV Resistant Individuals.

Figure 15: SSCP Analysis of IL-4 Promoter Region in HIV Resistant Individuals. A region corresponding to the first 452 nt of the IL-4 promoter was amplified in the presence of α^{32}-ATP, digested with *Hae* III and loaded onto a non-denaturing polyacrylamide gel. The above figure is an autoradiograph of the gel. The control lane (U) shows a non-denatured sample, while the experimental lanes are denatured samples from 12 HIV resistant individuals (lanes A-L) and 6 low risk controls (lanes M-R).

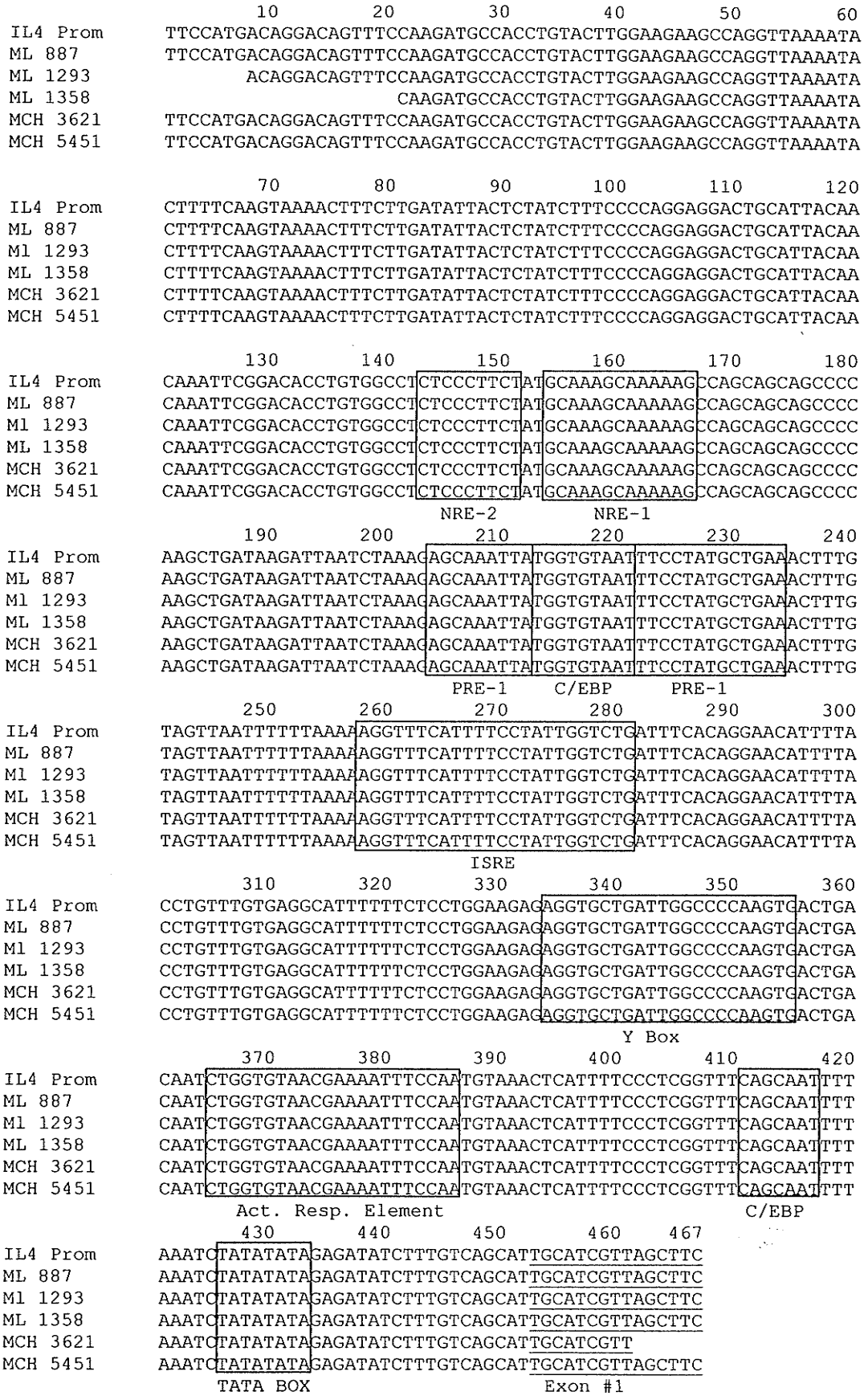

Figure 16: Sequence Analysis of IL-4 Promoter Region.

Figure 16: Sequence Analysis of IL-4 Promoter Region. The sequence derived for the IL-4 promoter region for 5 samples and the published IL-4 promoter. Regions believed to be important in the transcriptional regulation of IL-4 are indicated by the boxed areas. (Brown and Hural, 1997).

Microsatellite Analysis of the IL-4/Th2 Gene Cluster

The IL 4 or Th2 gene cluster contains a number of genes believed to be important in the regulation of type-1 immune responses. Genetic polymorphism in this area has been associated with altered susceptibility to a number of infectious diseases including Schistosomiasis and Leishmaniasis and has been associated with altered pathogenesis to asthma and atopy (Gorham et al., 1996; Marquet et al., 1996; Marsh et al., 1994; Meyers et al., 1994; Walley and Cookson, 1996). Pathogenesis of these diseases has been linked to type-1 and type-2 immune responses. A diagram of the IL-4 cluster is shown in Figure 17, indicating the immune response genes that reside within in this region.

We decided to carry out a candidate gene-like case control study on six different microsatellites that span this region (D5S666, D5S1984, IL-4 ms, IRF-1 ms, D5S2115, and D5S399, also shown in Figure 17). We chose these markers for our analysis as they provided the best coverage of this region, to further investigate other known IL-4 polymorphisms, and as an initial investigation of some of the other genes that lie in this region.

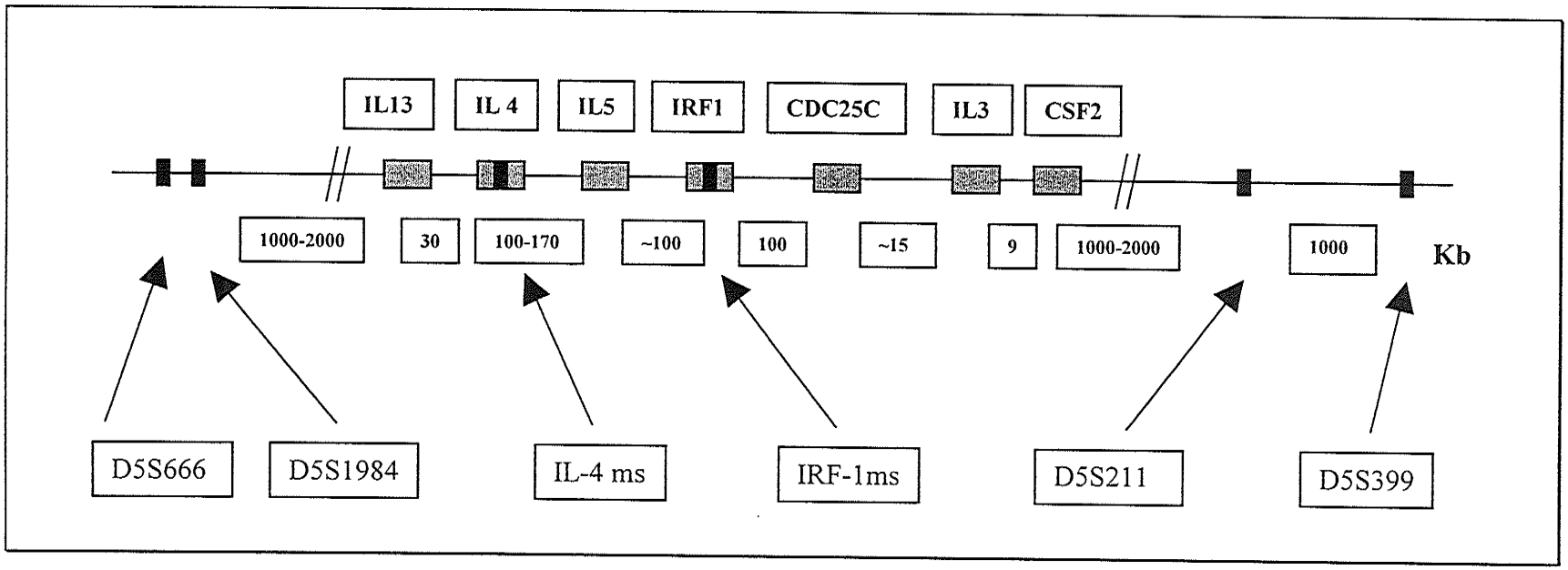

Figure 17: Depiction of IL-4 Gene Cluster.

Figure 17: Depiction of IL-4 Gene Cluster. The diagram shows a representation of the human IL-4 gene cluster located between 5q23 and 5q31. The topmost boxes indicate the genes located within this region, Interleukin 13, Interleukin 4, Interleukin 5, Interferon Regulatory Factor 1, CDC 25, Interleukin 3, and Colony Stimulation Factor 2. The boxes immediately below the figure indicate the approximate distance (In kilobases). The last boxes indicate the location of the 6 microsatellite markers chosen for analysis.

Allele frequencies for the microsatellite markers D5S666, D5S1984, IL-4, IRF-1, D5S2115 and D5S399 markers were determined for between 447-591 susceptible women, and between 70-86 HIV resistant women. The number of patients typed for each microsatellite varied because of lack of biological material to type all 6 markers. The results of the typings are compiled in Table 11. Initial chi-square analysis was carried out to determine whether there were any differences in allele distribution between the two study groups. The initial analysis was carried out by setting up contingency tables for each allele of a particular marker. However, there are some concerns about the appropriateness for using chi-square analysis for observed or expected values of less than 5 per cell of the contingency table. For that reason we grouped less frequent alleles into their own category and re-constructed the contingency tables. Either analysis indicated that there was a significant difference in the allele distribution for the D5S1984 marker and the IRF-1 ms marker (p values 0.002, and 0.009 respectively, see Table 11). There were no significant differences in the allele distribution for any of the other markers.

We examined allele distribution in both the D5S1984 marker and the IRF-1 ms marker. Although the allele distribution in the 1984 marker appeared to differ significantly between the two study groups it was difficult to clearly identify particular alleles associated with the resistance phenotype. For D5S1984 the 219 and 227 alleles were increased in frequency in the resistant women, while the 215 and 225 alleles were found at a decreased frequency. In contrast the IRF-1 ms marker clearly only had one allele (allele 179) specifically increased in the resistant population, while others, especially the 181 allele were found less often in this population.

Table 11: Genotype Distribution, and Allele Frequencies of TH2 Cluster Microsatellite Markers in HIV Susceptible and Resistant Individuals.

D5S666	Susceptible		Resistant	
Allele	n	%	n	%
*224	7	0.6		
*228	13	1.2	1	0.6
*230	4	0.4		
232	42	3.7	11	6.5
234	50	4.4	8	4.7
236	102	9	18	10.6
238	298	26.3	53	31.2
240	35	3.1	5	2.9
242	154	13.6	21	12.4
244	116	10.3	14	8.2
246	101	8.9	8	4.7
248	153	13.5	19	11.2
250	44	3.9	9	4.7
*252	5	0.4	1	0.6
*254	7	0.6	3	1.8
*256	1	0.1		
Total	1132		170	
[1]Chi-Sqr 15df, X^2= 14.10, p = 0.52				
[2]Chi-Sqr 10df, X^2= 9.26, p = 0.51				

D5S1984	Susceptible		Resistant	
Allele	n	%	n	%
*209			1	0.7
*211	2	0.2	1	0.7
213	115	12.9	20	14.3
215	262	29.3	31	22.1
217	128	14.3	20	14.3
219	46	5.1	16	11.4
*221	10	1.1	1	0.7
*223	41	4.6	3	2.1
225	142	15.9	16	11.4
227	108	12.1	29	20.7
*229	12	1.3	0	
*231	10	1.1	1	0.7
*233	12	1.3	1	0.7
*235	3	0.3		
*237	3	0.3		
Total	894		140	
[1]Chi-Sqr 14df, X^2= 31.39, p = 0.005				
[2]Chi-Sqr 6df, X^2= 21.46, p = 0.002				

IL-4 ms	Susceptible		Resistant	
Allele	n	%	n	%
*144	12	1.1		
*146	6	0.6	2	1.3
*148	16	1.5	2	1.3
150	446	41.7	73	47.4
*152	23	2.2	4	2.6
*154	3	0.3		
156	38	3.6	8	5.2
158	63	5.9	15	9.7
160	72	6.7	10	6.5
*162	6	0.6	2	1.3
*164	33	3.1	2	1.3
*166	31	2.9	1	0.6
168	79	7.4	11	7.1
170	79	7.4	11	7.1
172	95	8.9	8	5.2
174	56	5.2	5	3.2
*176	12	1.1		
178	67	6.3	10	6.5
*180	5	0.5	3	1.9
*182	2	0.2		
*184	1	0.1		
Total	1070		154	
[1]Chi-Sqr 20df, X^2= 23.50, p = 0.26				
[2]Chi-Sqr 9df, X^2= 9.68, p = 0.38				

IRF-1 ms	Susceptible		Resistant	
Allele	n	%	n	%
179	424	35.9	82	49.4
181	404	34.2	40	24.1
*183	36	3	1	0.6
*185			1	0.6
*187	12	1	3	1.8
*189	100	8.5	9	5.4
191	132	11.2	17	10.2
*193	8	0.7	2	1.2
195	6	0.5		
*197	7	0.6		
*199	11	0.9	4	2.4
*201	10	0.8	2	1.2
*203	16	1.4	3	1.8
*205	9	0.8	1	0.6
*207	7	0.6	1	0.6
Total	1182		166	
[1]Chi-Sqr 14df, X^2= 30.18, p = 0.007				
[2]Chi-Sqr 4df, X^2= 13.53, p = 0.009				

D5S2115	Susceptible		Resistant	
Allele	n	%	n	%
*151	2	0.2		
*153	11	1	4	2.4
155	158	13.6	14	8.3
157	26	2.3	5	3
161	48	4.1	11	6.5
*163	44	3.8	1	.6
*165	27	2.3	3	1.8
167	24	2.1	6	3.6
169	126	10.8	19	11.3
171	72	6.2	18	10.7
173	217	18.6	31	18.5
175	200	17.2	27	16.1
177	103	8.9	14	8.3
179	42	3.6	5	3
181	61	5.2	8	4.8
*183	3	0.3	2	1.2
*185	1	0.1	1	0.6
Total	1164		168	
[1]Chi-Sqr 16df, X^2= 25.18, p = 0.07				
[2]Chi-Sqr 11df, X^2= 11.93, p = 0.37				

D5S399	Susceptible		Resistant	
Allele	n	%	n	%
*115	28	2.4	3	1.9
117	133	11.5	22	13.1
119	550	47.7	67	41.4
121	223	19.3	36	22.2
123	49	4.3	10	6.2
*125	17	1.5		
127	40	3.5	11	6.8
129	44	3.8	8	4.9
131	50	4.3	9	5.4
133	17	1.5	6	3.7
*135	1	0.1		
*137	2	0.2		
Total	1154		172	
[1]Chi-Sqr 11df, X^2= 14.33, p = 0.22				
[2]Chi-Sqr 8df, X^2= 13.32, p = 0.10				

Table 11: Genotype Distribution, and Allele Frequencies of TH2 Cluster Microsatellite Markers in HIV Susceptible and Resistant Individuals. This Table gives the allele frequencies for each allele in each of the 6 microsatellite markers examined. Allele frequencies show the distribution on either chromosome (2n), so the study number equals total/2. Chi square analysis was carried out on the entire table[(1)], or by grouping less frequent alleles[(*)] into their own category and carrying out the Chi square analysis again[(2)]. Allele distribution appears to be altered in D5S1984 ($p<0.002$) and in IRF-1 ms ($p= 0.009$).

Specific IRF-1 ms Polymorphisms are Associated with Resistance to HIV-1

We further examined the genotypic and allelic frequencies of the IRF-1 ms 179 allele in 83 HIV-1 resistant and 591 HIV susceptible women. As can be seen in Table 12 the IRF-1 179 genotype allele is strongly associated with the resistance phenotype at both the allelic and gene frequency levels exerting a modest protective effect ($p = 0.0051$, OR 0.53 (95% CI 0.32-0.87)). To determine if the association may be due to a decreased frequency of a susceptibility allele we examined the distribution of the IRF-1 ms 181 allele (which appeared to be decreased in the resistant population) in 83 resistant and 591 susceptible women. As can be seen in Table 13 the IRF-1 181 ms allele was not significantly decreased in the resistant population.

We also analyzed the effect that the IRF-1 ms 179 allele had on seroconversion. As can be seen in Figure 18 those individuals who had at least one copy of the IRF-1 allele had a significantly decreased risk of seroconversion than those who lacked the IRF-1 ms 179 allele (log rank test $p<0.021$). The protective effect of IRF-1 ms 179 was also calculated using a Cox linear regression model to generate an incidence rate ratio (IRR) and showed that IRF-1 ms 179 provided a protective effect against seroconversion (IRR=0.69, 95%CI 0.51 – 0.96, $p<0.03$). Those individuals with the 181 allele did not appear to have a significantly increased risk for seroconversion (Figure 19).

The distribution of the IRF-1 ms allele was also examined in susceptible groups exhibiting differential disease progression and was not found to be associated with disease progression in any manner (data not shown).

Table 12: IRF-1 ms 179 Allele is Associated with Resistance to HIV-1 at Both Allelic and Gene Frequency Levels.

IRF-1 Alleles	Allele frequencies				Genotype frequencies			
	Susceptible		Resistant		Susceptible		Resistant	
	n	%	n	%	n	%	n	%
179 present	249	42.1	48	57.8	424	35.9	82	49.4
179 absent	342	57.9	35	42.2	758	64.1	96	50.6
total	591		83		1182		166	
X^2, p value	6.65 2df, p = 0.009				10.79 2df p = 0.001			
FET p value	0.0051				p < 0.00000001			
OR (95%CI)	0.53 (0.32-0.87)				0.57 (0.41-0.80)			

Table 12: IRF-1 ms 179 Allele is Associated with Resistance to HIV-1 at Both Allelic and Gene Frequency Levels. Allele frequencies show the frequency of a copy of the IRF-1 179 allele on either chromosome, while the genotype frequency shows the overall frequency of 179 alleles present on both chromosomes. FET p value designates Fischers Exact Test comparing the protective 179 allele vs non-protective genotypes (any other allele other than 179). Relative risk is computed comparing resistant and susceptible allele and genotype frequencies respectively.

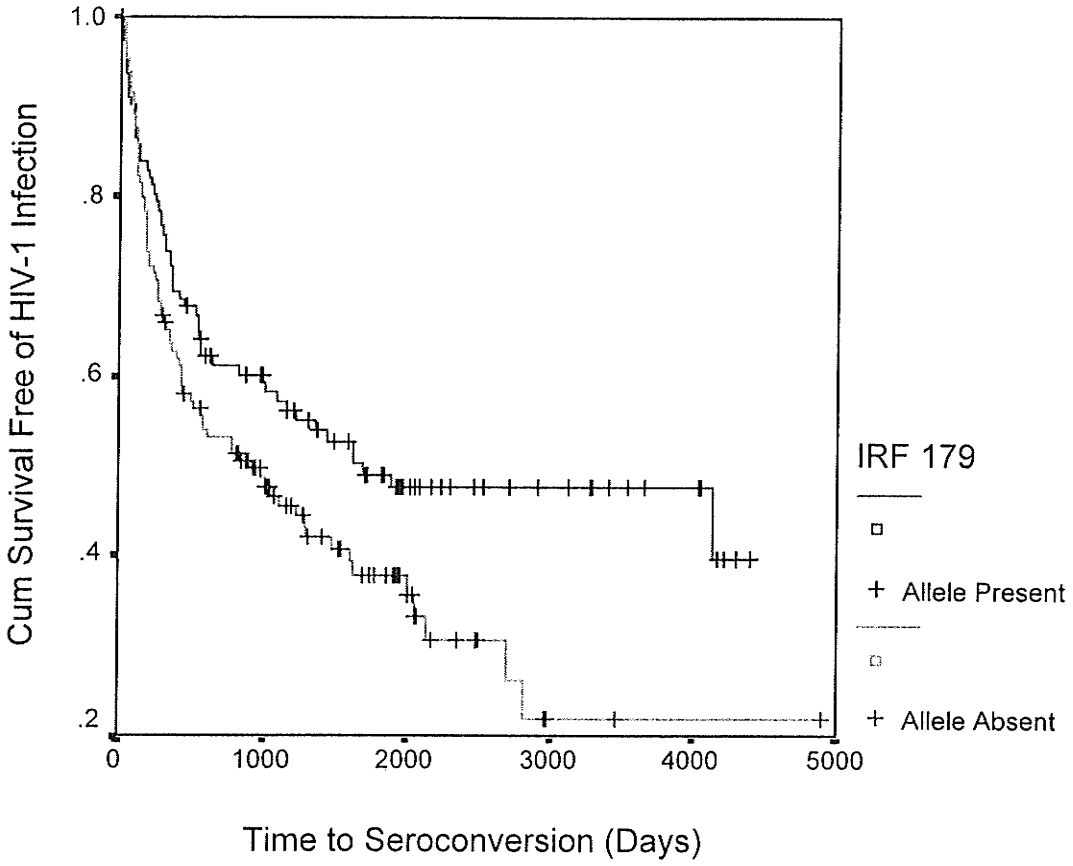

Figure 18: Kaplain-Meier Survival Analysis Comparing Individuals With and Without the IRF-1 ms 179 Allele.

Figure 18: Kaplain-Meier Survival Analysis Comparing Individuals With and Without the IRF-1 ms 179 Allele. Kaplain-Meier survival plots for those with and without the IRF-1 179 ms allele comparing HIV-1 free survival (seroconversion). All available seronegative individuals typed for IRF-1. Those individuals who had at least 1 copy of the IRF-1 ms allele were significantly less likely to seroconvert (log rank test $p<0.021$).

Table 13: IRF-1 ms 181 Allele is Not Associated with Susceptibility to HIV-1 at the Allelic Level, but is Weakly Associated at the Genotype Frequency Level.

IRF-1 Alleles	Allele frequencies				Genotype frequencies			
	Susceptible		Resistant		Susceptible		Resistant	
	n	%	n	%	n	%	n	%
181 present	243	41.1	26	31.3	404	34.2	40	24.1
181 absent	348	58.9	57	68.7	778	65.8	126	75.9
total	591		83		1182		166	
X^2, p value	2.51 2df, p = 0.11				3.96 2df p = 0.012			
OR (95%CI)	1.3 (0.9-1.61)				1.64 (1.1-2.42)			

Table 13: IRF-1 ms 181 Allele is Not Associated with Susceptibility to HIV-1 at the Allelic Level, but is Weakly Associated at the Genotype Frequency Level. Allele frequencies show the frequency of a copy of the IRF-1 181 allele on either chromosome, while the genotype frequency shows the overall frequency of 181 alleles present on both chromosomes. Relative risk is computed comparing resistant and susceptible allele and genotype frequencies respectively.

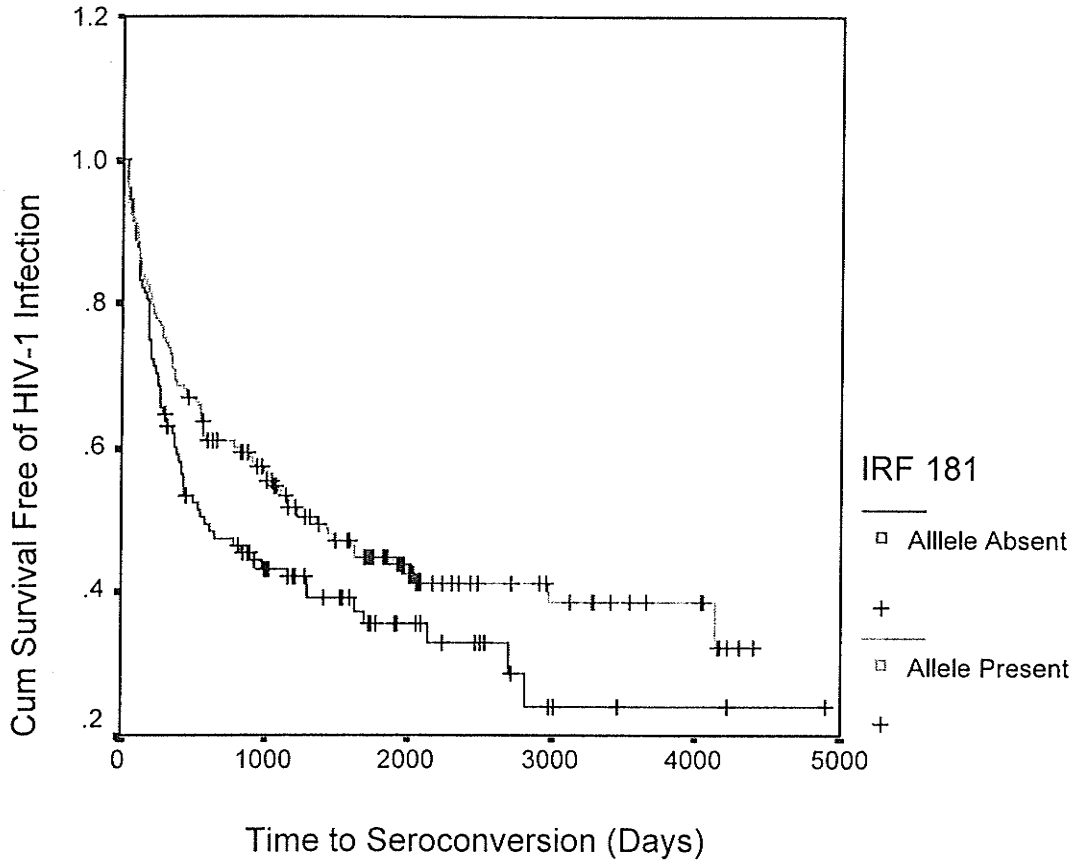

Figure 19: Kaplain-Meier Survival Analysis Comparing Individuals With and Without the IRF-1 ms 181 Allele.

Figure 19: Kaplain-Meier Survival Analysis Comparing Individuals With and Without the IRF-1 ms 181 Allele. Kaplain-Meier survival plots for those with and without the IRF-1 181 ms allele comparing HIV-1 free survival (seroconversion). All available seronegative individuals typed for IRF-1. Those individuals who had at least 1 copy of the IRF-1 ms 181 allele were not significantly more likely to seroconvert (log rank test p=0.059).

Specific D5S 1984 ms Polymorphisms are Not Strongly Associated with Resistance to HIV-1

We further examined the genotypic and allelic frequencies of the both the D5S 1984 219 and 227 ms 179 alleles in 83 HIV-1 resistant and 591 HIV susceptible women. As can be seen in Table 14 the D5S 1984 219 genotype allele is was not associated with the resistance phenotype at the allelic level ($p = 0.07$), although it was associated with resistance at the gene frequency level exerting a modest protective effect ($p = 0.007$, OR 0.41 (95% CI 0.22-0.80)). The D5S 1984 227 genotype allele was also not associated with the resistance phenotype at the allelic level ($p = 0.23$), although it was associated with resistance at the gene frequency level exerting a modest protective effect ($p = 0.007$, OR 0.53 (95% CI 0.33-0.85))

We also analyzed the effect that the D5S 1984 alleles had on seroconversion. The presence of either the D5S 1984 219 or 227 alleles had no effect on the risk of seroconversion (log rank test $p = 0.266$ and $p = 0.378$ respectively, data not shown). The distribution of the D5S 1984 219 or 227 alleles was also examined in susceptible groups exhibiting differential disease progression and was not found to be associated with disease progression in any manner (data not shown).

Table 14: D5S 1984 219 and 227 Alleles are Not Associated with Susceptibility to HIV-1 at the Allelic level, but are Weakly Associated at the Genotype Frequency Level.

D5S 1984 Alleles	Allele frequencies				Genotype frequencies			
	Susceptible		Resistant		Susceptible		Resistant	
	n	%	n	%	n	%	n	%
219 present	46	10.3	13	18.6	46	5.1	16	11.4
219 absent	401	89.7	57	81.4	848	94.9	134	88.6
total	447		70		894		140	
X^2, p value OR (95%CI)	3.33 2df, p = 0.07 0.51 (0.26-1.05)				7.39 2df p = 0.007 0.41 (CI 0.22-0.80)			
227 present	101	22.6	21	30.0	108	35.9	29	49.4
227 absent	347	77.4	49	70.0	786	64.1	96	50.6
total	447		70		896		111	
X^2, p value OR (95%CI)	1.46 2df, p = 0.23 0.68 (0.38-1.23)				10.79 2df p = 0.007 0.53 (CI 0.33-0.85)			

Table 14: D5S 1984 219 and 227 Alleles are Not Associated with Susceptibility to HIV-1 at the Allelic level, but are Weakly Associated at the Genotype Frequency Level. Allele frequencies show the frequency of a copy of the D5S 1984 219 and 227 alleles on either chromosome, while the genotype frequency shows the overall frequency of 179 alleles present on both chromosomes.Relative risk is computed comparing resistant and susceptible allele and genotype frequencies respectively.

Discussion:

Within a large sex worker cohort in Nairobi, Kenya we have identified groups of women who exhibit differential susceptibility to infection by HIV-1. Epidemiologic evidence suggests that a subset of these women are resistant to infection by HIV-1. Resistance correlates with cellular immune responses to HIV-1 as measured by proliferative responses to HIV-1 antigens and the appearance of HIV-1 specific, MHC class I restricted CTL. This presence of these responses in a group that can be epidemiologicaly defined as resistant clearly suggests that these woman have developed acquired immunity to HIV-1. A number of hypotheses have been generated as to how these women may be protected against infection by HIV-1. These hypotheses include the possibility that these women are able to mount protective primarily cellular (type-1) immune responses to the virus, and/or the possibility that these women have protective HIV specific IgA in their genital mucosa. There are a number of alternate explanations, but we believe that these women have developed acquired immunity to HIV-1.

The question remains however as to why do these women appear to be able to mount an effective immune response to HIV-1 while the vast majority of individuals are unable to? In accordance with the idea that cellular immune responses may be important in protecting against infection it has been observed certain HLA alleles are associated with resistance suggesting that there may be genetically defined mechanisms responsible for conferring resistance. Further, perhaps more exciting evidence to suggest that there may be a genetic component to resistance has come from anecdotal reports that a number of resistant women appear to be related to one another. These preliminary observations led us to question if there is a familial basis for resistance to HIV-1, and if this resistance

could be due (at least in part) to a genetic component. These questions and our belief that resistant women have developed acquired immunity to HIV-1 led us to develop the hypothesis that was tested in this thesis. That is, that resistance to infection by HIV-1 is mediated by genetic factors that are involved in the regulation of protective immune responses to HIV-1.

Demonstrating a Familial Basis for Altered Susceptibility to HIV-1.

If we are to prove our hypothesis that resistance to infection by HIV-1 is mediated by genetic factors we have to clearly demonstrate that there is a genetic component involved in this phenomena. To do this we showed that the likelihood of infection by HIV-1 was in some manner dependent on the relatedness to a resistant woman in two different cohorts.

Sex Worker Relatives of Resistant Women are Less Susceptible to Infection by HIV-1.

Our strict definition of resistance requires that these women are active sex workers, remain persistently seronegative for HIV-1 antibodies (as detected by HIV-1 EIA), and have no evidence of integrated proviral DNA as detected by sensitive HIV-1 PCR assays. Women who did not meet these criteria were not considered for the purposes of this study. For the majority of the studies presented we were interested in comparing individuals within the cohort with whom we could define their susceptibility to infection

by HIV-1 (i.e. resistant or susceptible). Susceptibility of a population to an infectious disease depends upon the route and degree of exposure to the causative pathogen. In our study we are able to determine sufficient exposure to HIV-1 that would result in infection only in individuals who have been active SWs for over 3 years and exhibit risk factors similar to others within the cohort, (i.e. condom use, numbers of partners per day, etc.). This results in us only being able to define women from the ML cohort who have been enrolled in the study for more than three years as resistant.

The assignment of the susceptible phenotype was much easier. The presence of antibodies that recognize HIV-1 antigens is indicative of infection by HIV-1. Therefore our susceptible group consists of all seropositive individuals from either the ML or Kindred cohort.

The third phenotype that we considered was HIV-1 uninfected or HIV negative. This phenotype was not ideal for determining differential susceptibility, but provided an ability to consider individuals who did not meet our definition of resistance. The HIV uninfected group would include individuals who if under enough infection pressure would either go on to be considered resistant (a small percentage, <13% based upon the current rate of individuals that become resistant in the ML cohort, (Fowke et al., 1996)). Or, those that would eventually become infected by HIV-1 and therefore fall into the HIV-1 susceptible group. Analyses that used the HIV negative phenotype were done primarily to determine what effect being related to a resistant prostitute had on susceptibility to HIV-1. We used this phenotype in analysis within the ML cohort so that we could include data from as many members of the cohort as possible. This analysis is

looking at the susceptibility of the two groups to HIV-1, and not the clustering of the resistance phenotype.

In an analysis comparing HIV prevalence in sex workers we were able to demonstrate that those related to a resistant woman were significantly less likely to be infected with HIV-1 than those related to a susceptible woman (15/33 infected or 45.5% prevalence compared to 93/103 or 90.3 %, OR 0.1, 95%CI 0.07-0.26, $p<0.00001$). This strongly suggested that being related to a resistant woman provided a protective effect against HIV-1 infection and provides evidence to suggest that there is a genetic basis for resistance, in agreement with our hypothesis.

If our hypothesis that a genetic basis for resistance is correct then we should be able to see a decreased risk of infection by HIV-1 in non-prostitute relatives of these women as well. However, the best approach would be to look at HIV resistant individuals and see if their numbers were increased in the population related to the resistant women. Obviously as previously discussed, only women from the ML cohort can be epidemiologically defined as resistant. If we were able to identify a marker for resistance that we could apply to non-sex workers or women who have been sex workers for less than 3 years than we would be able to re-analyze the data to investigate resistance to HIV-1 and not just the absence of infection.

Kin of Resistant Women are less Susceptible to Infection by HIV-1

The Kindred cohort was established in 1996 as part of this study to investigate what effect being related to an HIV resistant woman had on susceptibility to HIV-1 in a

largely non-sex worker cohort. Based upon current HIV prevalence rates in Kenya (over 15% in some areas) we theorized that we would be able to identify a significant difference in susceptibility to HIV-1 in a large scale epidemiologic investigation. We compared HIV susceptibility in individuals related to a resistant woman, and in individuals not related to a resistant woman. Initial investigations showed strong evidence that those individuals related to a resistant woman were less likely to be infected by HIV-1 (data not shown). This lead to an expansion of the original study and a wider recruitment of study subjects. The epidemiological investigation initiated for this study was completed in co-operation with Dr. Joshua Kimani. Partial results of this study (extracted from Kimani et al.) are shown in table 5B. They clearly show that individuals related to a resistant prostitute were significantly less likely to become infected with HIV-1. HIV prevalence was significantly less in those related to a resistant women (37/276 or 13%) compared to those related to a susceptible women (101/303 or 33%) (OR.31, 95%CI 0.199-0.48), $p<0.00001$).

Thus, both in the ML cohort and the Kindred cohort we were able to show that being related to a resistant woman led to a less likely chance of becoming infected. These results strongly suggest that a genetic factor or factors are involved and would be consistant with an autosomal dominant or co-dominant gene being responsible for this phenomenon. This finding strongly supports our hypothesis that there is a genetic basis for resistance to HIV-1. As previously described there is ample evidence both from studies on HIV and on other infectious diseases that there is a genetic component responsible for altered susceptibility to these diseases. This study is unique in that it is the first to identify a familial basis for resistance to infection by HIV-1 that may be mediated

by mechanisms responsible for acquired immunity to HIV-1. Our observations were made possible due to the number of women enrolled in the HIV resistant group, and the length of time that they have been under investigation. This cohort is one of the few in the world in which members of the same family are under comparably high infection pressure by HIV-1. This finding is of utmost importance for understanding the role that acquired immunity may play in natural immunity to HIV-1. Further design and development of HIV vaccines must be predicated on models of naturally acquired immune responses to HIV-1.

However, to further characterize genetic factors that may be responsible for resistance to HIV-1 and provide proof of our thesis, it is first necessary to identify the gene or gene products that may be mediating this protection. To map and identify these genes it would be ideal to identify all individuals who are resistant to infection by HIV-1. To do this we needed to find a "marker" for resistance that we could apply to members of the ML and Kindred cohorts who are either not prostitutes, or who do not meet our epidemiologic definition of resistance. The identification of a "marker" for resistance would allow us to focus our investigation and hopefully identify further functional differences in the immune responses of resistant women.

Immunologic Studies of Resistant Women and their Kin

These studies were carried out in order to try to determine a "marker" for the resistance phenotype that would aid in the identification of genes that may be responsible for resistance. To do this we focussed our attention on immunologic factors that may

either reflect or be involved in cellular immune responses to HIV, such as natural killer cells, proliferative responses to other antigens besides HIV, and cytokine responses by monocytes and macrophage. In the event that we would be unable to identify a surrogate marker for resistance we were prepared with alternate strategies that would be employed to determine genetic factors that may be involved in conferring resistance to infection by HIV-1. A secondary objective of these experiments was to obtain a further understanding of the immunobiology of HIV and its relationship to the resistance phenotype. The results of these investigations will allow us to better focus the subsequent genetic investigations by either identifying immunologic processes that may be important in resistance, or alternately allow us to discount immunologic processes that do not appear to be important for resistance.

Natural Killer Cells and Resistance to HIV-1

NK cells are believed to play an important role in the immune regulation of CTL, and NK cell activity can be up-regulated by a number of type-1 cytokines (i.e. IFN-γ and IL-12) (Raulet, 1999). If the hypothesis that resistant individuals have protective type-1 immune responses against HIV-1 is correct, than it is possible that this may be reflected in increased NK and or LAK activity.

Our data shows that NK and LAK activity is not associated with resistance to HIV-1. Clearly Figure 4B and 5B show that there is no significant difference in NK or LAK activity of the 4 Kenyan groups, indicating that NK activity (Figure 4B) and LAK activity (Figure 5B) remains similar for resistant women as well as for control groups.

Thus NK activity does not appear to serve as surrogate marker for the resistance phenotype.

The lack of an association with NK activity and resistance does not prove or disprove the hypothesis that resistance to HIV-1 may be due to a primarily cellular immune response. NK cells are an important part of the innate immune response, and their role in controlling HIV infection and pathogenesis is still unclear. It has been suggested that NK cells may help to control HIV replication by ADCC-like mechanisms (Ahmad and Menezes, 1996), which requires a humoral immune response to HIV-1, which we do not observe in the resistant individuals. We hypothesized that if we did observe an increased NK response in the resistant women, it would be the result of a general upregulation of type-1 cytokines and NK cell activity would not be directly responsible for conferring resistance. The role of NK cells in differential susceptibility to HIV-1 is poorly studied at best (Ullum et al., 1999), and though the results of these experiments were negative, they do provide some information on the role of NK cells in HIV-1 infection. However, it is still clear that NK or LAK activity provides a poor correlate for the resistance phenotype and further genetic investigations should not be focussed on genes involved in these responses.

Resistant Women Exhibit Normal Proliferative Responses to Recall Antigens

The ability to mount an effective secondary immune response, either cellular or humoral generally depends on CD4+ T-cell help (Abbas, Murphy and Sher, 1996). Resistant women appear to have cellular immune responses directed towards HIV but do

not mount a systemic humoral response to this pathogen (Fowke et al., 2000). We assayed for the ability of these women to mount effective helper responses to a panel of recall antigens that should elicit a strong secondary immune response. If these women have a primarily type-1 response to HIV then it is possible that these women may have strong type-1 responses to other pathogens as well. Preliminary data had also suggested that resistant women had an increased IL-2 response in unstimulated or resting cells, and upon activation with the mitogen PHA (Fowke et al., 2000). For these reasons we assayed for the production of IL-2, a type-1 cytokine, in response to a panel of recall antigens and mitogens.

The IL-2 response of subjects to mitogen and various recall antigens is shown in Figures 6 to 11. It is apparent from these Figures, and from statistical analysis that the resistant women do not respond to these antigens differently than any of the control groups. Depressed IL-2 responses to some recall antigens were observed in the low risk Kenyan controls compared to resistant women, resistant kin, and susceptible kin. These depressed responses may be due to recent pregnancies in some of the members of this group. It has been demonstrated in some studies (Reinhard et al., 1998) that pregnancy can alter immune responses resulting in a decrease in IL-2 and type-1 cytokines. In the event that depressed IL-2 responses were observed, we were prepared by the inclusion of other control groups such as the susceptible kin study group which also served as a control for these experiments.

The most consistent observation made was that the HIV susceptible or infected group had significantly depressed responses to the antigens and stimuli used. This is not unexpected, as numerous studies have shown that even asymptomatic infected

individuals can have severely altered immune responses. Therefore it is not surprising that we would observe these depressed responses in the HIV infected or susceptible group.

Resistant women do not appear to have different IL-2 responses to recall antigens compared to control groups. This does not mean that the hypothesis of a general type-1 immune response in resistant women is not valid. Although IL-2 may be considered a type-1 cytokine, it is produced by a wide variety of cell types and not exclusively by type-1 CD4+ T-cells. Thus our finding of a lack of IL-2 production may not accurately reflect an increase in other type-1 cytokines. Concurrent with this study, a series of experiments optimized to look for differential type-1 and type-2 cytokine production in this population were undertaken (Trivedi et al., 2000). Although they were unable to show a difference in type-1 responses in resistant women, they were able to show that resistant women appeared to have a global deficit in IL-4 production (a prototypic type-2 cytokine). Therefore the primarily cellular immune response to HIV observed in the resistant women may be due to the lack of ability to generate type-2 responses, rather than the ability to preferentially develop a type-1 immune response. The lack of a difference in IL-2 response would suggest that genes involved in IL-2 responsiveness would not be ideal for further investigation. However, the IL-4 data suggests that polymorphisms in genes responsible for IL-4 production, or in type-1/2 differentiation would be of more interest for further investigations.

Monocyte/Macrophages Studies of Resistant Women.

Initial contact between a pathogen and the host's immune system usually occurs via interaction with professional antigen presenting cells, (APC's) such as dendritic cells of the monocyte lineage (Sousa, 1999). There is considerable evidence to suggest that the initial interaction between an APC and CD4+ T-cells is important in the development of type-1 and type-2 immune responses. Dendritic cells of the monocyte lineage are thought to be one of the first cell types encountered by HIV-1 during natural infection by the virus. Thus it seems likely that the cytokine response of these cells may be an important marker of type-1 or type-2 responses, and possibly resistance. In this study IL-10 and IL-12 cytokine production from SAC activated monocytes was assayed by ELISA.

Our data suggests that IL-10 levels stimulated by media alone or in response to SAC stimulation are similar in resistant women and control groups (Figure 12). However the HIV susceptible group had significantly depressed IL-10 responses upon SAC stimulation compared to all of the other groups. This may be a further example of the general immune dysfunction observed in some HIV infected individuals.

The IL-12 data (Figure 13) is much more intriguing. This data suggests that resistant women produced more IL-12 unstimulated, and upon SAC activation than control groups. However, caution should be applied when analyzing this data. Based upon the results of Figure 13, it does not appear that the SAC activation has any effect on IL-12 production. However, it was obvious from the IL-10 and IL-12 p40 data that SAC stimulation in these cultures did occur. Why then was this activation not apparent when analyzing the production of the IL-12 p70 heterodimer? The production of complete

(p70) IL-12 is a tightly regulated process. It appears that p40 is generally produced in excess, while the production of the p35 subunit is under tight transcriptional control (Cowdery et al., 1999). This may explain why we could see a SAC effect when looking at the p40 subunit, but a less apparent effect when looking at the complete IL-12 molecule. It is also apparent that the detection limits for this assay were not low enough to accurately determine differences in IL-12 p70 production. The limit of detection of the assay was 16 pg/ml. Only a few samples gave IL-12 (p70) values greater than this range, almost all of them from resistant women (8 out of 21 tested, compared to 2 out of 23 tested in the control group, $p=0.02$). This made it difficult to determine differences between the resistant group, and the control group. It does suggest however that the resistant women may produce higher levels of IL-12 than control groups.

This finding is in agreement with the suggestion that resistant women have preferential type-1 immune responses to HIV-1. IL-12 is an important type-1 cytokine and may be responsible for the induction of cellular responses to HIV-1. Whether increased IL-12 production by monocytes is responsible for a type-1 response, or if this could be due to the decreased IL-4 production previously reported (Trivedi et al., 2000). Clearly further investigation is necessary. The inability to clearly identify differences in IL-12 production suggests that this assay is not optimum for the identification of the resistance phenotype and that genes involved in IL-10/12 regulation may not be ideal for consideration in further genetic studies.

Conclusions Regarding Immunologic Assays

The immunologic assays chosen were unable to clearly define an adequate marker for the HIV resistant phenotype. NK and LAK activity proved to be similar in resistant and control groups. The IL-2 proliferation studies also failed to provide a means to identify resistant individuals. The data obtained by the monocyte studies suggests that resistant women had increased IL-12 production by both resting, and stimulated monocytes. This data was not clear however, and IL-12 production was unlikely to serve as a good marker for the resistance phenotype. Further investigation is necessary to explore the role that the increased IL-12 responses may play in resistance to HIV-1. Although these assays were unable to clearly define a marker for the resistance phenotype they were able to address some important issues concerning resistance to HIV-1. It is clear that these women do not have altered NK or LAK activity, nor do they differ significantly in their IL-2 responses to recall antigens or mitogens. They may however have altered IL-12 responses, and this may be important in mediating cellular immune responses and presumably resistance to HIV-1.

Without a complete understanding of the immunobiology of resistance to infection by HIV-1 and the lack of an easily testable phenotype for resistance the identification of genetic factors that may mediate protective immune responses to HIV-1 is more difficult, but not impossible. To either prove or disprove our thesis we will have to rely on alternate genetic methods (candidate gene analysis) to try to identify genes that may help confer a protective immune response to HIV-1. The information provided by the immunologic assays allow us to better focus the search for candidate gene or genes

that may be involved in the resistance phenotype. Clearly genes that may be involved in NK responsiveness are less likely to be important in determining the resistance phenotype. Genes involved in IL-12 responses may be of more interest to consider, however this data was inconclusive. While IL-2 responsiveness was not a marker for resistance the IL-4 data provided evidence to suggest that IL-4 responsiveness may be altered in resistants (Trivedi et al., 2000). Thus we decided to focus further investigation on genes such as IL-4 that may be more important in regulating/developing type-1 and type-2 immune responses.

Candidate Gene Studies

A candidate gene approach is one of the most powerful tools for identifying relationships between disease and genetic factors responsible for pathogenesis or altered susceptibility to that disease. An association between a disease state and a polymorphism in a gene of known, or suspected function immediately suggests a role for that gene in that specific disease state. The drawback of this approach is that for certain diseases there are a wide array of genes that may be involved some of which have yet to be identified. To look at associations between each gene and the disease state that one is interested in, may involve looking at potentially hundreds of known polymorphisms, in hundreds perhaps thousands of genes. In our case we are looking at a group who are resistant to infection by HIV-1, and resistance may be mediated by protective cellular immune response to HIV-1. Clearly, if a genetic basis for resistance does exist, it could be due to polymorphism in any gene or genes involved in cellular or humoral immune response to

HIV-1, or in a gene affecting HIV's replicative cycle. For this reason we have chosen to investigate polymorphisms in genes known to be involved in resistance/altered susceptibility to HIV-1 and to other infectious diseases.

The study groups used in these analyses were exclusively from the sex worker or ML cohort. We compared the prevalence of genetic polymorphisms between HIV susceptible individuals and HIV resistant individuals. Thus we can look at two readily defined groups who exhibit documented differential susceptibility to infection by HIV-1. A number of studies have been carried out on the role that genetic polymorphisms play in HIV susceptibility and pathogenesis. These studies commonly compare the prevalence of a particular polymorphism in an HIV infected or susceptible group and compare that to the prevalence in an HIV uninfected group. One major concern about these studies is that the exposure to HIV-1 in the uninfected group has been difficult to quantify. This means that it is difficult to determine if the uninfected group is uninfected simply because they have not had exposure to the virus, or if they are actually differentially susceptible to infection. Our study is unique in that we have strong epidemiologic data on HIV-1 exposure, and can demonstrate that these women are indeed differentially susceptible to HIV-1. Thus it is possible for us to be able to determine the potential role of genetic variation in altered susceptibility to HIV-1.

The Vitamin D Receptor is not Associated with Resistance to HIV-1.

Dihydroxy-cholecalciferol is the most active metabolite of vitamin D and is one of the principal factors involved in calcium and phosphate metabolism (MacDonald,

Dowd and Haussler, 1994). The effects of vitamin D are mediated through the vitamin D receptor (VDR) which is expressed on diverse cell types. VDR is part of the steroid receptor superfamily and is encoded by a gene on chromosome 12q13-q14 (Taymans et al., 1999). Vitamin D has wide pleiotropic effects on cells of the immune system, acting through the VDR which is expressed on monocytes, macrophage, and on activated T and B cells (Provvedini et al., 1983). It is thought that Vitamin D acts to suppress T and B cell activity, while upregulating monocyte and macrophage function (Manolagas et al., 1994). Vitamin D has also been shown to upregulate HIV replication in cultured monocytes and macrophage, however this effect may be due to Vitamin D's role in monocyte growth and differentiation and not through direct effects on HIV's replicative cycle (Locardi et al., 1991; Skolnik et al., 1991).

A T to C polymorphism in the VDR gene at codon 352 in exon 9 causes the formation of a *Taq I* restriction endonuclease site (Morrison et al., 1994). This polymorphism has been associated with low bone density in post-menopausal women and more importantly has been shown to be responsible for increased VDR transcription levels in reporter-gene assays (Durrin et al., 1999). This polymorphism may be relevant in susceptibility to HIV-1 in that increased VDR expression may effect Vitamin D's proposed roles in regulating immune responses, and/or its effects on HIV-1's replication.

This polymorphism has been associated with altered pathogenesis to some infectious diseases including Hepatitis B, Tuberculosis, and Leprosy (Bellamy and Hill, 1998; Roy et al., 1999). It is interesting to note that altered pathogenesis to these diseases has also been associated with dichotomous type-1 or type-2 immune responses. We

believed that this *Taq I* polymorphism may be involved in the regulation of type-1 or type-2 responses and would be an ideal "candidate" to investigate in our population.

It is obvious from analysis of the 69 resistant and 322 susceptible individuals studied indicated in Table 6 that the *Taq I* polymorphism was found equally within the two groups. Further analysis (gene frequency, rate of HIV infection, and association with individuals who show altered disease progression) all failed to show any effects of this polymorphism. The lack of an association with VDR and resistance suggests that in this population at least, VDR polymorphisms do not play a significant role in HIV pathogenesis. Even if VDR polymorphisms do play a role in type-1 and type-2 immune responses and the pathogenesis of other diseases it is not necessarily true that the same mechanisms would be important in protection against HIV infection. HIV pathogenesis and replication strategies are considerably different that bacterial infections, and even hepatitis B infections. These findings suggest that any role that VDR polymorphisms may play in regulation of type-1 or type-2 responses are not likely to be important in HIV infection in this population.

Lack of Association Between Secretor Status and Resistance to HIV-1

Lewis blood group antigens are generally present in secretions from mucosal surfaces, including intestinal secretions, vaginal secretions, bronchial secretions, and saliva. Twenty to thirty percent of individuals in many populations do not secrete their blood group antigens, and are referred to as non-secretors (Blackwell, 1989). Non-secretor status is due to an inactivating G to A mutation (Trp to stop codon) in the *FUT-2*

gene on chromosome 19q. The *Fut-2* gene encodes a fucosyltransferase enzyme. The lack of this enzyme prevents the further modification of a glycolipid precursor by the A, B and Lewis transferases that would act upon this precursor to produce the secreted ABO and Lewis antigens (Kelly et al., 1995). The secretor phenotype has been linked to altered susceptibility to a wide variety of bacterial and viral infections (Blackwell, 1989). Non-secretors appear to be at an increased risk for bacterial and fungal infections, but are protected from viral infections. Non-secretor status has been associated with decreased risk of HIV infection in a study of two African populations, one from Senegal, and the other a large cohort of Ugandans (Ali, 1998).

The examination of secretor status in 57 resistant and 267 susceptible individuals from our cohort (Figure 7) failed to show any signs of association with resistance, altered seroconversion rates, or altered disease progression. The role of secretor status in HIV pathogenesis is likely to be quite complicated *in vivo* due to the wide interplay of the host, concurrent bacterial infections (either commensal microorganisms or STIs), and the infecting HIV virions. It is well established that STI's such as *H ducreyi* can increase the risk of acquiring an HIV-1 infection (Plummer et al., 1991; Tyndall et al., 1996). Thus any role for secretor status in HIV infection must also consider the role of secretor in acquisition of other infections as well. One could hypothesize that non-secretors may be more susceptible to infection by HIV-1 because they would be more likely to acquire bacterial infections that increase the risk of HIV-1 infection. Thus any protective effect of non-secretor status in HIV-1 infection must be capable of compensating for the increased risk of acquiring a harmful bacterial infection. Previous findings of non-secretor susceptibility to HIV have failed to adequately consider the possible roles of bacterial

infections in HIV pathogenesis. In our population due to their work as sex workers, both cases and controls are at similar risks for bacterial and other viral STI's. Unlike the sex worker cohort studied by our group the populations studied in Senegal and Uganda were not sex workers. These studies were done on individuals in rural populations who would presumably be at a much lower risk for acquiring an HIV-1 infection and presumably at a lower risk of acquiring other STIs compared to the women from a prostitute cohort. Thus the protective effects (if any) of non-secretor status may vary considerably in the different populations.

Secretor status and its effects on HIV-1 infection are still unclear. Our data would suggest that in our population at least, non-secretor status does not contribute significantly towards HIV resistance. Previous associations with non-secretor and altered susceptibility to HIV-1 are from cohorts on whom little data are available on other risk factors for HIV-1 infection. The observation made in our cohort may be the most valid as we know that our two groups (resistant and susceptible) all exhibit similar risk taking behavior and therefore are under similar infection pressures, and are among the best controlled group available for these sorts of studies.

CCR2b-64I Polymorphism is Associated with Slower HIV-1 Disease Progression but not Resistance

Polymorphisms in chemokine receptor genes were the first identified host genetic factors shown to effect the natural history of HIV-1 infection (Rowland-Jones, 1999). An inactivating 32 bp deletion mutant in CCR5 has been shown to protect against both HIV

infection and disease progression, presumably due to lack of expression of the HIV-1 co-receptor molecule. A polymorphism in the CCR2b gene, located in the chemokine cluster on chromosome 6 encoding a Valine to Isoleucine change has been shown to be associated with long-term nonprogression to AIDS in some populations (Kostrikis et al., 1998), but not in others (Smith et al., 1997). The functional role of this mutation has not been elucidated. Recent evidence has suggested that this polymorphism is in linkage disequilibrium with a number of point mutations in the promoter region of the adjacent CCR5 gene (Mummidi et al., 1998). These promoter variants have been associated with altered CCR5 expression and HIV disease progression. The mechanisms and credibility of these associations is still under considerable debate. A number of these polymorphisms appear to be in linkage disequilibrium with one another and with the CCR2-64I polymorphism. These polymorphisms appear to be highly population dependent. For example the del-32 CCR5 mutation is found fairly often in those descended from Europeans but rarely found in those descended from African populations, and the CCR2b-64I polymorphism appears to be associated with altered disease progression in African populations and not European populations.

Our data strongly supports the observation of an association between altered disease progression and the CC2b-64I allele. An examination of the 52 long term non-progressors, the 352 "normal" progressors, and the 15 rapid progressors in Table 9 clearly shows an association of the 64I allele with non-progression. This allele had no effect on acquisition of HIV-1 and was not associated with the resistance phenotype in any way. Upon initial investigation it would appear that the polymorphism was in fact associated with an increased risk of HIV infection. However, the overrepresentation of

the mutant allele observed in the HIV susceptible group could be explained if the CCR2b-64I allele was associated with increased survival after infection. Thus you would observe an artificial "amplification" of this genotype in the susceptible group due to the increased presence of LTNP's within this group. The subsequent analyses showed that this was indeed the case.

Originally it had been postulated that CCR2 which can also act as a coreceptor for HIV-1 was involved in evolution of the virus from the less virulent M-tropic strain to the more virulent T-tropic strain. This idea has found less favor in light of evidence suggesting that the CCR2-64I polymorphism is linked to one or more presumably functional polymorphisms in the CCR5 gene (Mummidi et al., 1998). Studies have also shown that virus evolution from M to T tropic strains does not require the promiscuous use of chemokine receptors like CCR2b (de Roda Husman et al., 1999). There are at least 5 major different promoter alleles in the CCR5 gene with widely different distributions in different ethnic populations. Further efforts to establish a mechanism by which these promoter polymorphisms effect HIV-1 disease progression will require further functional studies to completely elucidate the role these polymorphism's may play in chemokine receptor expression and evolution of different HIV phenotypes.

In any case it is clear that genetic polymorphisms in CCR2 are present in this cohort and that these polymorphisms may have an effect on HIV disease progression. But, it is also apparent that this polymorphism is not associated with the resistance phenotype. Further investigation into the role of the various CCR5 polymorphisms is currently underway.

IL-4 Promoter Polymorphisms and HIV-1 Resistance

Recent evidence from our group has suggested that resistant women have a global deficiency in the production of IL-4. IL-4 is an important type-2 cytokine responsible for B-cell activation and the production of IgG and IgE, as well as a negative regulator of a number of type-1 immune responses (Lucey, 1999). Obviously a deficit in IL-4 production would tend to favor type-1 immune responses. Recent data from mouse models have shown that IL-4 deficient mice have enhanced CTL activity (Schuler et al., 1999).

Evidence that the IL-4 gene contains functional polymorphic regions has come from the study of asthma and atopy. Both asthma and atopic responses have been linked to increased IgE and high levels of IL-4 transcription. The first reported polymorphism in the IL-4 gene is a C to T substitution at position -590 of the IL-4 promoter. This polymorphism has been associated with increased IL-4 transcription levels and an increase in measures of asthma and atopy in some subjects (Walley and Cookson, 1996).

We were unable to show any association with resistance and the IL-4 promoter polymorphism in our cohort. Table 11 shows the results from 77 resistant and 464 susceptible subjects. Further analysis of the IL-4 promoter region by sequence analysis and SSCP also failed to show any association with the resistant phenotype and altered IL-4 promoter regions. It was interesting to note that the prevalence of the IL-4 -590 promoter mutation in our population was much higher than that of the European populations previously reported (gene frequency of 0.27 in "normals", and 0.33 in asthma patients compared to roughly 0.46 in the entire ML cohort). If this polymorphism is

associated with increased type-2 responses than this finding may support the hypothesis that African populations have higher type-2 responses.

The lack of an association between HIV-1 resistance and the IL-4 polymorphisms we selected for this study does not mean that other unknown polymorphisms not in the IL-4 gene may be responsible for the resistance phenotype. This data only provides evidence that this particular polymorphism is not important.

Microsatellite Analysis of the IL-4 Cluster

The IL-4 or Th2 gene cluster is a well-characterized group of immune response genes found near 5q31-5q33. Polymorphisms in this region have been associated with a wide variety of diseases in which dichotomous type-1 and type-2 immune responses are believed to play an important role. Studies have shown that genetically mediated differences in susceptibility to asthma and atopy as well as genetically mediated differences in *S. mansoni* infection are linked to this region (Marquet et al., 1996; Marsh et al., 1994). This cluster contains many of the genes involved in the generation of type-2 immune responses including IL-3, IL-4, IL-5, IL-13, CSF2, CSF-1R and IRF-1.

We began an investigation of genetic differences in the IL-4 gene cluster between two groups differentially susceptible to HIV-1. As described in Table 12 we investigated the distribution of 6 markers in 402-591 susceptible women, and between 65-97 HIV resistant women. We identified statistically significant differences in the allele distribution for the microsatellite markers D5S1984 and IRF-1. The difference in allele distribution between HIV resistant and susceptible women was difficult to distinguish in

the D5S1984 marker. Multiple alleles such as 219 and 227 were found at an increased frequency in the resistant group, while the 215 and 225 alleles were found at a decreased frequency. This distribution does not appear to clearly distinguish a particular allele or alleles as being associated with resistance. It is possible that an allele of D5S1984 is in linkage disequalibrium with a functional polymorphism nearby. However, there is little evidence for known genes encoded near this marker that might effect HIV susceptibility. The differences in the IRF-1 marker were much easier to distinguish, with a single allele (IRF-1 179) appearing to provide a protective effect. Furthermore the IRF-1 gene is a particularly attractive candidate to explain the resistance phenotype.

IRF-1 179 ms Allele is Associated with Resistance to HIV-1

We concentrated further analysis on the IRF-1 microsatellite as it is found within the IRF-1 gene, which as will be discussed later is an attractive candidate to possibly explain (at least in part) the HIV-1 resistant phenotype. We showed that a particular IRF-1 allele (IRF-1 179) was significantly increased in the HIV-1 resistant group compared to the HIV susceptible group. As can be seen in Table 13, 48/83 or 57.8% of resistant women had at least one copy of the IRF-1 179 allele compared to 249/591 or 42.1% of the susceptible women (OR 0.53, 95%CI 0.39-0.99, $p<0.009$). This allele appears to provide a protective effect in that it was associated with the resistance phenotype at both the genotypic and allelic level. This suggests that the polymorphism can be considered dominant as either one or two copies of this allele provided the same protective effect in our analysis.

One possibility that cannot be discounted is that the HIV resistant women may in fact be resistant due to the lack of a susceptibility allele rather than the overrepresentation of a resistance allele. For example, one could argue that the IRF-1 ms 181 allele might be a marker for such a susceptibility allele as it appears to be underrepresented in the resistant group. Although this possibility cannot be ruled out, it appears to be a less likely explanation. The statistical associations with IRF-1 ms 179 are much stronger than any associations with the IRF-1 ms 181 at the allelic level ((OR 0.53, 95%CI 0.39-0.99, $p<0.009$) compared to (OR 1.3, 95% CI 0.9-1.61, $p=0.11$)) and at the genotypic level ((OR 0.57, 95% CI 0.41-0.80, $p = 0.001$) compared to ((OR 1.64, 95% CI 1.1-2.42, $p = 0.012$). Thus the protective effects of the IRF-1 ms 179 allele are stronger than the risk generated by having the IRF-1 ms 181 allele. Further investigation into these findings will still have to take into account the possibility that a susceptibility allele may exist.

IRF-1 179 ms Allele is Associated with a Decreased Risk of Seroconversion

If the IRF-1 179 allele has any effect on contributing to the resistance phenotype then one would expect that individuals that have this allele would be less likely to be become infected, and show a reduced likelihood of seroconverting to HIV-1. Figure 18 shows that individuals with at least one copy of the IRF-1 179 allele were significantly less likely to seroconvert (i.e. become infected by HIV-1) than those who lacked this allele ($p <0.02$, log rank test). If we compare the survival benefit of the IRF-1 ms 179 allele (Figure 18) to increased risk due to the the IRF-1 ms 181 allele (Figure 19) this again suggests that the most likely explanation for these results is that the IRF-1 ms 179 allele is protective, rather than the 181 being a marker for susceptibility.

An analysis using Cox proportional hazards modelling seroconversion data also shows the protective effect of the IRF-1 ms 179 allele in resistance to infection (Hazard Ratio=0.69, 95%CI 0.51 – 0.96, $p<0.03$). Although the effects of this allele appears to be modest (OR=0.53, Hazard Ratio= 0.69) the fact that the two values are in agreement provides strong evidence that the observed protective effect is indeed real. These data together suggest that the IRF-1 179 allele is important in mediating susceptibility to infection by HIV-1. The question remains as to why would a polymorphism in this particular gene be important in altered susceptibility to HIV-1?

IRF-1 is Necessary for Type-1 Immune Responses

IRF-1 was the first identified member of a group of transcription factors known as the interferon regulatory factor family. This group of proteins is responsible for regulating and mediating the antiviral, growth regulatory and immunoregulatory properties of interferons. The IRF-1 gene encodes a transcription factor that binds to, and positively regulates the virus inducible interferon regulatory response element (ISRE) originally identified in IFN-β and found in the majority of IFN inducible genes such as IFN-α/β, MHC class I, TAP/LMP, and IL-12 p40 (Taniguchi, 1997).

IRF-1 has been shown to be important in the generation of type-1 immune responses. IRF-1 knockout mice are unable to generate CD8+ T cells (Penninger et al., 1997) most likely due to their inability to express MHC Class I molecules (Hobart et al., 1996). IRF-1 knockout mice show a normal ability to generate CD4+ T cells, however these T cells exhibit altered cytokine production. They produce lower levels of IL-2 and IFN-γ but show an increased production of IL-3, 4, 5 and 6 (McElligott et al., 1997). This

has lead to speculation that IRF-1 knockouts are only capable of type-2 immune responses. Consistent with these observations IRF-1 knockout mice are susceptible to viral and bacterial infections (Dutia et al., 1999; Grieder and Vogel, 1999), but are more resistant to nematode infections (Lohoff et al., 1997; Penninger et al., 1997). These studies provide strong evidence that IRF-1 is required for the development of type-1 immune responses.

It is not surprising that IRF-1 is critical for the generation of cell mediated immune responses. Recent data has shown that IRF-1 is a critical transcription factor that regulates IL-12 p35 and p40 expression, and is responsible for maintaining functional IL-18 levels through its role as a transcription factor for interleukin-1 converting enzyme (ICE) a molecule responsible for post-translational production of IL-18. Both IL-12 and IL-18 play a crucial role in the development of Th-1 CD4+ cells (Tominaga et al., 2000). Thus it appears that IRF-1 plays an important role in the development and regulation of cellular immune responses.

Interestingly, IRF-1 appears to be coordinately regulated with the polymeric immunoglobin receptor (Blanch, Piskurich and Kaetzel, 1999). This molecule is responsible for the translocation of secretory IgA across epithelial surfaces. Recently, some groups have reported HIV-1 specific secretory IgA in HIV exposed uninfected individuals. This has also been observed in HIV resistant women from this cohort (Kaul et al., 1999). The role that secretory IgA may play in mediating resistance to HIV-1 is currently unknown. It is possible that IRF-1 may play a role in this immunologic process as well.

IRF-1 179 ms Allele may be in Linkage Disequilibrium with a Functional Polymorphism in IRF-1.

It seems likely that a functional polymorphism within the IRF-1 gene could be responsible for altering the cellular immune response to a particular pathogen or pathogens. The existing data suggests that cellular immune responses, particularly type-1 T helper cell responses and CTL to HIV-1 are important in mediating protection against HIV-1. With the identification of an association between the HIV resistant phenotype and a microsattelite marker in a gene responsible for regulating type-1 immune responses we believe that the IRF-1 179 allele is in linkage disequilibrium with an a yet unidentified functional polymorphism within or near to the IRF-1 gene. Thus, we propose that there is a currently unknown functional polymorphism in the IRF-1 gene that may be responsible for mediating a protective, cellular immune response to HIV-1. It is possible that the IRF-1 microsatellite repeat itself is responsible for functional differences in the IRF-1 gene. However, this is unlikely because the microsatellite repeat lies within the seventh intron of the IRF-1 gene, (Cha et al., 1992), and not within a coding region. It is possible that this region could lie within a transcriptional regulatory region, however this is unlikely, as it is uncommon to find a transcriptional regulatory region within an intron. The most likely hypotheses is that this particular allele is in strong linkage disequilibrium with an as yet unknown, functionally significant polymorphism within the gene.

Significance and Further Direction

The finding of an association between an IRF-1 polymorphism and resistance to infection by HIV-1 may provide insight to potential protective immune responses to HIV-1. This is the first report of a genetic polymorphism in an immunoregulatory protein that may have an effect on HIV-1 pathogenesis. Previous reports of genetic polymorphisms having an effect on HIV-1 susceptibility and disease have been mostly described in genes that encode co-receptor molecules involved in the attachment and binding of the virus to host cells, and in some cases in the natural ligands for these co-receptor molecules. These mutations appear to protect against HIV-1 by purely innate mechanisms. Either by the production of non-functional co-receptor molecules due to inactivating polymorphisms, regulating the amount of co-receptor expressed on a cell, or perhaps by production of excess natural ligand, thus blocking HIV's access to such molecules. It is likely that the mechanism by which the IRF-1 179 allele protects against infection is due to modulation of the immune response to HIV-1. This would be of enormous importance in the future design and development of a successful HIV-1 vaccine. If the mechanism by which IRF-1 alters the immune response to HIV-1 towards a more protective response could be duplicated this would aid immensely in the development of effective HIV vaccines.

One concern is that not all of the resistant women have this protective allele. If the hypothesis that protective responses to HIV-1 are mediated by type-1 immune responses is correct, then women who have the IRF-1 allele may be protected due to its (as yet unknown) influence on type-1 and type-2 response. Women who do not have this allele may have a type-1 bias towards HIV-1 due to some other mechanism. The IRF-1

polymorphism may represent just one of a number of potential differences that allow these women to mount a protective immune response to HIV-1.

The association with resistance to HIV-1 and the IRF-1 179 allele may be due to linkage disequilibrium with another polymorphism in a nearby gene. The IL-4 cluster contains a number of genes that may play a role in resistance/susceptibility to HIV-1. This is a possible, although unlikely explanation, as association studies such as this are rarely able to detect an association with nearby genes further than 100 Kb away (Hill, 1998). In our study we were unable to detect any associations between the IRF-1 and IL-4 microsatellite markers (data not shown). These markers lie 200-270 Kb apart, and appear to segregate independently suggesting that it would be unlikely to detect linkage between the IRF-1 microsatellite and any other gene in this area. However, we could readily detect an association between specific IL-4 microsatellite alleles and the IL-4 – 590 promoter polymorphism (data not shown).

The region of the IL-4 gene cluster has been associated with differential susceptibility to a number of infections and pathologic conditions including schistosomiasis and allergy/atopy believed to be mediated by type-2 responses. It is interesting to note that pathogenesis due to these two conditions appear to dependent on the regulation of the type of T-cell response to particular antigens. Thus, it seems that this genomic region may be important in the regulation of type-1 and type-2 immune responses. If type-1 responses are responsible for a protective immune response to HIV-1 it is not unreasonable to hypothesize that potential genetic mechanisms for resistance to HIV-1 may be found in this region. Although the regions believed important in schistosomiasis and allergic responses map to other regions in the TH2 cluster and not to

the IRF-1 gene it provides further evidence to suggest the importance of this genomic region in the coordinate regulation of the type-1 and type-2 responses.

Further studies are necessary to identify whether or not the observed protective genotype is associated with a functional change in the IRF-1 gene, or if there are any phenotypic changes in immune responses in general, or specifically to HIV-1 in individuals with this polymorphism. We believe that these individuals differ in their immune response and are able to mount stronger cellular responses, resulting in protective immune responses to HIV-1. Studies also need to be conducted to investigate whether the effect of this polymorphism occurs in all populations, or is specific to certain ethnic groups. These factors have led to difficulties in deriving a clear interpretation of previous reports discussing the effects of genetic variation on HIV-1 pathogenesis. Some polymorphisms have only been either observed in certain ethnic groups (i.e. the protective effects of a 32 bp deletion in the CCR5 molecule is only observed in those of European descent), or the protective effects of these polymorphism do not appear to be observable in all groups (i.e. variations in CCR2 and the CCR5 upstream promoter do not appear to be consistent in different cohorts). It is unknown whether or not the IRF-1 179 allele will provide a protective effect in other groups. It is possible that the protective effect of IRF-1 may only be observed in African populations, or may only protect in sexual transmission of the virus. This may be due to underlying differences in the immune response found in African individuals, or may be due to interaction with other confounding factors such as mucosal immune responses. If for example IRF-1 helped to elicit strong type-1 responses in the mucosal immune response, this would not protect individuals infected by intraveinous drug use for example. These results may be hard to

duplicate in other populations for a number of other reasons. The effect of the IRF-1 179 allele appears to be fairly subtle, and may be difficult to observe in all but the largest studies. As well, other studies may be unable to observe such a large group of highly exposed yet uninfected individuals in which protection against infection can clearly be demonstrated.

In conclusion, we hypothesized that there was a genetic basis for resistance to HIV-1, and this may be mediated by mechanisms that may effect protective immune responses to HIV-1. We have clearly demonstrated a familial basis for resistance to infection by HIV-1 in both the ML sex worker cohort, and in the Kindred cohort as well. This was in complete agreement with our hypothesis. Although unable to identify a readily testable resistance phenotype we were able to identify an association between a specific microsatellite allele and a group of women relatively resistant to infection by HIV-1. The allele is associated with resistance and a decreased likelihood of becoming infected with HIV-1. This association was found in an immunomodulatory gene (IRF-1) known to be important in the regulation of type-1 immune responses that we and others believe are important in protecting against HIV pathogenesis and infection, again in agreement with our hypothesis. We believe that this microsatellite allele is in linkage disequilibrium with an as yet unknown functional polymorphism within the IRF-1 gene, and this polymorphism is involved in regulating cellular immune responses to HIV-1. These data are the first to describe an association of a polymorphism within a gene involved in the transcriptional regulation of the immune system and differential susceptibility to HIV-1. These findings may have an impact on the study of cellular

immune responses to HIV-1 and may play a role in the design of future HIV-1 vaccines, and/or any vaccine designed to elicit cellular immune responses.

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Appendix: Abbreviations Used.

ABS	Human AB Serum
ADCC	Antibody Dependent Cellular Cytotoxicity
AIDS	Advanced Immunodeficiency Syndrome
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
ATCC	American Type Culture Collection
B-lcl	B lymphoblastoid Cell Line
CAF	Cellular Antiviral Factor
CAND	*Candida Albicans* Antigen
CD4	Cluster Differentiation molecule 4
CCR	Chemokine Receptor
cDNA	Cloned Deoxyribonucleic Acid
CTL	Cytotoxic T Lymphocyte
CXCR	Chemokine Receptor
DIG	Digoxygen
DNA	Deoxyribonucleic Acid
dNTP's	Deoxyribonuleotides
DTH	Delayed-Type Hypersensitivity
ELISA	Enxyme-Linked Immunosorbant Assay
env	Envelope
EtBr	Ethidium Bromide
EU	Exposed, Uninfected
FLU	Influenza Antigen
g	Gravity
gag	Group Specific Antigem
gp	Glycoprotein
HIV-1	Human Immunodeficiency Virus-1
HTLV	Human T-Lymphotrophic Virus
IFN	Interferon
Ig	Immunoglobin
IL	Interleukin
IN	Integrase
IRF-1	Interferon Regulatory Factor-1
KW	Kruskal-Wallis Statistical Test
LAK	Lymphokine Activated Killers
LTNP	Long Term Non-progressor
mCi	Millicurri
MCH	Mother to Child Transmission Study Group
mL	Millilitre
ML	Nairobi Prostitute Study Group
ML Kindred	Family Members of ML Group
mM	MilliMolar
MW	Molecular Weight
NK	Natural Killer

nM	NanoMolar
NRAMP	Natural Resistance Associated Monocyte Protein
PBMC	Perpheral Blood Mononuclear Cells
PCP	*Pneumocystis carinii* pneumonia
PCR	Polymerase Chain Reaction
Pol	Polymerase
PR	Protease
RFLP	Restriction Fragment Length Polymorphism
RPR	Rapid Plasma Reagin
ul	Microlitre
uM	Micromolar
RT	Reverse Transcriptase
SAC	*Staphylococcus aureus* Cowen Strain
SI	Stimualtion Index
SIV	Simian Immunodeficiency Virus-1
STI	Sexually Transmitted Infection
SW	Sex Worker
TB	Tuberculosis
Th1	T Helper Type-1
Th2	T Helper Type-2
TNF-α	Tumer Necrosis Factor alpha
VDR	Vitamin D Receptor