Exploring CD4+ T cell function in HIV-1 infection and resistance

By

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A thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
In partial fulfillment of the degree of

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Department of Medical Microbiology
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THE UNIVERSITY OF MANITOBA

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Abstract

It is well accepted that not all individuals who are exposed to HIV will necessarily become infected. The University of Manitoba/University of Nairobi Collaborative Group has identified a cohort of commercial sex-workers in the Pumwani district of Nairobi who, despite constant unprotected sexual exposure to HIV, remain uninfected.

Previous studies by this group have identified several immunological and genetic correlates of protection against HIV. Therefore, the focus of this thesis has been to explore both of these aspects in CD4+ T cells, which are crucial in coordinating the adaptive immune response and the major cell type targeted by HIV. Immune and genetic contributions to HIV resistance were studied in greater detail than previous studies through the use of microarray technology to monitor global gene expression. Early studies demonstrated the need to focus gene expression analysis specifically on CD4+ T cells rather than the mixed cell population. This strategy also exposed dramatic gene dysregulation in CD4+ T cells early on in HIV infection.

Studies comparing gene expression profiles of CD4+ T cells of HIV resistant (HIV-R) women to those of HIV low-risk negative (HIV-LRN) and HIV high-risk negative (HIV-HRN) women showed that the CD4+ T cells of HIV-R women expressed lower baseline levels of genes involved in cellular activation and host genes involved in HIV replication. As well, several genes involved in the T cell receptor signaling and cytokine signaling pathways were expressed at lower levels in HIV-R women. Furthermore, we show that this lower baseline gene expression can be used to characterize HIV-R individuals. Analysis of levels of soluble immune mediators support these findings, as unstimulated

immune cell cultures of HIV-R women show significantly lower levels of key pro-inflammatory cytokines. Taken together, these data support a model where HIV-R individuals have a high proportion of circulating CD4+ T cells that are at a lower level of activation. Since HIV preferentially infects activated cells and activation is required for establishment of early infection, this heightened level of immune quiescence likely contributes to HIV resistance. These findings should be informative for the design of an HIV vaccine which is so urgently needed.

Dedication

This thesis is dedicated to all the women, past and present, enrolled in the Pumwani cohort. It is their gifts of friendship and cooperation that made this work possible. It is our responsibility to ensure that these gifts are not given in vain.

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Here's to the few who forgive what you do, and the fewer who don't even care – L. Cohen

If it takes a village to raise a child it takes much more than that to make a thesis, particularly this one. I have been helped in countless ways by countess individuals over the years and would like to take this opportunity to say thank you all. Please forgive me if your name does not appear below, omissions in these brief lines are a necessary evil and not reflective of a lack of gratitude on my part.

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Section 1.0: Introduction

1.1 Global Impact of HIV-1

Despite unparalleled research into treatment, prevention and vaccination, the global pandemic of the human immunodeficiency virus type-1 (HIV-1) has grown every year since its discovery in the early 1980s[1, 2], and shows no signs of slowing down. It is currently estimated that 39.5 million people are infected with HIV-1 globally and that more than 25 million have already died from acquired immunodeficiency syndrome (AIDS), bringing the total number of lives impacted by HIV to well over 60 million[2]. In 2006 alone it is estimated that 4.3 million people were newly infected by HIV-1 while another 2.9 million people died of AIDS. These numbers serve not only to underline the magnitude of the impact of HIV-1 on the human population, but as a reminder that despite our best efforts to date the net number of infections continued to grow in 2006 by approximately 1.4 million infections. This is truly a global trend, as over the past 2 years the number of HIV-1 infections has increased in every region of the world, with some of the greatest increases being seen in eastern Europe and eastern and central Asia[2].

By far the hardest hit areas of the world are those countries in the region of Africa south of the Sahara desert, where the average adult HIV-1 prevalence is 5.9%. Currently in sub-Saharan Africa, it is estimated that 24.7 million people are living with HIV-1, this number accounts for approximately 63% of all infections worldwide[2]. Recently, there has been some evidence of decline in rates of infection in several sub-Saharan African nations; notably Zimbabwe, Tanzania and Kenya[2]. This recent decline has been attributed to behavioral change and condom programs[3, 4], however, there is some

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question as to whether these declines are significant when morbidity and mortality rates are considered.

Since the discovery of HIV-1, considerable research has been conducted into possible treatment strategies with some success. There are currently 21 antiretroviral (ARV) compounds licensed by the American Food and Drug Administration (FDA) that act against various stages in the virus life cycle[5]. As well, combinations of these drugs into a regimen known as Highly Active Antiretroviral Therapy (HAART) has been successful in reducing morbidity and mortality in industrialized nations[6, 7, 8]. However, despite global initiatives aimed at introducing ARVs into developing nations, such as the World Health Organization's 3 by 5 initiative and the United States government's President's Emergency Plan For AIDS Relief (PEPFAR), it is estimated that less than 20% of the world's population in need of treatment are actually receiving therapy[2]. Scaling up of treatment would not only reduce morbidity and mortality, but may play a role in prevention, as decreases in HIV-1 viral load may also prevent new infections[9, 10].

Prevention strategies such as scaling up of condom programs[11], treatment of other sexually transmitted infections[12] and male circumcision[13, 14] have all been shown to lower HIV-1 transmission rates. Several other non-vaccine preventative strategies are also currently being investigated as to their ability to prevent HIV-1 transmission, such as treatment and prevention of Herpes Simplex Virus 2 (HSV-2) infection[5] and the use of topical microbicides[5, 15]. However, the efficacies of these strategies remain to be seen and despite these known and proposed prevention strategies, the rates of HIV infection continue to rise.

There is no question that the best hope to stop the HIV-1 pandemic is the discovery of a preventative vaccine. To date only one vaccine candidate has completed phase III trials, with disappointing results[16]. This vaccine focused on inducing neutralizing antibodies to the HIV-1 major antigen gp120, and showed no reduction in infection rates in the test population. As well, a phase IIb trial focusing on eliciting protective cell mediated immune responses was recently halted after initial results showed no protective effect[17]. There are currently 2 other ongoing clinical trials of possible HIV-1 vaccines, these focus on eliciting both anti-HIV-1 antibodies and cellular immune responses. This new strategy has been proposed to be more effective than the previous strategies[16], however, early immunogenicity data suggest that the current constructs may not be effective in preventing infection[18]. Invaluable to design of a successful HIV vaccine will be studies of individuals who remain uninfected even after multiple exposures to the virus.

Understanding the possible mechanisms of naturally occuring resistance to HIV-1 infection in human populations has become the greatest hope for informing both vaccine design and treatment strategies. Several studies from around the world have defined groups of individuals who, despite extensive exposure to HIV-1 remain uninfected[19, 20, 21, 22, 23]. Several models of resistance to HIV-1 infection have been proposed. However, there remains no one overall theory describing HIV-1 resistance in all populations. Innate antiviral responses, HIV-specific immune responses, and genetic polymorphism have all been proposed to explain reduced susceptibility to HIV[19, 24, 25, 26, 27, 28]. However, one fact remains clear; naturally occurring resistance to HIV-1

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infection is likely a sophisticated, multi-factorial trait involving both immune and genetic factors. In order to fully understand possible mediators of protection against infection by HIV-1, studies examining the function of key immune cells in these individuals need to be carried out. The aim of this thesis is to define and describe possible mediators of protection against infection by HIV-1 in the CD4+ T cell population of a well-defined cohort of HIV-1 resistant individuals.

1.2 HIV-1 Discovery and Transmission

1.2.1 HIV-1: the causative agent of AIDS

In 1981 a cluster of 5 cases of a rare pneumonia caused by an opportunistic fungi known as *Pneumocystis carinii* (now known as *Pneumocystis jiroveci*) were described in gay men in Los Angeles[29]. These cases were accompanied by a low T cell count and the syndrome was originally dubbed Gay-Related Immune Deficiency (GRID). Later that year, several more cases of *Pneumocystis carinii* pneumonia (PCP) as well as other opportunistic infections accompanied by severe immune deficiency were described[29]. As the major risk factors included intravenous drug use, hemophilia and Haitian origin, in addition to homosexuality, the syndrome was renamed Acquired Immunodeficiency Syndrome (AIDS)[30]. Analysis of the dynamics of this new syndrome lead epidemiologists to the conclusion that its root cause was an infectious agent that was primarily transmitted through blood and other body fluids.

Laboratories from around the world began investigating the causative agent of this immune deficiency. In 1983, a group working from *L'Institut Pasteur* in Paris, France, headed by Luc Montagnier, isolated a retrovirus from a lymph node biopsy of a patient

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displaying lymphadenopathy. This virus could not be immunoprecipitated from culture supernatants with antibodies to the only other known human retroviruses, Human T cell Leukemia Viruses 1 and 2 (HTLV-1/2). Therefore, it was determined that this was a novel virus that they termed Lymphadenopathy Associated Virus (LAV)[1]. A short time later, a group working at the National Cancer Institue in the United States of America, headed by Robert Gallo, described a retrovirus isolated from blood samples of a patient with AIDS. Due to similarities between this virus and others studied in their lab, they named the virus HTLV-III[31]. The following year a group from the University of California at San Francisco, headed by Jay Levy, published their data on the isolation of a retrovirus from a patient with AIDS that they termed AIDS Associated Retrovirus (ARV)[32]. In 1986 it was determined that all three newly identified viruses were in fact the same virus, and the only known member of a new viral species known as the Human Immunodeficiency Virus[33]. That same year a second retrovirus was isolated from a patient with AIDS in Western Africa. It was shown that this virus was sufficiently different in genetic sequence and protein structure from the originally described HIV (now known as HIV-1) and thus was named HIV-2[34].

Since the initial events in the mid 1980s extensive research has been carried out describing the nature of both viruses. It is now well accepted that HIV-1 is the causative agent of AIDS and its subtypes are responsible for the HIV-1 pandemic.

1.2.2 HIV-1 transmission

Some of the earliest research on HIV was focused on transmission dynamics, in hopes of discovering means by which to prevent infection. HIV-1 can only be transmitted through

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contact with infected body fluids. The major forms of transmission include unprotected sexual contact, sharing of needles during injection drug use, blood transfusion, and from mother to child during birth or through breast-feeding. It is also clear that different populations exhibit differing transmission dynamics. However, heterosexual contact accounts for an estimated 85% of transmission worldwide[11].

1.3 HIV Origins, Structure and Replication

1.3.1 HIV virology and diversity

HIV-1 is classified into the genus *Lentiviridae* within the *Retroviridae* family of viruses, so named for their ability to transcribe their RNA genome into DNA, a process known as reverse transcription. HIV-1 is further divided into three groups; M (main), N (non-M, non-O) and O (outlier) based on their genetic makeup[5]. Pandemic HIV-1, caused by group M, has been further classified based on genetic sequence into 9 subtypes known as clades; named A, B, C, D, F, G, H, J and K, with subtype C being responsible for 55-60% of infections worldwide[35, 36]. Both clades A and F have been further broken down into sub-subtypes known as A1, A2 and F1 and F2 respectively. In the past few years, recombinant forms of the virus have also been identified, and are likely the result of dual infections of different viral subtypes within a single individual. Both unique recombinant forms, those arising in a single individual with no proof of transmission, and circulating recombinant forms, those with strong epidemiologic evidence for transmission, have been described[37, 38, 39]. Globally, subtype distribution is diverse with several regions being impacted by different subtypes. For example subtype B predominates North America and Western Europe, while subtype C is responsible for the bulk of infections in Southern Africa[5, 40]. Even within the continent of Africa the epidemic is heterogenous with the

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subtype distribution being vastly different in the eastern and central regions than it is in the south[40]. The functional consequences of these genetic differences have been investigated, and it has been shown that there are some differences in the ability of particular subtypes to pass from person to person and to progress to disease. However, the impact of these observed differences on the pandemic are unclear[35]. The ability of HIV-1 to recombine between subtypes, as well as the high mutation rate of its replication machinery (it is estimated that the viral polymerase introduces approximately 2 errors per round of replication[41]) is of great significance. Not only does this ability provide protection for the virus against the host adaptive immune system, but is considered a major factor complicating the development of a preventative vaccine[42].

1.3.2 HIV origins

It is currently believed that all 3 HIV-1 groups (M,N,O), as well as HIV-2, originated from related yet geographically distinct strains of Simian Immunodeficiency Virus (SIV), via separate cross-species transmission events[43]. Recently, studies in fecal samples of wild chimpanzees of the subspecies *Pan troglodytes troglodytes* showed that HIV-1 groups M and N likely arose from SIVcpz carried by geographically distinct populations of chimpanzees in Cameroon[44]. The origins of group O are not as well defined, although epidemiological evidence suggests cross-species transmission may have occurred in Gabon[43]. HIV-2 on the other hand, likely originated from SIVsm, and entered the human population as a result of several transmission events from a different genus of primates known as *Cercocebus atys*, more commonly known as the sooty mangabey[45, 46].

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1.3.3 HIV viral structure

HIV-1 is a single-stranded, enveloped RNA virus that measures approximately 110-120

nm in diameter (Figure 1). The viral envelope is acquired from the host cell upon exit of

the virus and contains both viral proteins and several host proteins important for

infection[47]. The HIV-1 genome is encoded by an approximately 9.5 kilobase, single-

stranded, positive sense RNA, of which two copies are carried in the viral capsid[48].

The genome encodes 9 open reading frames that produce 15 proteins[49]. The three

major structural polyproteins; *gag* (group specific antigen), *pol* (polymerase) and *env*

(envelope), are proteolytically processed into 9 distinct structural proteins. The *gag*

polyprotein is processed into four proteins known as p24, p17, p9 and p7. The p24

protein, also known as the capsid (CA) protein, forms the typical retroviral cone-shaped

core. The p17 or matrix (MA) protein forms a shell around the p24-derived core that

closely associates with the viral envelope. The remaining two *gag*-derived proteins, p9

and p7, bind to the RNA genome of the virus to form a protective nucleocapsid (NC).

The *pol* gene products are all enzymatically active and consist of the viral protease (PR,

used to proteolytically process the viral polyproteins), the reverse transcriptase (RT, used

to make DNA copies of the RNA genome) and integrase (IN, necessary for integration of

the viral DNA genome into the host chromosome). The *env* gene encodes two surface

glycoproteins known as gp41 and gp120. These proteins combine to form trimeric

structures in the viral membrane that are involved in attachment and entry of the virus

into the host cell[48, 50].
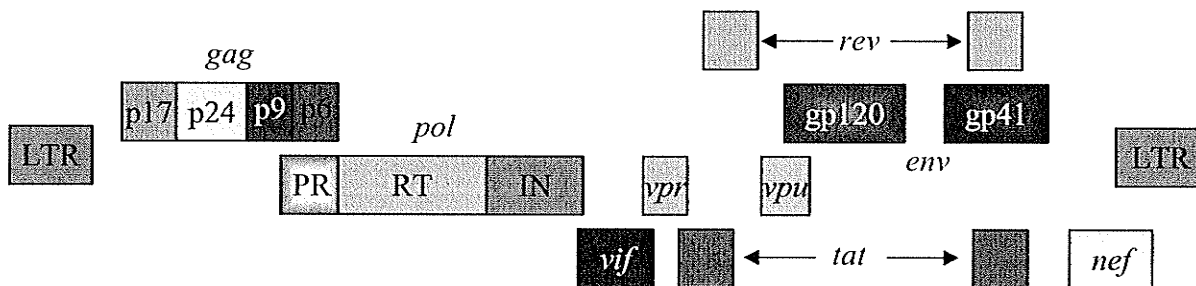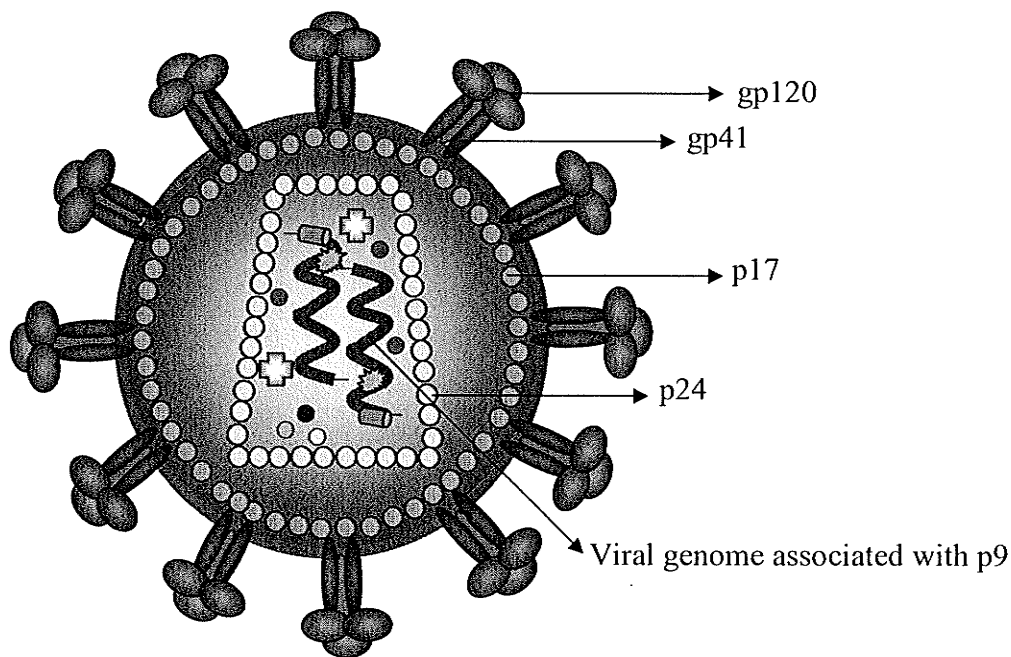
Figure 1: Cartoon diagram of HIV-1 virion structure and genomic arrangement. A review of viral structure can be found in Turner et al[**51**]

HIV-1 also produces three regulatory proteins known as Nef, Rev and Tat. The Nef protein has been studied extensively and acts to modify both the cellular and extracellular environment in order to improve HIV-1 survival and infectivity. Functions of Nef include; immune evasion in the form of downregulation of CD4 and MHC class I, allowing the infected cell to escape killing by CD8+ cytotoxic T cells[52, 53], and by inducing the expression of Fas ligand (FasL) which binds to Fas on the surface of bystander cells and induces apoptosis[54]. Both the Rev and Tat proteins are essential to viral replication with Tat regulating much of viral gene transcription and Rev acting to stabilize and shuttle long transcripts and the viral genome out of the nucleus[55, 56]. As well, HIV-1 encodes three accessory proteins known as Vif (for Viral infectivity Factor), Vpr (Viral Protein R) and Vpu (Viral Protein U) that aid in viral infectivity, replication and release[48].

1.3.4 HIV viral life cycle

Early on in HIV-1 research, studies were carried out to elucidate the mechanism of infection of HIV-1. It was soon determined that HIV primarily infected cells expressing the surface molecule CD4, specifically CD4 + T lymphocytes and macrophages[57]. Soon after this discovery the CD4 molecule was determined to be the primary cellular receptor for HIV-1[58]. Since those initial discoveries, much work has been done to describe the entry and replication strategy of this virus.

The initial step in HIV-1 replication is the interaction of the viral glycoprotein gp120 with CD4 on the cell surface. This interaction induces a conformational change in the gp120 molecule that promotes its binding to a secondary receptor also on the cell

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surface[49]. The two most important secondary receptors in HIV-1 pathogenesis belong to a group of G-protein coupled chemokine receptors[59] and are known as CCR5 and CXCR4[60]. Several other chemokine receptors, most notably CCR2 and CCR3[61] have also been implicated in binding and entry of HIV-1, although the clinical importance of these interactions is unclear[49]. In fact, circulating virus is often classified as either R5 or X4 tropic, depending on the co-receptor it binds. The so called R5 tropic viruses are the major transmitted form of HIV-1, both mucosally and intravenously, and predominate in early and latent infection, with X4 tropic viruses arising late in infection[60].

As well, gp120 has been shown to interact with a molecule on the surface of dendritic cells (DCs) known as dendritic-cell-specific ICAM3-grabbing non-integrin (DC-SIGN)[62]. It is thought that when HIV-1 encounters sub-mucosal DCs during sexual exposure, the virus binds to the surface of these cells and may either be internalized or remain attached to the cell surface and can be carried by these cells to the lymph nodes where it is presented to a susceptible population of CD4+ T cells[49]. Thus DCs act as a 'Trojan horse' for shuttling of HIV-1 to the lymph nodes[63]

Once bound to the cell surface, HIV-1 may enter the host cell by both membrane fusion, through direct interaction of gp41 with the host cell membrane, or endocytosis, although it is thought that membrane fusion events lead to the bulk of productive infection[49]. Once inside the cell, the viral structure un-coats, exposing the reverse transcriptase (RT) complex. The un-coating process is not well understood but it is thought to require interaction between several viral and host proteins[64, 65, 66, 67]. This newly exposed RT complex is composed of a copy of the viral RNA genome as well as the viral proteins

RT, IN, MA (p17), NC (p7 and p9), Vpr and Vif as well as host molecules, most importantly tRNAlys3, which is responsible for priming reverse transcription[49, 68, 69]. Reverse transcription is initiated at the long terminal repeat (LTR) regions of the viral genome and is carried out by the RT complex and results in a double-stranded cDNA genome that associates with IN, MA, Vpr, and several host proteins to form what is known as the pre-integration complex (PIC)[70]. It is at this stage where the bulk of diversity is introduced into the viral genome. As mentioned previously the viral RT is extremely error prone and mutations are introduced into the viral genome at a rate of 1 error for every 5×10^5 bases[41]. As well, during replication the RT molecule may jump from one copy of the RNA genome to the other several times, it is likely that this mechanism creates recombinant viruses in dually infected cells[71, 72]. This replication strategy, although inefficient, only 1 in 10,000 progeny viral particles are infectious, likely improves overall survival of the virus by modifying epitopes recognized by the adaptive immune system, introducing drug resistance mutations and increasing diversity, complicating the construction of a preventative vaccine[42].

The newly formed pre-integration complex travels to the nuclear membrane along microtubules[73] where the virus meets its next challenge, entry into the nucleus. In dividing cells, where the nuclear membrane has dissolved, there is no blockade for viral entry, thus the life cycle may progress unabated. However, HIV-1 also has the ability to infect non-dividing cells, and must therefore deal with the fact that the PIC is roughly twice the size of the nuclear pore. How the virus crosses the nuclear membrane remains a debated issue. One model suggests that binding of a canonical nuclear localization signal (NLS) found on the HIV-1 MA protein by the host proteins importin-alpha and beta is

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sufficient to provide entry into the nucleus[74]. Another theory states that direct interaction of Vpr and IN (both of which contain non-canonical NLSs) with the nuclear pore, in association with host proteins such as importin 7, may be necessary for entry[75, 74, 76]. Regardless of the mechanism(s) employed, the viral PIC is efficiently transported across the nuclear membrane and the life-cycle continues.

Once in the nucleus, a linear form of the double-stranded DNA viral genome is inserted into the host chromosome using the viral IN protein[77]. In addition to the linear genome, the viral cDNA may also exist in the host cell nucleus in a circular form containing either 1 or 2 copies of the viral LTR. These copies of the genome are not themselves infectious. However, they do produce mRNA transcripts and may aid in the early events of viral gene transcription[66, 78]. Once inserted into the host genome, the 5' LTR acts as both a promoter and enhancer region for viral gene transcription. The promoter region contains both a TATA box element as well as SP1 binding sites that are used to recruit host RNA polymerase II to begin transcription[79]. Upstream of the promoter region, the LTR contains a transcriptional enhancer region that binds nuclear factor kappa B (NF-κB), nuclear factor of activated T cells (NFAT) and members of the Ets family[80]. The binding of these host proteins creates a fully functional transcription enhancement complex. It is likely that this early requirement for host cell proteins found primarily in dividing cells, allows the virus to remain latent in non-dividing cells, both creating a viral reservoir and allowing the virus to escape eradication by treatment, since most antiretrovirals block replication[49].

Initial transcription of viral mRNAs, induced by host proteins, produces several transcripts that are heavily spliced by host machinery, and produce the proteins Tat, Rev and Nef[78]. Once sufficient levels of Tat are accumulated in the nucleus this viral protein takes over induction of transcription. Tat, in association with several host proteins including cyclin T1 and cyclin dependant kinase 9 (cdk9)[81], binds to transactivation response (TAR) elements in the HIV genome and is a very strong transcriptional regulator[55]. Similarly, before any of the larger transcripts can be stably produced, a certain threshold of the viral protein Rev is required. Rev functions to stabilize longer gene transcripts for the remaining HIV-1 genes, particularly *gag, pol* and *env*, preventing them from being spliced by host machinery. Rev is also essential for stabilization and shuttling of the complete single-stranded RNA viral genome out of the nucleus and to the cell membrane[82, 56]. Once these requirements are met, translation of all viral proteins and genomic replication can occur.

Translation of all viral transcripts occurs in the cytoplasm utilizing host machinery. After translation, viral structural proteins begin to accumulate at the cell membrane. The envelope proteins, gp120 and gp41, are initially translated as a polyprotein known as gp160. This protein is trafficked through the endoplasmic reticulum where it is glycosylated and shuttled through the golgi where it is processed by host proteases into the gp120 and gp41 sub-units, which are then inserted into the cell membrane[49]. Gag, and Gag-Pol polyproteins, also accumulates in the cytoplasm at the cell surface where they are cleaved into their various subunits by the viral protease through both autocatalysis (freeing PR from the Gag-Pol polyprotein) and through enzymatic interaction with PR dimers. Assembly of the viral particle is directed by Gag[83, 84]. The

remaining components of the virus, including the RNA genome, Vpr and tRNALys3 are also included at this point[85]. Once assembly is complete the virus buds from the host cell membrane where it collects its envelope including viral glycoproteins and host proteins, such as MHC class II[47], and an infectious virion is produced.

1.4 HIV Disease Course and Classification

Over the years there have been several attempts to classify clinical disease stages in HIV infection. As the loss of CD4+ T cells is the hallmark of HIV disease, and is thought to be the primary cause of immunodeficiency[50], CD4+ T cell count, in association with other clinical symptoms, are generally used in some combination to classify disease stages. The primary purpose of any classification system is to guide and inform treatment decisions made in the care of HIV infected patients[86]. Although there have been several disease staging systems proposed[87, 88, 89], the method developed by the Centers for Disease Control and Prevention[86] is primarily used. This system uses a combination of three categories of CD4+ T cell counts (or CD4+ T cell percentages) and three categories based on clinical symptoms. These categories are outlined in Table 1. This system also provides a definition of AIDS, as any condition that is predictive of defects in cell-mediated immunity in a person infected by HIV. This definition includes the occurrence of several clinical syndromes or a CD4+ T cell count below 200, the point at which most AIDS defining illnesses are thought to occur[86].

Table 1: CDC classification system of HIV disease

CD4+ T Lymphocyte categories

Category	CD4+ T cell count	CD4+ T cell %
1	>500 cells/ul	>28%
2	200-499 cells/ul	14-28%
3	<200 cells/ul	<14%

Clinical Condition Category

Category	Description	Example Conditions*
A	Acute/Asymptomatic infection (conditions listed in B and C must not have occurred)	- Persistent generalized lymphadenopathy - Acute HIV infection with accompanying illness
B	Symptomatic infection. Displaying a condition not in Category C that meets 1 of the following conditions: 1) Attributable to HIV infection or indicative of a defect in cell mediated immunity 2) Requires clinical management that is complicated by HIV infection	- Oral pharyngeal candidiasis - Herpes Zoster (shingles) - Peripheral neuropathy
C	Symptomatic infection including an AIDS defining illness	- Candidiasis - Cervical Cancer - Cytomegalovirus disease - Encephalopathy - Histoplasmosis - Kaposi's sarcoma (Human Herpes Virus 8) - Lymphoma, Burkitt's - Lymphoma, immunoblastic *Mycobacterium avium* complex or *M. kansasii* *Mycobacterium tuberculosis*, any site - Progressive multifocal leukoencephalopathy - Pulmonary/extra-pulmonary tuberculosis *Pneumocystis jiroveci* pneumonia - Toxoplasmosis of brain - Wasting syndrome (due to HIV)

*Full list of conditions for all categories available in MMWR #92, 1993 [86]

1.5 Stages of HIV infection

The initial (acute) stage of HIV infection occurs quite rapidly and has a large impact on the host immune system. Sexual exposure to HIV-1 results in infection of resident CD4+ T cells, macrophages and dendritic cells[90, 91]. As mentioned previously, the infected dendritic cells act to shuttle the virus across the mucosal barrier and into local lymph nodes where the virus encounters a high concentration of susceptible cells[90]. At this stage there is also a large amount of viral replication in susceptible cells at the site of exposure that helps to establish broader systemic infection, including establishment of viral reservoirs in the peripheral lymph nodes and other organ systems[92, 93].

Several studies of HIV-1 [94, 95] and SIV[96, 97] have shown that at this early stage of disease, ~2-3 weeks after infection, there is a massive replication of virus, up to 10^7-10^8 copies/ml of blood[98], that results in a large, if not total, depletion of a subset of CD4+ T cells known as effector-memory T cells (T_{EM}) in the extra-lymphoid tissue, particularly in the gastrointestinal tract. However, this is mirrored by a much smaller decline in the blood levels of CD4+ T cells[96, 99]. This stage can correspond to the first clinical symptoms of HIV infection that may include fever, mononucleosis-like illness, rash, oral ulcers and lymphadenopathy[100, 101, 102]. After this initial replication and T cell depletion, T cell numbers in the blood begin to rebound and viral loads drop to a much smaller level known as the viral set-point[103]. Immunologically, this corresponds to the establishment of an HIV-specific cytotoxic T lymphocyte (CTL) response which functions to partially control viral replication. It has been shown that an individual's ability to mount an effective cell-mediated immune response and control viral replication can influence the course of disease[104]. As well, around this time, a humoral (antibody)

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response can be detected and an individual is said to have seroconverted. Seroconversion to HIV can take up to 3 months[103].

After acute infection, when the host immune response has been generated and viral replication has been controlled, an asymptomatic phase of chronic infection begins. In the absence of treatment this stage typically lasts anywhere from 2-10 years depending on a variety of host, viral and environmental factors[105, 106]. However, the virus is not inactive during this phase, as it has been shown that replication on the order of 10^{10} copies per day may still occur[91]. Generally speaking at this stage blood viral loads are controlled, due in large part to HIV-1 specific cell-mediated immunity. Throughout this period CD4+ T cell levels slowly decline and immune control eventually fails. This immune failure results in an increase in viral load and sharp decline in the number of CD4+ T cells.

The final stage of infection, AIDS, occurs when the host CD4+ T cell count falls below 200 cells/ul of blood[86]. At this point the viral load increases sharply, owing to a loss of cell-mediated immunity, and the host becomes susceptible to a large range of opportunistic infections which, in the absence of proper treatment, inevitably leads to death. Of particular note is *Mycobacterium tuberculosis* infection in people with AIDS, as it is thought that this infection is responsible for one third of AIDS deaths worldwide[107].

1.6 Altered Disease Progression

It is well known that variability exists on both an individual and population level in terms of rate of progression of disease after HIV-1 infection. In North America and Europe, the median time to AIDS after HIV-1 infection is 8-10 years[108]. However, the picture is not as clear in developing nations. Although some studies have suggested faster rates of disease progression in African nations as well as Asia and India[109, 110, 111, 112], many studies, particularly in Africa, have suggested the median time to AIDS is the same as in untreated North American populations[113, 114, 115, 116, 117]. Despite this controversy, studies have shown that within geographic areas certain sub-populations, such as the study done in a cohort of commercial sex-workers in Kenya[118], progress faster than the general population.

At the host level there is also very well documented variability in disease progression. Several studies have described individuals who, in the absence of treatment, remain relatively healthy for long periods of time. These individuals are known as long-term non-progressors (LTNP). Though the precise definition varies between studies, the general features include low levels of viral RNA in plasma, and maintenance of a CD4+ T cell count greater than 400 cells/ul, in the absence of treatment, for longer than 7 years after infection[119, 120, 121].

It is clear from several studies that there is no one underlying mechanism to explain all instances of long-term non-progression. However, several mechanisms including viral fitness, host genetics and immune function have been proposed. The ability of the infecting virus to tolerate mutations that lead to escape from the immune system[122],

deletions in viral genes, particularly *nef* [123, 124, 125], and subtype-specific differences

in virulence[126, 127, 128] have all been shown to influence disease progression.

However, these viral factors can only explain a small percentage of LTNP.

Polymorphisms in host genes involved in the HIV-1 life cycle and the immune response

have also been implicated in control of infection. Several host genetic factors including

heterozygosity for a mutation in the viral co-receptor CCR5 known as CCR5

delta32[129, 130], polymorphism in CCR2 known as CCR2 V64I [131], and high

genomic copy number of the chemokine gene CCL3L1[132] have all been associated

with slower disease progression. As well, several human leukocyte antigen type 1 (HLA

I) alleles have been associated with slower disease progression. Overall, heterozygosity at

all HLA class I loci, and the presence of rare HLA alleles, seems to have a protective

effect against HIV-1 disease progression[133, 134, 135]. Specific HLA alleles have also

been associated with protection, most notably the alleles known as B27[136, 137] and

B57[137, 136, 138] have consistently been associated with slow disease progression,

although recent studies suggest that cell function and epitope specificity within the

context of a particular HLA type may be truly what is important in controlling

infection[139, 140].

Immune cell function, particularly cell-mediated immunity, has also been investigated as

a contributor in control of infection. Breadth of HIV-specific CD8 T cell responses[141],

and their ability to produce interferon gamma (IFNγ)[142, 141] or proliferate[143, 144]

in response to HIV-1 antigens have been associated with slowed progression. As well, the

role of CD4+ T cells have been investigated and suggest that the ability of HIV-1 specific

CD4+ T cells to proliferate, produce interleukin 2 (IL-2) or IFNγ is associated with slower disease progression[145, 146, 147].

Recently studies have revealed a subset of LTNP known as elite suppressors[148]. These individuals are unique among the LTNP population in that they maintain exceptionally low viral loads (<50 copies/ml). This phenomenon is not well understood but it is thought to be due, at least in part, to characteristics of their CTL response, as the HLA B57 allele is over-represented in this group[149, 148].

In contrast the phenomenon of long-term non-progressors, there is a subset of individuals known as rapid progressors (RP) whose CD4 counts fall below 200 cells/ul within 2 years of infection[118]. Similar to the LTNP group several host genetic factors[150, 151, 152], HLA types[153, 154, 155] and immune factors[138, 156, 157] have been associated with this phenomenon.

Furthermore, there are several examples of individuals who, despite consistent exposure to HIV-1, never become infected. As with those individuals that progress to disease in an altered manner, several immune and genetic factors have been proposed to explain this resistance to infection. As a group of these individuals are the major focus of this thesis, these factors will be discussed in further detail later on in this section.

What is clear from all of these studies is that the rate of disease progression and even the level of susceptibility, in an individual is a dynamic interaction between the virus and the

host. Studies of individuals that seem to control or those that resist infection are seen as a

great hope in designing new anti-HIV therapies and therapeutic or preventative vaccines.

1.7 Introduction to the Human Immune System

The human immune system can essentially be dichotomized into two separate yet

intertwined arms, innate and adaptive. The major differences defining these two arms are

the capacity of the adaptive response to recognize specific antigens and develop memory,

whereas the innate immune response is non-specific and does not change with re-

exposure.

1.7.1 Innate immunity

The innate immune system is comprised of several different cell types that secrete a

variety of antimicrobial compounds that act non-specifically to inhibit viral, bacterial,

fungal and parasitic infection. The major cell types of the innate immune system are;

dendritic cells (DC), granulocytes, monocytes, macrophages, natural killer cells (NK),

natural killer T cells (NKT) and gamma-delta T cells[158, 159]. A critical component of

innate immunity is its ability to recognize common microbial patterns, such as

lipopolysaccharide (LPS) or CpG rich DNA, through the use of pattern recognition

receptors (PRR) such as Toll-like receptors (TLRs)[160]. Once the innate immune system

is activated, the cells involved in this response can, depending on their type; process and

present foreign antigen to cells of the adaptive immune system, non-specifically induce

cell death in infected cells, and secrete a variety of cytokines and chemokines that shape

the adaptive response[160].

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As well, the complement system comprises a major part of innate immunity. This system involves several small proteins normally circulating in the blood that can be activated using either the classical, alternative or mannose-binding lectin pathways. These proteins aid in innate immunity in several ways such as enhancement of phagocytosis and formation of the membrane attack complex[161].

1.7.2 Adaptive immunity

Subsequent to the activation of cells of the innate immune system is the induction of adaptive immunity. The major characteristic of this response is that it is antigen-specific and has the capacity to create memory cells that aid in secondary immune responses upon re-exposure[162]. The adaptive immune response begins when exogenous antigens or infectious agents are engulfed, processed and presented in the context of host proteins known as human leukocyte antigen class II (HLA II) on the surface of antigen-presenting cells (APC) such as macrophages and dendritic cells[163]. Lymphocytes known as CD4+ T helper cells (Th) are capable of recognizing antigens presented in the context of HLA II through the T cell receptor (TCR), a complex of integral membrane proteins found at the cell surface. Once an antigen-specific T cell recognizes a foreign antigen it becomes activated and can produce a variety of different cytokines that shape the adaptive immune response.

The adaptive immune response itself can be broken down into humoral and cell-mediated arms. The humoral arm is comprised of antigen-specific antibodies produced by B cells that, after engagement and activation through the B cell receptor, rearrange their immunoglobulin gene segments through a process known as affinity maturation. These

antibodies can be secreted and aid in opsonization of extra-cellular foreign particles, direct lysis of infected cells through a process known as antibody-dependant cellular cytotoxicity or direct neutralization of pathogens[163].

Cell-mediated immunity involves the recognition of infected cells by CD8+ T lymphocytes through interaction of the TCR and HLA class I molecules, which are present on the surface of all nucleated cells. HLA class I molecules serve to sample the intracellular environment and present peptide epitopes on the surface of the cell. When a peptide from an infectious agent is presented in an HLA I molecule on a cell surface it can interact with the TCR of a CD8+ T cell, in an antigen-specific manner, and result in a cytotoxic T lymphocyte (CTL) response. This interaction ultimately leads to the death of the infected cell either through cell directed lysis, secretion of cytolytic compounds such as perforins and granzymes, or through Fas/FasL mediated apoptosis[163]. Both the humoral and cell-mediated arms of the immune system function in an antigen-specific manner to remove infected cells or eliminate pathogens. These arms also maintain what are known as memory cells, specific for a given pathogen that may persist well after the infection is cleared.

1.7.3 CD4+ T cells in adaptive immunity

The major cell type mediating the differentiation of the adaptive immune response into either the humoral and/or cell-mediated arm is the CD4+ Th cell[163]. After antigen recognition, depending on signals received from innate immune cells, CD4+ T cells can differentiate into either Th1 or Th2 cells responsible for initiation of either the cell-mediated or humoral response respectively. Th1 type cells typically produce IFNγ and act

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to stimulate CTL and some antibody production and are generally directed towards intracellular pathogens. Conversely Th2 cells predominantly secrete interleukin (IL) 4 or IL-5 and aid in development of an antibody response generally directed towards extracellular pathogens and allergy[163].

The initial event in CD4+ T cell activation is the interaction of the TCR complex on the surface of the T cell with HLA II molecules on the surface of an APC. A result of this interaction is the activation of the protein kinase p56^{lck} bound to the intercellular domain of the CD4 molecule. Active p56^{lck} then binds to immunoreceptor tyrosine-based activation motifs (ITAMS) on the CD3 molecule within the TCR complex resulting in a signaling cascade involving several other molecules such as ZAP70 and LAT. This signaling eventually results in induction of transcription factors such as nuclear factor of activated T cells (NFAT) or nuclear factor kappaB (NF-κB). It is this signal, in association with proper costimulation through either CD28 or CD40L on the T cell (when engaged to their cognate molecules CD80/86 or CD40 on the surface of an APC) that induces expression of the cytokines that serve to direct the activation of either CTL and/or B cells[163]. Thus the CD4+ T cell is indispensable to the establishment of an appropriate adaptive immune response, and it is their dysfunction during HIV-1 infection that leads to the eventual failure of the immune system.

1.8 Innate Immunity and HIV-1

The study of innate immunity throughout the course of HIV-1 infection has revealed both a level of immune dysfunction not previously understood and innate molecules that may act to inhibit infection and replication. A major cell type mediating innate immunity,

known as dendritic cells (DCs), are literally found on the front lines of sexual exposure to HIV-1. As mentioned previously DCs can be infected and play an important role in infection by shuttling HIV-1 across mucosal barriers to local lymph nodes where the virus can come into contact with a large pool of susceptible cells. Immature, tissue-residing myeloid dendritic cells (mDCs), and circulating plasmacytoid dendritic cells (pDCs) are both impacted during HIV-1 disease course. Initially it was shown that DC levels fall throughout HIV-1 infection[164, 165]. Further studies demonstrated that the pDC compartment declines throughout chronic infection, at least partially because of CTL mediated killing, while the mDC compartment falls drastically during AIDS[166, 165]. Importantly to the host's ability to fight off other infections, these pDC are not recovered after initiation of HAART[167]. This finding is underscored by the observation that levels of pDC correlate inversely with viral load[168]. This depletion and subsequent defective reconstitution has a profound effect on the host's ability to mount an effective innate immune response late in HIV-1 disease.

NK cells are non-specific cells of the innate immune system that function to eliminate virally infected and tumor cells[169]. Activation of NK cells is mediated through the interaction, or lack of interaction, of HLA I on an infected target cell, and the killer immunoglobulin-like receptor (KIR) on the surface of the NK cell. The KIR family, like the HLA family, are encoded by a large number of polymorphic genes[170]. Cells expressing lower levels of HLA I on their surface, due to cellular dysfunction or infection, lack interaction with KIR molecules resulting in destruction of the target cell by the NK cell. In addition to this function, NK cells may also produce IFNγ, involved in adaptive immunity[171]. Studies in chronically HIV-1 infected individuals have shown a

loss of IFNγ producing NK cells throughout infection[172]. As well, HIV-1 Env and Tat have been shown to directly inhibit the activity of NK cells[173, 174]. Finally, population-based studies have shown that the interaction of specific KIR and HLA I alleles is associated with delayed disease progression[175].

Monocytes and macrophages, key antigen-presenting cells of the innate immune system, also play a large role in HIV-1 infection. They express both the CD4 and CCR5 receptors necessary for HIV-1 infection, although only macrophages can be productively infected[176]. Tissue macrophages are a major reservoir for HIV-1 throughout chronic infection[177, 176] and, due to the non-lytic nature of their infection, are maintained even when levels of CD4+ T cells are undetectable[178].

Toll-like receptors comprise a major part of the innate immune response to infection by activating cells of the innate immune system to present antigen and secrete cytokines that direct adaptive immunity[160]. Recently it has been shown that single-stranded RNA from HIV-1 can bind to and trigger TLR7 and TLR8 expressed by macrophages and DCs[179]. This activation may have separate and contradictory effects during acute versus chronic infection. In acute infection, the stimulation of TLRs 7/8 initiates an antiviral state that can inhibit HIV-1 replication. However, during chronic infection the activation mediated through TLR 7/8 signaling serves to increase viral replication through activation of latently infected cells[180].

There have also been a variety of studies done on soluble molecules that act to either inhibit or activate HIV-1 replication. The cytokines interferon alpha (IFNα) and beta

(IFN β), as well as the chemokines macrophage inflammatory protein 1 alpha (MIP1α) and beta (MIP1β), regulated upon activation, normal T-cell expressed, and secreted (RANTES) and stromal-derived-factor 1 (SDF-1) have all been shown to inhibit HIV-1 infection[181, 182, 183, 184]. In contrast, several proinflammatory cytokines, including the interleukins (IL) IL-1, IL-6 and tumor necrosis factor (TNF) have been shown to upregulate HIV-1 replication, likely by induction of NF-κB[185]. As well, it has been shown that HIV-1 infection itself induces TNF expression thus initiating a paracrine loop that promotes viral replication. Furthermore, a group of small antimicrobial peptides known as defensins have been shown to directly inhibit HIV-1[186, 187, 188].

Perhaps the most interesting discovery in recent years, in terms of innate immunity to HIV-1 infection, has been the description of anti-retroviral factors in cells that are not permissive for retroviral replication. Two such molecules are apolipoprotein B mRNA editing enzyme catalytic peptide-like 3G (APOBEC3G) and tripartite motif 5 alpha (TRIM5α). APOBEC3G was initially isolated from cell lines found to be non-permissive

to *in vitro* HIV-1 replication[189]. This protein is a member of a family of cytidine deaminases and acts during reverse transcription by inducing several C to U mutations in nascent retroviral DNA[190]. However, the antiviral function of APOBEC3G is overcome in natural infection by the HIV-1 Vif protein which acts to both specifically downregulate APOBEC3G translation and target the protein for ubiquitination and degradation[191, 192]. However, the effect of Vif can be saturated, as over-expression of APOBEC3G can inhibit even Vif containing virus[193], providing hope for a possible treatment strategy based on the action of APOBEC3G.

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TRIM5α was initially discovered as a factor that restricts HIV-1 replication in African green monkey cells[194]. Although the exact mechanism has yet to be elucidated, it is thought that this protein acts to prematurely disassemble the viral capsid rendering it unable to replicate[195]. Orthologues of this gene have been found in several other primate species including humans. However, human TRIM5α is not effective against HIV-1[196].

Despite the wealth of information that has arisen in the last few years as to the interaction of the innate immune system with HIV-1, there is still much to be known. Specifically more work into the subtle ways in which HIV-1 exploits innate immune cells and discovery and understanding of possible inhibitory molecules may serve to inform treatments as well as prevention methods.

1.9 Adaptive Immunity to HIV-1

1.9.1 Humoral responses to HIV-1

HIV-1 infected individuals mount both humoral and cell-mediated adaptive immune responses with varying success. A circulating HIV-specific antibody response can usually be detected in infected individuals anywhere from 1-3 months post infection[197, 198]. The presence of HIV-specific antibodies is the basis for clinical HIV-1 testing, as it is seen as definitive proof of infection. Neutralizing antibodies (nAb), Ab that can prevent *in vitro* infection by HIV-1, are found in almost all HIV-1 infected individuals and generally target the Env protein. However, several studies suggest it is unlikely that the

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antibodies generated have any impact on the course of acute infection, as the response is only mounted well after the initial viral replicative burst is contained[197, 198]. As well, primate studies have shown that depletion of B cells in rhesus monkeys leads to delayed production of nAb but had no impact on viral kinetics in early infection[199]. Although associations between the presence of nAb and control of infection have been demonstrated in some populations[200, 201], the relative contribution of this response may be limited despite its ability to place immune pressure on the virus[202, 203]. Studies in macaques have shown that pre-existing nAb can protect against HIV-1 infection[204] leading to hopes for the creation of a vaccine. However, all attempts to create vaccines that elicit these responses in humans have failed[205]. Newly designed vaccine constructs still aim to elicit nAb, although now the focus is on producing these antibodies in concert with an effective CTL response[206].

1.9.2 HIV-1-specific cytotoxic T cell responses

In contrast to the lack of efficacy of the HIV-specific humoral response, the HIV-specific cell-mediated response has been associated with control of infection. Early studies began to elucidate this role by demonstrating the ability of HIV-1 specific CTL to suppress viral replication and lyse infected cells *in vitro*[207, 208]. Cytotoxic T lymphocytes have also been shown to secrete anti-HIV-1 soluble factors such as MIP1α, MIP1β and RANTES.

In vivo, control of replication during acute infection has been correlated with emergence of HIV-specific CTL[209, 210, 211]. In SIV models of retroviral infection, depletion of CD8+ cells prior to infection lead to an inability to control primary viremia. As well, transient depletion of CD8+ T cells during chronic infection lead to a rise in plasma viral

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loads that was reversed when the CTL re-emerged[212, 213]. Furthermore, primate

studies focusing on vaccination methods to elicit CTL responses have shown that CTL

levels in immunized animals prior to infection with SIV or a simian-human

immunodeficiency virus hybrid (SHIV) correlated with higher levels of CD4+ T cells,

lower viral loads and longer survival[214, 215, 216] fueling hopes that a human vaccine,

directed at creating HIV-specific CTL, may act to prevent or control infection.

As mentioned previously, evidence continues to mount that presentation of HIV-1

epitopes in the context of certain HLA I alleles, such as HLA B27 and B57[137, 148,

138, 217, 136], to virus-specific CTL has a great impact on the progression of infection.

Recently, several studies have shown that the ability of virus-specific CTL to detect and

respond to epitopes within the HIV-1 Gag protein, in the context of several different

HLA types, correlates strongly with slow disease progression and control of viral

infection[218, 139, 140, 219, 220]. Whether this knowledge can be applied on a large

scale to improve disease outcome globally through creation of a therapeutic or

preventative vaccine is not yet known.

1.9.3 CD4+ T cells in HIV-1 infection

Decline of CD4+ T cells in HIV-1 infection is well documented and seen as the hallmark

of HIV disease. These cells are the primary target of the virus and their loss is responsible

for the immune deficiency seen in AIDS[61]. Despite this, considerably fewer studies

have been conducted into the role of HIV-specific CD4+ T cells in controlling infection.

It is known that HIV-specific IFNγ secreting CD4+ T cells are generated during acute

infection, however, their numbers decline in chronic infection. Those cells that do remain

seem to lose function, as proliferative responses wane throughout the course of HIV infection[221, 222]. A reason for this may be the ability of HIV-1 to preferentially infect and destroy CD4+ T cells that are HIV-specific, this no doubt leads to loss of control of infection[223, 221].

The overall loss of CD4+ cells during HIV-1 infection also impacts the CTL compartment's ability to control viral replication, as CD4+ T cell help is crucial for persistence, differentiation and maintenance of CTL memory[224, 225, 226]. In studies of rhesus monkeys vaccinated against SIV or SHIV, prolonged survival and good CTL responses were correlated with the presence of virus-specific IL-2 and IFNγ-producing CD4+ memory T cells. In HIV-1 infection, several studies have shown that the presence of Gag-specific CD4+ T cells that secrete either IL-2 alone or IL-2 and IFNγ correlate with low viral load and delayed disease progression[222, 227, 228]. In fact, one study showed that the presence of Gag-specific CD4+ T cells that proliferate and produce IFNγ correlated better with long-term non-progression than do CTL responses[146]. A better understanding of how CD4+ T cells function to interact with CTL to restrict viral replication would greatly aid vaccine design.

Despite the presence of HIV-specific humoral and cellular responses, in the absence of treatment this immune control inevitably fails. Although it is not entirely understood why this happens, the leading candidate mechanism is viral mutation resulting in immune escape. The error-prone RT enzyme, used by HIV-1 to replicate, introduces mutations into the viral genome. If the virus is able to tolerate a given mutation and it occurs within an epitope recognized by the immune system, for example in an anchor residue for HLA

binding, the virus can escape the immune response, begin to replicate to high levels and cause immune dysfunction[229, 230, 231]. It is imperative to HIV-1 treatment and vaccine design that we understand not only mechanisms of immune failure, but mechanisms of protection from disease progression and perhaps most importantly natural resistance to infection.

1.10 Natural Immunity to HIV-1 Infection

It is a fundamental tenet of infectious disease biology that there is heterogeneity within the population with respect to susceptibility to infection, and once infected, the extent of development of disease. Natural immunity against infection has been described in several populations with both presumed and definitive exposure to HIV-1. This phenomenon of resistance to infection has been described in groups of HIV-1 exposed seronegative (ESN) commercial sex-workers (CSW)[232, 20, 233], HIV-1 sero-discordant couples[234, 235, 236, 237], HIV-1 negative babies born to positive mothers[238, 239], healthcare workers exposed to HIV-1 infected blood[240, 241] and injection drug users known to have shared needles with HIV-1 infected people[242]. Among these groups, HIV-1 resistant CSW, such as those under study by this group at the University of Manitoba, are unique in that they have the highest levels of exposure to the most diverse population of viral variants, and thus may be the best model to inform preventative vaccine design. A variety of mechanisms have been proposed to explain this phenomenon of natural resistance to HIV-1 infection including several immunological and genetic paradigms. However, no one factor completely explains resistance in all populations. It is likely that the phenomenon of HIV-1 resistance is a multi-factorial trait involving complex interplay between immune and genetic factors that may vary within and between

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exposed seronegative populations. It is also unclear whether resistance represents an inability of these individuals to become infected at all, or an ability to clear the virus after infection. In fact, one study has shown that in some exposed seronegative individuals there is evidence of extremely low-level infection[243], in the form of pro-viral DNA. Complete understanding of both immunological and genetic contributions to HIV-1 resistance is the best hope for designing a preventative vaccine.

1.10.1 Immune correlates of HIV-1 resistance

1.10.1.1 Innate immune factors in HIV-1 resistance

Efforts to understand the existence of natural resistance to HIV-1 have focused on many aspects of immune control, including both innate and adaptive factors. The best-studied innate factors have been the chemokines RANTES, MIP-1α, MIP-1β and SDF-1 due to

their interactions with the HIV-1 entry co-receptors CCR5 (RANTES, MIP-1α and MIP-1β) and CXCR4 (SDF-1)[181, 244, 245]. The ability of culture supernatants from activated CD8+ T cells obtained from peripheral blood of HIV infected individuals to inhibit *in vitro* HIV-1 infection[246, 208] lead to the search for the identity of these HIV suppressive factors (HIV-SF). Studies of both CD8+ T cell clones and primary cells identified the beta chemokines RANTES, MIP-1α and MIP-1β as being highly expressed

by these cells. Antibody experiments further showed that the HIV suppressive ability of CD8+ cell supernatants could be completely inhibited by addition of a combination of antibodies against RANTES, MIP-1α and MIP-1 β [183]. Further studies demonstrated that the inhibitory function of these chemokines was due to their ability to interact with

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CCR5 and thus inhibit infection[247, 248, 249]. In studies of HIV exposed seronegative individuals several groups have demonstrated higher expression levels of these chemokines both *ex vivo*[249, 250, 251] and circulating[252]. However, this over-expression is not seen in all groups of HIV resistant individuals[253].

Recently, a genomic study of HIV-1 discordant couples revealed an over-expression of IL-22, an inducer of innate immunity produced by T cells, and concurrent over-production of acute-phase serum amyloid A (A-SAA) protein, that resulted in reduced susceptibility of DC to uptake of HIV[24]. This demonstrates the ability of innate factors to act both directly and through induction of other proteins to prevent HIV infection.

1.10.1.2 Adaptive immunity in HIV-1 resistance

Studies of HIV-specific adaptive immunity have also been extensively carried out in exposed seronegative cohorts. The vast majority of studies designed to detect HIV-1 specific immunity in exposed seronegative people have been successful in showing HIV-specific CTL, CD4+ and/or immunoglobulin A (IgA) in a proportion of ESN individuals. The production of HIV-1 specific humoral responses in the form of IgA has been detected in several ESN populations[232, 254, 255, 256, 257, 237]. These antibodies are generally specific for gp120 and seem to correlate best with evidence of recent exposure[257]. Analyses of these antibodies *ex vivo* have shown their ability to inhibit HIV-1 infection in culture and transcytosis in transwell experiments[254, 255]. Although these findings provide hope for development of mucosal vaccines, their kinetics and lack of persistence make it unlikely that they mediate resistance.

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Due to their importance in clearance of other viral infections and proven association with delaying disease progression in HIV-1 infection, CTL specific for HIV-1 were one of the first detected immune correlates of protection. Circulating HIV-specific CTL, detected either by cytotoxic ability or ability to secrete IFNγ, have been found in ESN individuals by a variety of studies[233, 258, 259, 260, 261, 262, 263, 264, 265, 266]. These responses seem to be detectable in anywhere between 30-70% of ESN depending on the study and, when specificity was measured, were most commonly directed towards Gag protein, although responses have also been seen directed towards Env, Nef, Pol, Pro, Rev and RT. One study involving discordant couples showed CTL responses persisted even up to 34 months post-exposure, although the strength of these responses did wane[260]. Another study, from our research group done in exposed seronegative CSW, showed HIV-specific CTL in the blood and at the cervix and that their presence correlated with duration of exposure (measured as years involved in commercial sex-work). This study further went on to show that CTL present in ESN CSW differed in epitope specificity from HIV positive CSW, despite having the same HLA restriction, thus proposing a possible mechanism by which these CTL might mediate protection[265]. However, whether the presence of HIV-1 specific CTL is what is mediating protection, or if they are merely a marker of exposure, remains controversial[267].

Studies of HIV-specific CD4+ T cells have been less frequent. However, the presence of HIV-specific CD4+ T cells has been demonstrated in ESN individuals[268, 269, 234, 262, 251, 237]. These cells have most often been shown to secrete IL-2 in respones to Env protein or peptides. However, a recent study done by our group showed some ESN CSW had strong proliferative responses to p24[268]. This study and others have also

shown cellular markers of immune activation to be expressed at lower levels in CD4+ T cells of ESN individuals[270, 271, 272]. Interestingly, kinetic studies of SIV infection have demonstrated that although initial infection is established in resident resting CD4 T cells, sustained infection and seroconversion requires infection of activated cells[273]. Epidemiological studies in humans have also shown that transmission of infection is increased in instances of heightened activation at mucosal surfaces, such as during concurrent non-HIV sexually transmitted infection (STI)[274, 275, 276]. Thus it is possible that activation state of CD4+ T cells may have an impact on susceptibility to HIV-1.

1.10.2 Genetic correlates of HIV-1 resistance

In addition to the above immune correlates, several genetic factors have been shown to associate with resistance to HIV-1 infection. Perhaps the best described of these is a 32 base-pair deletion in the CCR5 co-receptor known as CCR5delta32. This polymorphism was originally described in ESN individuals whose CD4+ cells could not be infected *in vitro* by M-tropic strains of HIV-1[23]. It was determined that these individuals were homozygous carriers of a deletion in their CCR5 gene that lead to no expression of the protein on the surface of their CD4+ cells[277, 278]. This deletion confers resistance to primary infection by sexually transmitted R5 tropic viruses. However, exposure to X4 tropic virus, generally parenterally, can still lead to infection. The CCR5delta32 gene seems to provide no protective advantage against infection in heterozygous individuals, although there is some evidence of delayed disease progression associated with carrying one copy of the polymorphism[279, 280, 281]. To date, homozygosity for CCR5delta32 is the only proven mechanism of resistance to HIV-1 infection in a human population.

However, it only explains a small number of ESN individuals, as the mutation is most prevalent in Caucasian individuals and is rare to absent in Asian and African populations.

Several other polymorphisms in chemokines and cellular receptors for HIV-1 have been discovered, although their role in preventing infection is less clear. A deletion in the minor co-receptor for HIV-1 entry CCR2, known as CCR2 delta 64I, has been shown to be associated with protection in discordant couples and in mother to child transmission[282, 283]. However, the major effect of this mutation seems to be in delaying progression to disease[131, 284]. A promoter polymorphism in RANTES, RANTES -28G, has been associated with both delayed progression[285] and reduced susceptibility[286]. Studies of the alpha chemokine stromal derived factor 1 (SDF-1), the natural ligand for CXCR4, have shown a possible role for a polymorphism in the 3' untranslated region (UTR), known as SDF-1 3'A, in reduced susceptibility, possibly due to increased translation and occupation of the CXCR4 receptor[286, 287]. A rare genotype of the gene encoding the DC-SIGN molecule, called the 7/6 genotype (based on number of repeats in the C-terminal transmembrane domain), has been found at higher levels in ESN, and is hypothesized to act by limiting DC binding of HIV-1[288]. However, even all of these polymorphisms together only describe a small portion of ESN individuals, and none but CCR5 delta32 are completely protective.

As with delayed disease progression, several MHC class I and II genotypes have been found to associate with lowered susceptibility to infection. In discordant couple studies HLA mismatch, i.e. members of the couple having different HLA types, has been associated with protection. As well, the class I allele B53 and the class II alleles DR5,

DR1 and DQ4 have been shown to be over-represented in the HIV negative members[289, 290]. In studies of HIV ESN CSW the class I allele B18[232] and the class II allele DRB1*01[286] have been associated with protection. However, the strongest association has been demonstrated in our HIV-1 resistant CSW cohort from Nairobi, Kenya with the so-called HLA A2/6802 supertype[291].

Furthermore, certain alleles of the KIR genes, expressed on NK cells, have been associated with lowered susceptibility to infection, even independent of HLA type. Heterozygosity for the alleles KIR2DL2/KIR2DL3 and homozygosity for KIR3DL1 are over represented in a population of HIV ESN CSW in Côte d'Ivore[292]. Mechanisms by which HLA or KIR genes may mediate protection include a heightened allogenic immune response, in the case of increased protection against virus from a partner having a different HLA type[289], the ability of certain HLA types to present conserved epitopes not presented by HIV positive individuals[265], or by enhanced ability of CTL or NK cells (in the case of the KIR alleles) to eliminate infected cells. However, it should be mentioned that these associations seem to be population dependant, and thus may be linked to other genetic traits or may only be specific for circulating viral variants. In summary, it is likely that the majority of cases of reduced to susceptibility to HIV-1 involve a multi-parametric combination of host genetic and immunologic factors. Further understanding of what mediates protection in ESN individuals, particularly CSW, will be critical in designing a preventative vaccine that is effective against multiple viral subtypes.

1.11 HIV-1 Resistance in the Nairobi CSW cohort

In Nairobi, Kenya, where the population prevalence of HIV-1 is 6%[2], a group of CSW have been described that, despite high levels of sexual exposure to HIV-1, remain persistently seronegative and are thus defined as HIV-1 resistant (HIV-R)[20]. This group of HIV-R CSW exists within a larger cohort located in the Pumwani district of Nairobi and named the ML cohort (after Malaya, the Swahili word for prostitute). The cohort has been active since 1985 and was designed to study factors associated with HIV-1 transmission[293, 294]. To date more than 2700 women have been enrolled. Of these, approximately 600 are in active follow-up. The women are resurveyed semi-annually, which includes a physical examination, testing for bacterial STIs, and collection of sexual-behavioral data, such as number of partners and frequency of condom use. The women participate in several studies and receive primary health-care and are welcome to attend the clinic year-round for any acute conditions. Upon enrollment, a majority of women are seropositive for HIV-1 infection, and of those who enroll negative, most seroconvert within 1-3 years of enrollment. However, a proportion of women (~5-10%) remain HIV-1 negative despite more than 15 years of follow-up (Figure 1). These women are considered to be relatively resistant to HIV-1 infection if they meet the following criteria[20]:

1) HIV-1 negative by both serology and PCR

2) Active in commercial sex-work

3) Follow-up in the cohort for greater than 3 years
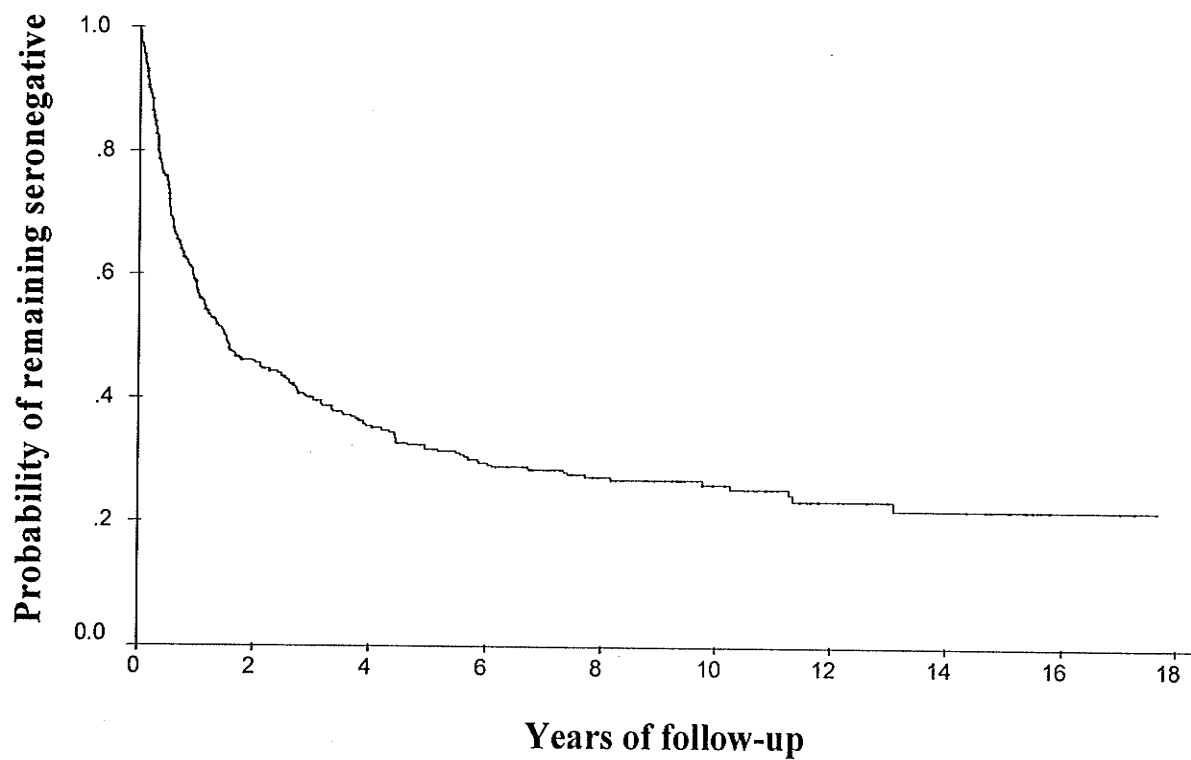
Figure 2: Kaplan-Meier survival plot of probability of remaining HIV-1 seronegative with years of follow-up in the Pumwani commercial sex-worker cohort. Importantly, not all individuals necessarily seroconvert over time. (Modified from Fowke et al 1996[**20**]. Permission obtained February 2007)

This cohort is unique amongst other groups of ESN individuals in that the women who remain HIV negative are exposed at a very high level to what is likely a variety of different viral subtypes. In addition to this, the size of the cohort, the length of follow-up (over 20 years for some members) and the wealth of behavioral, clinical, demographic, epidemiological, genetic and immunological data obtained on these women make it an excellent model for studying naturally occurring immunity to infection by HIV-1. Since the initial identification of this population as HIV-1 resistant, several studies have been carried out to answer the fundamental questions: what is the nature of this resistance and why does it develop?

Early studies in this cohort showed that this resistance was not due to differing sexual behavior, such as lower number of partners, fewer sexual encounters, increased reported condom use or lower susceptibility to other STI[20]. It was also shown that these women do not possess the CCR5delta32 mutation, decreased *in vitro* cellular susceptibility to HIV-1 infection or increased *in vitro* production of RANTES, MIP-1α and MIP-1β by PBMC[253]. However, a later study in this cohort showed HIV-R women had ten fold higher levels of RANTES in cervical-vaginal lavage samples, indicating the possibility of a compartmental anti-HIV environment[295].

1.11.1 Immune correlates of HIV-1 resistance in the Nairobi CSW cohort

As in many other cohorts, several immunological and genetic parameters have been investigated in order to help explain the HIV-R phenotype. Mucosally, a proportion of HIV-R women have been shown to have higher levels of both CD4+ and CD8+ T cells, as well as higher levels of RANTES than HIV negative, non-resistant CSW[295]. Also,

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HIV-1 specific IgA[256] and CTL[264] have been detected in the vagina and at the cervix.

Circulating HIV-specific CTL have also been found in a number of studies from our lab and the larger research group, both by specific lysis of transformed cells expressing HIV-1 epitopes[262, 131] and by IFNγ production in response to a variety of HIV-1 proteins and peptides[259, 265, 262, 131]. Overall, these responses seem to be directed to a variety of HIV-1 proteins including Env, Gag, Nef and Pol. Interestingly, these responses seem to be qualitatively different from those seen in HIV-1 positive individuals in that HIV-R women seem to present epitopes that are either subdominant or not present in HIV-1 positive women with the same HLA I restriction[265] and they have been shown to be of narrower breadth and lower magnitude than HIV-1 positive women[259]. The hypothesis that epitope specificity may play a role in mediating protection is further supported by the observation that late seroconverters, defined as HIV-R women who later become HIV infected, tend to switch epitope specificity once they become HIV positive[265]. Whether these responses play a role in preventing infection, or are surrogate markers of exposure is unclear, as a prospective study showed no association between the presence of CTL responses and protection against infection[267].

Although less well characterized, our group has focused on HIV-specific CD4+ T helper cell responses and has shown that they can be detected in the HIV-R population and are specific against both HIV-1 envelope and p24 proteins, as measured by IL-2 production[262], proliferation and IFNγ production[268]. Differences have also been noted in the functional makeup of the CD4+ T cell compartment, as HIV-R women tend

to have a higher proportion of CD4+ central memory T cells (T_{CM}) than negative controls (S. Koesters unpublished data) and express lower levels of markers of immune activation, specifically HLA-DR and CD38, than HIV positive individuals[268] . The functional significance of these cells is also not clear. However, a variety of hypotheses can be generated to explain their role in HIV-1 resistance. For example, it is possible that they are needed to maintain an active and healthy CTL response that is responsible for limiting and clearing infection. The higher proportion of T_{CM} cells may be indicative of a lowered susceptibility to infection at the cellular level[296] or a different qualitative immune response upon exposure. Finally, the lowered levels of apparent immune activation may provide a limited pool of susceptible cells for establishing infection, as persistent infection requires activated T cells[273].

1.11.2 Genetic correlates of HIV-1 resistance in the Nairobi CSW cohort

In addition to the immune correlates of protection, several studies of host genetic background have been undertaken in this cohort. The theory that HIV-1 resistance may be, at least in part, linked to host genetic background is supported by the observation that both seronegative HIV-1 specific CTL responses and the phenomenon of resistance itself clusters in families[25]. Several class I and II HLA alleles have been associated with HIV resistance. The Class II allele DRB1*01 and the class I alleles A*0202 and A*6802 have been independently associated with resistance[291]. As well, several HLA alleles shown to form a group based on function, the so-called HLA A2/6802 supertype, consisting of the alleles A*0202, A*0205, A*0214 and A*6802, has been shown to be over-represented in the HIV-R population. Furthermore, candidate gene analysis of the IL-4 gene cluster demonstrated several polymorphisms in non-coding regions of the gene

interferon regulatory factor 1 (IRF-1), a gene involved host innate immune responses and in HIV-1 viral replication by induction through the HIV-1 LTRs, associate both independently and in a haplotype with HIV resistance. These polymorphisms also correlate with reduced expression of IRF-1 demonstrating both that host proteins involved in the HIV-1 life cycle may be altered in some HIV-R individuals[297] and providing further evidence of reduced immune activity in these women.

Overall, several associations have been made with the HIV-R phenotype in the ML cohort. However, none of these parameters are found in all HIV-R women and it is clear that the HIV-R phenotype is a multifactorial combination of both immune and genetic factors. Complete understanding of all parameters influencing reduced susceptibility to HIV-1 infection is critical for informing vaccine design.

1.12 Genomics in HIV-1 Infection and Resistance

The advent of large-scale gene expression monitoring has provided a wealth of new information to all aspects of biology. With the ability to monitor expression of thousands of genes simultaneously, through the use of expression microarrays (cDNA arrays and oligonucleotide arrays), it is possible to view changes in gene expression and cell function in a much more complete manner than was previously possible. In terms of infectious disease research, genomic technologies have allowed for probing genes and pathways impacted throughout all phases of infection. HIV-1 researchers have also taken advantage of this new technology through use of various genomics platforms and model systems to define early events in infection, monitor the impact of viral subunits on

affected cells, classify stages of disease progression and search for biomarkers of protection (reviewed in[298]).

Throughout these studies, several genes and pathways have been implicated in HIV-1 pathogenesis and disease progression. By far the two most common themes emerging have been the induction of immune activation and apoptosis by HIV-1 infection and its sub-units, either through increased expression of genes involved in these pathways or decreased expression of inhibitors. Studies of early events in HIV-1 infection in cell lines were the first to be completed, demonstrating induction of genes involved in T cell signaling and regulation of transcription and increases in genes normally associated with induced cell death[299, 300]. Direct infection *ex vivo* of monocyte derived macrophages (MDM) also showed an impact on cellular activation, as induction of several genes involved in inflammation, including IL-6 and TNF, and transcription, including NF-κB, JUN and NFAT was noted[301].

Further studies went on to examine the roles of specific HIV-1 proteins in pathogenesis. Treatment of peripheral blood mononuclear cells (PBMC) with either R5 or X4 tropic gp120 variants showed that, independent of infection or the presence of full virus, gp120 induced several genes involved in inflammation, specifically IL-6, MIP-1α and MIP-1β, as well as several genes in the mitogen activated protein kinase (MAPK) pathway. These same studies also revealed the ability of gp120 to induce apoptosis and decrease cell function in NK cells, suggesting a gp120 mediated impairment of innate immunity[302, 303].

Studies of more natural instances of infection have also been carried out in populations of HIV-1 infected people at various stages of disease progression. In studies comparing patients with high (viremic) versus low (aviremic) viral loads it has been noted that viremia correlates with heightened expression of genes involved in transcriptional regulation, RNA processing and protein trafficking, possibly implicating several host genes in viral replication[304]. As well, a study of mucosal lymphocyte function has shown dramatic differences in expression of genes involved in activation and inflammation in gut-associated lymphoid tissue (GALT), even demonstrating an association with disease status, as individuals with high viral load showed a much greater extent of upregulation of genes involved in immune activation than did LTNP[95]. A more recent study has also shown that both CD4+ and CD8+ T cells from aviremic individuals, as defined as viral loads <500 copies per ml of blood, function almost identically to uninfected individuals in terms of gene expression. However, when patients with more active viral replication were studied it was noted that a large number of interferon stimulated genes were activated in both acute and chronic cases of viremia[305]. These studies serve to confirm the more *in vitro* cell line models of infection and to show that disease progression and, in fact, HIV-1 infection itself is inherently tied in to immune activation.

Genomic studies in animal models of retroviral infection have also made contributions to our understanding of the immune impact of HIV infection. Recently, studies in mice of immunologic function after infection with the murine retrovirus LCMV noted an upregulation of a gene encoding the protein programmed death receptor 1 (PD-1) on the surface of functionally impaired CD8+ T cells[306]. Cells demonstrating increased

surface expression of this marker were less able to proliferate and secrete IFNγ in response to viral peptides. Following this discovery, two groups performed larger population-based studies on this marker in HIV-1 infection. Both showed that this receptor was also upregulated on CD8+ T cells with the same functional impairment as noted in the mouse model experiments[307, 308]. Furthermore, it was shown that by inhibiting the interaction of this receptor with its ligand, PD-L1, function of these CD8+ T cells could be restored. However, possible therapeutic implications of this are unclear as this receptor is also required for induction of tolerance and its dysfunction has been implicated in several autoimmune diseases[309]. Despite this, these studies serve to further demonstrate the power of genomic technology in uncovering gene products not previously known to impact immune function.

Relatively fewer genomics studies have been carried out on situations of reduced susceptibility to HIV-1 infection. To date, only 1 population-based study has been carried out on individuals with the HIV-1 exposed-seronegative phenotype. This study focused on gene expression in T cells of 21 sero-discordant couples, and implicated the innate immune mediator IL-22 in indirect protection against infection through induction of A-SAA which was further shown to inhibit uptake of HIV-1 virions by DC[24].

Overall, the field of genomics offers a powerful way to examine host cell function, in terms of gene expression and pathway regulation, in a variety of stimulation and disease conditions. Genomic studies of those individuals who, despite high levels of exposure to HIV-1 do not become infected, afford the opportunity to define the HIV resistant phenotype to a degree of detail that was not previously possible. Determination of genes

and pathways that are differentially regulated in these individuals will help guide the establishment of new treatments and inform rational vaccine design.

Section 2.0: Hypotheses

At the time this work was initiated, no studies were available describing the extent to which the gene expression profile of the mixed-cell PBMC population mirrored or differed from the T cell sub-populations. As considerable sample input is required for a microarray experiment we began by investigating if there was a large amount of information to be gained by separating out cell populations, limiting the amount of sample available, or if the PBMC population would provide similar information.

As well, our cohort provides a unique opportunty to study HIV-1 infected untreated (i.e. ARV naïve) asymptomatic, individuals and to compare the function of their CD4+ T cells to that of normal control individuals. The goal of such a study is to determine the levels of T cell dysfunction that exist even in the absence of severe disease.

Finally, understanding the mechanisms that mediate natural resistance to HIV-1 infection is the best hope for development of a preventative vaccine. Several immune and genetic factors have been associated with protection against HIV-1 in various models of reduced susceptibility to infection. Studies conducted by this group and others, as mentioned in the Introduction, have implicated HIV-specific responses in the form of IL-2 secretion and proliferation in CD4+ T cells as possible mediators of this protection. Furthermore, evidence exists to suggest that activation status and memory cell makeup of the CD4+ compartment may be altered both in the HIV-R population identified in Nairobi, and in other ESN cohorts. These factors, coupled with the obvious importance of CD4+ T cells in the immune response and the HIV-1 life cycle make them an ideal target for in-depth study in hopes of uncovering possible mechanisms of HIV-1 resistance.

Microarray analysis of differential gene expression in the CD4+ T cell population between HIV resistant, HIV negative non-resistant and HIV positive individuals provides a means by which to discover and describe genes and pathways that are involved in mediating protection against HIV-1 infection. With that in mind we have developed the following hypotheses:

- Within a given individual after antigenic stimulation, the gene expression profiles of the CD4+ and CD8+ T cell populations will differ from each other and from that of the mixed cell PBMC population.

- Relatively healthy (CD4 > 350) will show gene expression levels similar to those seen in uninfected individuals. The gene expression differences that are seen will confirm what is known about HIV-1 pathogenesis, specifically, higher expression of genes involved in immune activation will be observed.

- CD4+ T cells of HIV-1 resistant (HIV-R) commercial sex-workers function differently, both at baseline and in response to stimulation, than do those of HIV-1 low-risk negative (HIV-LRN) non-resistant and HIV-1 high-risk negative (HIV-HRN) non-resistant women. These differences can be measured at the level of gene expression.

- The resistance phenotype will correlate with a general under-expression of genes involved in T cell activation and genes known to contribute to HIV-1 replication and/or an over-expression of inhibitors of activation and viral replication compared to HIV-LRN and HIV-HRN women.

- This functional hypo-activation state will correlate with an under-production of soluble mediators of the immune response measured in supernatants of PBMC culture.

Section 3.0: Specific Objectives

With the above hypotheses in mind we developed the following specific objectives:

- Determine whether the expression profiles of the T cell sub-populations differ from the PBMC population.

- Compare gene expression profiles, both at baseline and after stimulation, between HIV+ and HIV-LRN populations and define differentially expressed genes.

- Compare gene expression profiles, both at baseline and after stimulation, between HIV-R, HIV-LRN and HIV-HRN populations and define differentially expressed genes.

- Characterize a sub-set of genes, both individually and in concert with other genes, on their ability to functionally distinguish HIV-R from control populations.

- Based on gene expression data measure levels of soluble immune mediators that could serve as biomarkers of resistance to HIV-1 infection.

- Define an HIV-1 resistance phenotype based on these data.

Section 4.0: Materials and Methods

4.1 General Materials

4.1.1 Solutions

Cell culture media:

RPMI, 10% fetal-calf serum (FCS), 2% pennicillin/streptomycin (all reagents obtained

from Invitrogen, Burlington Ontario)

Phosphate-buffered saline (PBS):

48.5 grams PBS powder (contains 137.93 mM NaCl, 2.67 mM KCl, 8.1 mM Na_2HPO_4.

1.47 mM KH_2PO_4) (Invitrogen, Burlington Ontario) per 1 litre of ddH_2O

PBS for cell separation:

PBS, 2% FCS (Invitrogen, Burlington Ontario)

Nylon array hybridization buffer:

4ml MicroHyb hybridization solution (ResGen distributed by Invitrogen, Burlington

Ontario), 10ul *cotI* DNA (Invitrogen, Burlington Ontario), 10ul Poly(A) (Sigma-Aldrich,

Oakville Ontario)

Radiolabeled nucleotides:

Alpha ^{33}P labeled deoxycytidine triphosphate (dCTP) (NEN distributed by PE

biosciences, Montreal Quebec)

20X saline sodium citrate (SSC):

175.3g NaCl (Sigma-Aldrich, Oakville Ontario), 88.2g sodium citrate (Fisher Scientific, Ottawa Ontario) in 1 litre of ddH$_2$O pH adjusted to 7.0 with 1M HCl (Sigma-Aldrich, Oakville Ontario)

Nylon array wash buffer:

2X SSC, 0.1% sodium dodecyl sulfate (SDS) (BioRad, Mississauga Ontario)

10N NaOH:

40g NaOH (Sigma-Aldrich, Oakville Ontario), 100ml ddH$_2$O

1M Tris HCl:

12.1g Tris base (Sigma-Aldrich, Oakville Ontario), adjust to pH 8.0 with concentrated HCl (Fisher Scientific, Ottawa Ontario)

Nylon array stripping buffers:

1) 5ml 10% SDS, 20ml 10N NaOH (Sigma-Aldrich, Oakville Ontario), 475ml ddH$_2$O

2) 5ml 10% SDS (Sigma-Aldrich, Oakville Ontario), 25ml 20X SSC, 100ml Tris HCl (Fisher Scientific, Ottawa Ontario), 370ml ddH$_2$O

12X MES stock solution:

64.61g MES hydrate, 193.3g MES sodium salt (both from Sigma-Aldrich Oakville Ontario), 1,000ml H$_2$O. Filter through 0.2 um filter (Fisher Scientific, Ottawa Ontario)

2X Hybridization buffer for Affymetrix GeneChips:

8.3ml 12X MES stock solution, 17.7ml 5M NaCl (Ambion, Austin Texas), 4.0ml 0.5M

EDTA (Sigma-Aldrich, Oakville Ontario)

Wash buffers for Affymetrix arrays:

Wash buffer A: 300ml 20X SSPE (Cedarlane Laboratories, Burlington Ontario), 1.0ml

10% Tween 20 (Fisher Scientific, Ottawa Ontario), 699ml H_2O, filter through 0.2 um

filter (Fisher Scientific, Ottawa Ontario)

Wash buffer B: 83.3ml 12X MES stock solution, 5.2ml 5M NaCl (Ambion, Austin

Texas), 1.0ml 10% Tween 20 (Fisher Scientific, Ottawa Ontario), 910.5ml H_2O, filter

through 0.2um filter (Fisher Scientific, Ottawa Ontario)

2X Staining buffer for Affymetrix GeneChips:

41.7ml 12X MES stock solution, 92.5ml 5M NaCl (Ambion, Austin Texas), 2.5ml 10%

Tween 20 (Fisher Scientific, Ottawa Ontario), 113.3ml H_2O, filter through 0.2um filter

(Fisher Scientific, Ottawa Ontario)

Streptavidin-phycoerythrin (SAPE) solution:

600ul 2X staining buffer, 48ul 50mg/ml bovine serum albumin (BSA) solution

(Invitrogen, Burlington Ontario), 12ul streptavidin-phycoerythrin (Molecular Probes

distributed by Invitrogen, Burlington Ontario), 540ul H_2O

Goat IgG stock solution:

50mg goat IgG (Sigma-Aldrich, Oakville Ontario 50ml 150mM NaCl (Ambion, Austin Texas)

Antibody solution for staining of Affymetrix GeneChips:

300ul 2X staining buffer, 24ul of 50mg/ml BSA (Invitrogen, Burlington Ontario), 6.0ul goat IgG stock solution, 3.6ul of 0.5mg/ml biotinylated goat anti-streptavidin (Vector laboratories, Burlingame California), 266.4ul H_2O

4.1.2 Antigens for stimulation

Phytohemagglutinin (Sigma-Aldrich, Oakville Ontario), *Candida albicans* (Greer Laboratories, Lenoir North Carolina), flu peptide, p24 (recombinant, produced in-house)

4.1.3 Microarray platforms used

Nylon immune microarrays:

Human immune microarrays containing 4,608 immunologically-relevant cDNAs spotted in duplicate were obtained from the National Institute on Aging (NIA, Bethesda Maryland). Information on all genes spotted on these arrays is available at http://www.grc.nia.nih.gov/branches/rrb/dna/array.htm)

Total genome arrays:

Human Genome U133 Plus 2.0 oligonucleotide arrays were obtained from Affymetrix (Santa Clara California). Information on all genes spotted on these arrays can be obtained at http://www.affymetrix.com/products/arrays/specific/hgu133plus.affx

4.1.4 Commercial kits

<u>Cell separation reagents:</u>

CD4+ T Cell Enrichment Cocktail (containing anti CD8, CD14, CD16, CD19, CD56 and

Glycophorin A antibodies), CD8+ T Cell Enrichment Cocktail (containing anti CD4,

CD14, CD16, CD19, CD56 and Glycophorin A antibodies), magnetic colloid, cell

separation columns and magnets were obtained from Stem Cell Technologies (Vancouver

Brittish Columbia)

<u>RNA isolation:</u>

Both the RNeasy Mini kit and RNeasy Mini kit Plus (Qiagen, Mississauga Ontario) were

used for total RNA isolation

<u>RNA labeling for nylon microarrays:</u>

For labeling of cDNA for nylon array experiments the LabelStar Array Kit (Qiagen,

Mississauga Ontario) was used. For experiments using the Human Genome U133 Plus

2.0 arrays the GeneChip Hybridization, Wash, and Stain Kit (Affymetrix, Santa Clara

California) was used for nucleotide labeling

<u>RNA quantity and quality analysis:</u>

Quantification and quality analysis of RNA samples prior to analysis on Affymetrix

GeneChips was performed using Agilent RNA 6000 Nano Kits (Agilent Technologies,

Mississauga Ontario).

Linear amplification and labeling of RNA:

Linear amplification and labeling of nucleic acids prior to hybridization to the Human Genome U133 Plus 2.0 arrays was accomplished using GeneChip Two-Cycle Target Labeling Kits (Affymetrix, Santa Clara California) and MEGA script T7 kits (Ambion, Austin Texas).

Real-time PCR reagents:

For generation of first-strand cDNA from total RNA for real-time PCR assays the Quantitect Reverse Transcription Kit was used. Gene-specific primers (various sequences as outlined in the results section) were designed using the proprietary Quantitect Primer Assay algorithm. Amplification of cDNA and detection of product was accomplished using the QuantiTect SYBR Green PCR Kit (all kits obtained from Qiagen, Mississauga Ontario)

Cytokine and chemokine quantification :

Quantification of soluble cytokines and chemokines in cell culture media was accomplished using the CBA Human Th1/Th2 Kit 2, CBA Human Inflammation Kit, CBA Flex Set Analystes; IL-1β, IL-2, IL-6, IL-10, IFNγ, TNF, and the CBA Master Buffer Kit for flex set analyte detection (all reagents from BD Biosciences, Mississauga Ontario)

4.2 General Methods

4.2.1 Sample source

4.2.1.1 Winnipeg donors

For initial studies of comparative analysis of gene expression between CD4+ T cells, CD8+ T cells and PBMC, samples were obtained from donors in Winnipeg. Blood was collected from an asymptomatic HIV+ individual (CD4 > 800 cells/ml) and an age and gender-matched HIV negative individual by venipuncture into vacuum tubes containing sodium heparin to the St. Boniface General Hospital Ambulatory Care Facility and the University of Manitoba respectively.

4.2.1.2 Pumwani CSW cohort

Blood samples from HIV resistant (HIV-R), HIV high-risk negative (HIV-HRN) and most HIV positive (HIV+) individuals were obtained from women attending the Pumwani commercial sex-worker clinic cosponsored by the University of Manitoba and University of Nairobi in the Pumwani district of Nairobi, Kenya. All women enrolled in this cohort are commercial sex-workers (CSW) and are thus considered to be at high risk for contracting HIV. HIV resistant women meet the following criteria:

1) HIV-1 negative by both serology and PCR

2) Active in commercial sex-work

3) Follow-up in the cohort for greater than 3 years

The HIV-HRN group consists of newly enrolled CSW who are HIV negative by serology but do not meet the definition of resistance. As HIV-1 resistance is a relatively rare

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phenomenon (~5% in this population), it is reasonable to assume most of the women in the HIV-HRN group are susceptible to infection by HIV-1.

The HIV+ group consists of women previously testing seropositive for antibodies directed against HIV-1. All women in this group were asymptomatic at the time of sample collection as measured by CD4+ T cell count (i.e. CD4>350) and had never received antiretroviral treatment. All individuals enrolled in this study received both testing for HIV-1 antibodies and CD4/CD8 enumeration at the time of sample collection.

4.2.1.3 MCH cohort

The Mother-Child Health (MCH) clinic also operates out of the Pumwani district of Nairobi. This cohort was established in 1986 and was originally designed to study HIV-1 transmission from mothers to their children, both perinatally and through breast milk[310]. Women attending this clinic are given free primary medical care and counseling in HIV-1 prevention. Demographic, social and clinical data are collected on these women at every visit. These women are not commmercial sex-workers and are considered to be at low risk for contracting HIV. All HIV low-risk negative (HIV-LRN) and a minority of HIV+ samples used in this study were collected from this cohort. As with the HIV-HRN group the HIV-LRN individuals are considered to be susceptible to HIV-1 infection (i.e. non-resistant). All HIV+ individuals had CD4>350 and were antiretroviral naïve at the time of sample collection. All women enrolled for this study from this cohort were not pregnant at the time of enrollment and received HIV-1 antibody testing and CD4/CD8 enumeration.

4.2.2 Sample collection and processing

All blood samples from individuals attending either of the Pumwani clinics were collected via veinipuncture into vacutainer tubes containing sodium heparin as an anticoagulant. Samples were then transferred to the laboratory where they were processed in biological safety cabinets using universal precautions. A separate tube, containing EDTA as an anticoagulant was also collected from each and was used for CD4/CD8 T cell counting.

Isolation of PBMC from all blood samples, regardless of collection location, was conducted in the same manner. Tubes containing blood samples were initially spun in a centrifuge for 5 minutes at 1500 RPM to separate plasma. Plasma was removed and aliquotted into cryovials, one aliquot was sent for HIV-1 serology and the rest were catalogued and stored at -80^0C for future studies. Remaining blood was then diluted approximately 1:1 with PBS and layered over 10-12ml ficol-hypaque (Invitrogen, Burlington Ontario). Layered bloods were spun for 25 minutes at 1700 RPM to separate PBMC. After separation, PBMC layer was removed using sterile transfer pipettes into a sterile tube. Cells were then diluted in PBS, mixed and centrifuged at 1500 RPM for 10 minutes. After this step, PBS was removed and discarded, cells were resuspended in sterile RPMI media and a 50ul aliquot was removed for counting and viability testing using a heamocytometer via trypan blue exclusion. Remaining cells were spun again at 1500 RPM for 10 minutes, supernatant was discarded and cells were resuspended in RPMI containing 10% FCS and 2% penicillin/streptomycin to a final concentration of $2x10^6$ cells per milliliter for culture.

4.2.3 HIV testing and confirmation

All samples obtained in the Nairobi clinics, ML and MCH, are routinely tested for HIV-1 sero-status at every visit. Plasma samples sent from the lab are initially tested using the Recombigen (Trinity Biotech, Carlsbad California) enzyme-linked immunosorbent assay (ELISA). Samples showing negative results in this assay are considered HIV-1 negative. All samples testing positive in the Recombigen assay are confirmed by a second, separate immunoassay Detect HIV1/2 (Adaltis, Montreal Quebec). Only those samples giving positive results in both assays are considered HIV-1 positive.

All women potentially meeting the definition of HIV-1 resistance (i.e. HIV-1 seronegative after 3 years of follow-up in the cohort while remaining active in sex-work) are also tested for the presence of proviral DNA by a nested polymerase chain reaction (PCR) assay. DNA samples are initially tested for the presence of *vif* and *nef* gene products using gene-specific primers. Should samples test positive for either of these, a third test using gene-specific primers specific for the *pol* gene is carried out. As this is not a clinically certified test, a sample is considered HIV-1 negative if two of three PCR results are negative.

4.2.4 CD4 and CD8 T cell counts

Tubes of blood collected from each individual with EDTA as an additive were used to obtain both CD4+ and CD8+ T cell counts. Whole blood was labeled using antibodies specific for CD4 and CD8 using the Tritest flow cytometry assay (BD Pharmingen, Mississauga Ontario). Samples were run on a FACSscan flow cytometer (BD, Mississauga Ontario) and both CD4 and CD8 cell counts were recorded.

4.2.5 Cell culture and stimulation

To allow normal antigenic processing, presentation and cell type interaction all antigenic stimulations were done on whole PBMC populations. Peripheral blood mononuclear cells obtained as described above were cultured in 6 well tissue culture plates at a final volume of 2.5ml corresponding to 5×10^6 cells per well in RPMI containing 10% FCS and 2% penicillin/streptomycin. PBMC from each study individual were divided into either 4 or 5 different aliquots for antigenic stimulation. Samples were either left unstimulated or treated with PHA at 5ug/ml, non-viable *Candida albicans* at 10ug/ml or recombinant p24 at 1ug/ml. Some samples also contained a 5th stimulation condition of flu peptide at a final concentration of 2ug/ml. These antigens were chosen to evaluate different aspects of the immune response, specifically; generalized activation by mitogenic PHA, recall response to non-HIV-1 antigen by *C. albicans* and flu peptide and recall response to recombinant HIV-1 p24 protein. Cells were stimulated for 24 hours at 37^0C and 5% CO_2. Following stimulation cells were removed from tissue culture plates using sterile transfer pipettes and transferred into sterile tubes. Cells were centrifuged at 1500 RPM for 10 minutes. Supernatants were then removed, aliquoted and stored at -80^0C for future study. Cells were then washed twice by resuspension of the cell pellet in PBS (first wash) or PBS + 2% FCS (second wash) and centrifugation at 1500 RPM for 10 minutes, after both wash steps supernatants were discarded. Following the second wash step, cells were resuspended in 200ul of PBS+2% FCS in preparation for T cell enrichment.

4.2.6 CD4+/CD8+ T cell enrichment by negative selection

Peripheral blood mononuclear cells obtained as above were subsequently enriched for CD4+ T cells by negative selection using magnetic bead technology (Stem Cell Technologies). Cells were first incubated in the presence of an antibody cocktail containing labeled antibodies specific for CD8, CD14, CD16, CD19, CD56 and Glycophorin A for 15 minutes at room temperature. After this incubation, secondary antibodies linked to iron bead particles were added and the cell mixture was incubated again for 15 minutes at room temperature. Cells were then passed over a column in the presence of a magnetic field and the column was washed with excess amounts of PBS+2%FCS. The CD4+ T cell enriched fraction was collected into sterile tubes. After enrichment, cells were collected by centrifugation at 1500 RPM for 10 minutes, the supernatant was discarded and cells were resuspended in PBS+2%FCS and counted using a heamocytometer using the trypan blue exclusion method. In our hands, purified cell populations have been shown to be >95% enriched for CD4+ or CD8+ Tcells by flow cytometry.

Early studies to obtain information on cell type differences in gene expression also involved enrichment for CD8+ T cells. This procedure is essentially the same as above with the addition of antibody specific for CD4 instead of CD8 added to the enrichment cocktail. All reagents (with the exception of PBS+2%FCS), columns and magnets used for cell enrichment were obtained from Stem Cell Technologies (Vancouver, Brittish Columbia).

4.2.7 RNA extraction

Subsequent to T cell enrichment cells were pelleted by centrifugation for 10 minutes at 1500 RPM, the supernatants were discarded and cells prepared for RNA isolation. In some cases an aliquot was removed for protein purification. Total cellular RNA was obtained using either RNeasy mini kits or RNeasy mini kits Plus (Qiagen) following manufacturers instructions. Briefly, cells were resuspended and lysed by addition of lysis buffer RLT+10ul/ml of beta-Mercaptoethanol. Cell lysates were homogenized by passing through a 21-gauge needle attached to a 1ml syringe 5 times. At this point lysates were frozen at -80^0C and transferred to the laboratories in Winnipeg for further processing. Continuation of RNA extraction involved an initial incubation of cell lysates at 37^0C for 15 minutes to ensure proper thawing of samples. Thawed lysates were then subject to column purification. Briefly, a volume of 70% ethanol equal to the volume of the lysate was added to each sample. Mixtures were then applied to purification columns. Columns were spun for 15 seconds at 8,000g and the flow through was discarded. Columns were then washed twice with supplied buffer RW1 and once with buffer RPE to remove protein and genomic DNA from the samples. Total RNA was then eluted using RNAse free water.

4.2.8 Nylon microarray procedures

Nylon immune microarrays, as outlined above, were used for initial assessment of gene expression differences between study groups. Human focused immune arrays were obtained from the National Institute on Aging (Bethesda Maryland).

4.2.8.1 Sample labeling and hybridization

Membranes to be used were pre-wet in 2X SSC and placed into hybridization bottles (Fisher). For blocking, to prevent non-specific binding, 4ml of a hybridization mixture containing 4ml MicroHyb buffer (ResGen distributed by Invitrogen, Burlington Ontario) plus 10ul human *cotI* DNA (Invitrogen, Burlington Ontario) and 10ul poly(A) (Sigma-Aldrich, Oakville Ontario) was added to each bottle. Prior to the addition of any sample material membranes were incubated in a rotating hybridization oven for 4 hours at 42^0C.

Approximately 1ug of total RNA per sample was prepared for hybridization to microarrays. Generation, labeling and purification of labeled cDNA was carried out using LabelStar array kits (Qiagen Mississauga Ontario) following instructions provided by the manufacturer. Briefly, total RNA was denatured by addition of denaturation solution followed by incubation at 65^0C for 5 minutes. Subsequent to denaturation, samples were stored on ice to prevent re-forming of secondary structure. Denatured RNA was reverse-transcribed using the LabelStar reverse-transcriptase in the presence of oligo dT primer, RT buffer, equal concentrations of dATP, dGTP and dTTP and an excess of alpha-^{33}P dCTP (NEN distributed by PE biosciences, Montreal Quebec). The RT mixture was incubated for 2 hours at 37^0C in a water bath followed by addition of stop solution. After incubation, excess primer and nucleotide were removed by column purification. Labeled cDNA was then denatured to remove secondary structure at 95^0C for 5 minutes and added to the hybridization cocktail already present in hybridization bottles. Hybridization of microarrays was carried out for 18 hours at 42^0C with rotation in a hybridization oven.

After hybridization, arrays were removed from hybridization bottles and washed twice in wash buffer containing 2X SSC and 0.1% SDS. All hybridization solutions and wash buffer were considered to be radioactively contaminated and were disposed of according to University of Manitoba institutional guidelines. After washing, arrays were fixed to development plates, created in-house, placed in a development cassette and exposed to Molecular Imager imaging screens (BioRad Mississauga Ontario) for 24 hours.

4.2.8.2 Microrray stripping for rehybridization

As previously described[311] nylon microarrays can be re-used by following a simple stripping procedure. In order to prepare arrays for re-use, arrays were washed twice for 30 minutes each time in a buffer containing 0.4N NaOH and 0.1% SDS at 65^0C. Secondary washing was carried out twice at room temperature in a buffer containing 0.2M Tris-HCl (pH 8.0), 1X SSC and 0.1% SDS for 10 minutes each time. Arrays were then air dried and stored for re-use. Quality control experiments using a single sample repeatedly on a single array showed reproducible signal quality of a given array after up to 5 uses. For the duration of the nylon array studies no single array was used more than three times.

4.2.8.3 Data acquisition and pre-processing

Microarray images were acquired after exposure by reading phosphorimaging screens on a Personal Fx phosphorimager (BioRad, Mississauga Ontario). Screens were scanned at the highest possible resolution (50 um) and image data were obtained using Quantity One software (BioRad, Mississauga Ontario). Image pre-processing included cropping and rotational alignment. Images were then imported as .tiff files into ArrayPro software

(Media Cybernetic, Bethesda Maryland) for spot quantification. Spots were detected using the automatic spot finding algorithm with subsequent visual inspection and manual adjustment of spot-finding grids to ensure optimum detection of signal. Spot intensities were calculated using the global background subtraction method. Quantified data files for each array were then imported into Microsoft Excel (Microsoft, Seattle Washington) for averaging of duplicate spots and prefiltering. As is standard for this array format, duplicate spots having a variance greater than 20% were removed from further analysis.

4.2.8.4 Principle components analysis and removal of batch effect

Prior to analysis for significant changes in gene expression, principle components analysis (PCA) was performed to visualize any non-sample related variation between arrays. Briefly, each quantified data set (i.e. gene values for a single array) was assigned all relevant experimental variables such as date of sample collection and isolation, array batch etc. A singular value decomposition is performed on the data's covariance matrix to compute the standard PCA[312]. Data were then visually inspected for batch-related effect by plotting of the top three principle components. Data were considered to have a batch effect if the top performing principle components were anything other than sample group or experimental condition. To correct for batch effect an algorithm based on calculation and correction of mixed-model analysis of variation was employed. Visualization of PCA plots and removal of batch effect were performed using Partek software (Partek inc. St. Louis Missouri).

4.2.9 Assessment of RNA quality for Affymetrix GeneChip analysis

Prior to sample amplification and application to Affymetrix GeneChips RNA quantity

and quality were measured using the Agilent Bioanalyzer 2100 using the RNA 6000

Nano kit (Agilent Technologies, Mississauga Ontario) following manufacturers

instructions. Briefly, a mixture of gel matrix, RNA 6000 dye and ladder are added to all

sample wells and control wells on an RNA nano chip. Samples to be analysed are

denatured at 70^0C for 2 minutes, briefly centrifuged and placed on ice. One microlitre of

sample is then added to each sample well of the RNA nano chip. Chips are then vortexed

for 1 minute. Chips containing all samples were run on the Agilent Bioanalyzer 2100

using Bioanalyzer 2100 Expert software (all from Agilent Technologies, Mississauga

Ontario). This analysis provides quantity and quality data of RNA samples based on

intensity and integrity of the 18s and 28s rRNA peaks. Each sample is given an RNA

integrity number (RIN) out of 10, only those samples with RIN values of greater than 9

were carried through for further analysis.

4.2.10 Affymetrix array procedures

<u>4.2.10.1 RNA amplification</u>

Due to significant input RNA requirement for standard sample labeling and hybridization

for Affymetrix arrays, it was necessary to perform signal amplification using the 2-cycle

cDNA amplification kit (Affymetrix, Santa Clara California) and the MEGAscript T7 kit

(Ambion, Austin Texas). This technique involves several stages, outlined below, to

produce amplified, biotinylated cRNA for hybridization to the Affymetrix GeneChips. As

these methods require specific equipment for setup, washing and scanning, and are

sensitive to variation in the procedure, the sample amplification, GeneChip hybridization

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and scanning were carried out at The Centre for Applied Genomics (http://www.tcag.ca), as this centre houses all necessary fluidics and scanning capabilities for running Affymetrix GeneChip experiments with minimum run to run variability. This centre uses all procedures as suggested by Affymetrix. The details of these procedures are outlined below.

First-cycle first and second-strand cDNA synthesis:

This stage uses the two-cycle cDNA synthesis kit (Affymetrix, Santa Clara California). A mixture of T7-oligo (dT) primer and non-eukaryotic control RNA is added to approximately 100ng of total RNA. Primer and sample mixture is denatured at 70^0C for 6 minutes, briefly cooled on ice and added to a master mix including reaction buffer, DTT, RNase inhibitor, dNTPs and Superscript II RT enzyme. This mixture is incubated for 1 hour at 42^0C, heated to 70^0C to inactivate the enzyme and cooled on ice.

A second strand cDNA synthesis master mix including *E. coli* DNA polymerase enzyme, RNase H, dNTPs, and reaction buffer is added to the first strand reaction. This mixture is then incubated for 2 hours at 16^0C, followed by enzyme inactivation for 10 minutes at 75^0C and then cooled on ice.

In vitro transcription of cRNA:

This stage uses the MEGAscript T7 IVT kit from Ambion (Austin Texas). A master mix containing reaction buffer, NTPs and enzyme mixture is added to the double-stranded cDNA generated in the previous section (4.2.10.1.1). This mixture is incubated at 37^0C for 16 hours and then cooled on ice.

Cleanup of cRNA:

This stage uses the GeneChip sample cleanup module (Affymetrix, Santa Clara California). A mixture of cRNA binding buffer, 96% ethanol and H_2O is added to the cRNA generated in section 4.2.10.1.2. The sample is then added to the provided column and centrifuged to bind sample to the column. The column is then washed once with the provided wash buffer, centrifuged, washed a second time with 80% ethanol, and then eluted in H_2O. The absorbance of the sample is then measured at 260nm and 600ng of cRNA is carried through to the next stage.

Second-cycle first and second-strand cDNA synthesis:

This stage uses the two-cycle cDNA synthesis kit (Affymetrix, Santa Clara California). A mixture of random primers is added to 600ng of cRNA generated in the previous section. The sample and primer mixture is denatured at 70^0C for 10 minutes and then cooled on ice. A master mix including reaction buffer, dNTPs, RNase inhibitor and Sunperscript II is added to the sample mix. The reaction is incubated at 42^0C for 1 hour. RNase H is then added to the sample and incubated for 20 minutes at 37^0C followed by enzyme inactivation at 95^0C for 5 minutes.

The second-cycle second-strand synthesis involves the addition of T7-oligo(dT) primer to the sample followed by denaturation at 70^0C for 6 minutes and cooling on ice. After this, a master mix containing reaction buffer, dNTPs, DNA polymerase 1 and H_2O is added to the sample and incubated at 16^0C for 2 hours. T4 DNA polymerase is then added and incubated for 10 minutes at 16^0C.

Cleanup of second-round double-stranded cDNA:

This stage uses the GeneChip sample cleanup module (Affymetrix, Santa Clara California). Binding buffer for cDNA is added to the sample and the mixture is then applied to the provided column and centrifuged for binding of cDNA. The column is then washed with the provided wash buffer and then eluted in the provided elution buffer.

Synthesis of biotin-labeled cRNA:

This stage uses the GeneChip *in vitro* transcription and labeling kit (Affymetrix Santa Clara California). Double-stranded cDNA produced in the previous section is added to a master mix including reaction buffer, NTPs containing biotinylated NTPs, enzyme mixture and H_2O and the mixture is incubated for 16 hours at 37^0C.

Cleanup and fragmentation of biotin-labeled cRNA:

This stage uses the GeneChip sample cleanup module (Affymetrix, Santa Clara California). The sample is column purified to obtain pure labeled cRNA as outlined in section 4.2.10.1.3. Following purification the sampled is quantified at 260nm. Fifteen micrograms of labeled, amplified cRNA is then added to a mixture of fragmentation buffer and H_2O. The mixture is then incubated at 94^0C for 35 minutes. The sample is now ready for hybridization the GeneChip.

4.2.10.2 Sample hybridization

Human Genome U133 Plus 2.0 oligonucleotide GeneChips (Affymetrix, Santa Clara California) are pre-incubated with hybridization buffer for Affymetrix GeneChips (see section 4.1 for recipe) for 10 minutes at 45^0C. A hybridization cocktail containing

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fragmented, bioinylated cRNA, control oligonucleotides (Affymetrix, Santa Clara California), herring sperm DNA, BSA (both from Invitrogen, Burlington Ontario), hybridization buffer for Affymetrix GeneChips, DMSO (Sigma-Aldrich, Oakville Ontario) and H_2O is created. The hybridization cocktail is then incubated at 99^0C for 5 minutes and then at 45^0C for 5 minutes. The pre-incubation hybridization buffer is then removed from the GeneChip. Hybridization cocktail containing sample is then added to the GeneChip and is incubated for 16 hours with rotation at 45^0C.

4.2.10.3 GeneChip washing and staining

GeneChip washing and staining is carried out using an Affymetrix fluidics station, operated using Affymetrix Microarray Suite GeneChip Operating System (GCOS) software (all from Affymetrix, Santa Clara California). Recipes for all washing and staining reagents can be found in section 4.1. Initially, GeneChips are washed for 10 cycles with 2 mixes per cycle with wash buffer A followed by washing for 4 cycles with 15 mixes per cycle with wash buffer B. The first round of staining is accomplished by addition of streptavidin-phycoerythrin (SAPE) solution and is incubated for 10 minutes at 25^0C. GeneChips are then washed for 10 cycles with 4 mixes per cycle with wash buffer A. Secondary staining is then carried out by addition of antibody solution for 10 minutes at 25^0C. Tertiary staining is then carried out by addition of SAPE solution and incubation for 10 minutes at 25^0C. A final wash step is then carried out by addition of wash buffer A for 15 cycles with 4 mixes per cycle at 30^0C. GeneChips can then be scanned

<u>4.2.10.4 Data acquisition and processing</u>

Scanning of Affymetrix GeneChips was carried out on an Affymetrix GeneChip scanner 3000 (Affymetrix, Santa Clara California) operated by Affymetrix microarray suite GeneChip operating software (GCOS). GeneChips are scanned at 570nm at a resolution of 3um. Files containing the raw quantification data were then exported to ArrayAssist software (Stratagene, La Jolla California). Raw data were log transformed, normalized by the probe logarithmic intensity error (PLIER) normalization method (see below). As well, a second algorithm, microarray suite 5 (MAS5) was applied to assess the validity of the spot data. This algorithm uses the Affymetrix perfect match mis-match data and applies a pass or fail score to each spot on each array. For group comparisons only spots showing greater than 50% pass scores (i.e. scored as present in at least half of all arrays) were carried through to statistical analysis. As a final transformation all data are then zero-centered to the mean intensity value of the control group (HIV-1 low-risk negatives) for each gene. Statistical analyses were then carried out as described below.

4.2.11 Array data normalization

As the field of microarray analysis is ever changing and evolving, several different normalization strategies were used throughout the course of this project. Brief descriptions of all normalization strategies with complete references are given below and normalization strategies employed for individual project stages will be given as necessary in the results section. The basic premise of all normalization strategies is to correct for intensity differences between arrays within and across experiments.

4.2.11.1 Z-score normalization

This relatively simple normalization method involves normalizing every array on a per-array basis for comparison. It can be carried out within an Excel spreadsheet and has been shown to perform as well as other global normalization methods[313]. Briefly, this method involves calculating a Z score for each spot on an array as a unit of standard deviation from the normalized mean of zero. Data for each array are first $\log_{10}$ transformed and then Z score values are calculated using the following formula: Zscore = (intensity$_G$ – mean intensity$_{G1...Gn}$)/SD$_{G1...Gn}$. where G is any single spot on the array, G1...Gn is the aggregate measurement of all genes and SD is the standard deviation. Application of this formula effectively sets the mean value of any given array to zero and the standard deviation as one. After this normalization has been applied, ratio and statistical confidence values can be calculated as described[313]. Genes were considered significantly differentially expressed between conditions or groups if they had a Z ratio of greater than 1.9 or a p value <0.05.

4.2.11.2 GeneSpring normalization

For early studies of gene expression differences between cell sub-populations, data were transferred to GeneSpring software (Silicon Genetics) after pre-processing and removal of variant spots. Individual arrays were normalized in two ways: The data for each array were first normalized to the distribution of all genes on that array (per-chip normalization) to account for variation across the individual arrays. The data were next normalized to the median value for each individual gene across all arrays (per-gene normalization) in order to control for variation between different arrays. Both methods are considered to be global normalization methods. Further details of these calculations

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can be found in Yang et. al. 2002[314]. Gene expression values were considered to be altered as a result of stimulation if normalized values from the stimulated condition were 2 fold greater than or less than the unstimulated condition.

4.2.11.3 Robust Multi-array Analysis (RMA)

This normalization method involves quantile-based normalization with the object of making the distribution of probe intensities the same for each array in a set and adds in a strategy of median polishing, essentially setting the median level of intensity of each array to zero[315]. Quantile-based normalization involves sorting each array by gene intensity, calculating a standard mean for each row, applying that value across the row and re-sorting back to the original gene order. This ensures the distribution of values across any given array is the same. This normalization strategy was applied to data-sets using Partek software (Partek inc. St. Louis Missouri).

4.2.11.4 Removal of non-biological variation by EMD and moving average correction

During the analysis of the focused immune arrays, some non-biological platform-specific variation became apparent. In order to separate the actual biological variation from this platform-specific variation it was necessary to design a custom algorithm for removal of this background variation from each array. This was accomplished by applying the techniques of Empirical Mode Decomposition (EMD)[316] and a moving average. Briefly, intensity values for each gene across a given array are plotted. The signal pattern is then decomposed by generating a series of Intrinsic Mode Functions (IMFs). Upon inspection, those IMFs corresponding to non-biological variation are removed and the signal is rebuilt. Separately, a moving average is calculated over all spots on the array.

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The correction function for each array is then built by combining data from the EMD calculation and the moving average function. The custom-built correction function is then applied to the array upon which it was built.

4.2.11.5 Probe logarithmic intensity error (PLIER) normalization

This normalization method, used exclusively on Affymetrix array data, is fundamentally similar to RMA. However, it improves upon this method by exploiting more sophisticated background correction methods by subtracting the so-called mismatch intensity (i.e. background signal for a given spot) from the perfect match value (i.e. actual signal). This normalization approach was carried out using ArrayAssist software (Stratagene, La Jolla California).

4.2.12 Determination of gene expression differences

For studies involving comparisons of CD4+ T cell function between HIV-1 positive and HIV-1 negative individuals, HIV-1 resistant and HIV-1 low-risk negative individuals (nylon arrays) and those comparing HIV-1 resistant and HIV-1 high-risk negatives (Affymetrix arrays) differential expression of genes both between groups and between stimulation conditions were evaluated both by fold change and p value.

Fold changes after stimulation were determined by dividing the mean expression value for a given gene within a sample group in the "treated" condition (i.e. after antigenic stimulation) by the mean expression value of that same gene in the "untreated" condition (i.e. media alone). Similarly, determination of differential expression between groups involved dividing the mean expression value of a gene in the "test" condition (i.e. HIV-1

positive or HIV-1 resistant) by the mean value of the "control" condition (i.e. HIV-1 low-risk negative or HIV-1 high-risk negative). Calculations of p values for each individual gene compared either between stimulation conditions or between groups, were accomplished by one-way analysis of variation (ANOVA).

Throughout the Results section, Tables are presented to outline genes of statistical and functional significance. In several cases these are not full lists of genes that were found to be significantly differentially expressed for a given comparison. However, in the interest of full disclosure complete lists of significantly differentially expressed genes containing gene symbol, geen description, fold change/difference and p value are available in individual spreadsheets for each Results section 5.1-5.5 from the following ftp site:

ftp://140.193.9.171
Username = picture
Password = paul2007

4.2.13 Cluster analysis

Basic class discrimination analyses were performed using Cluster and TreeView software freely available from the Eisen lab at University of California at Berkeley (http://rana.lbl.gov/EisenSoftware.htm and[317]). For clustering of both genes and arrays, complete linkage hierarchical clustering was performed using an Euclidean distance metric.

4.2.14 Predictive interaction analysis

The details of predictive interaction analysis (PIA) have recently been published [318]. This is a method of two-class discriminant analysis based on the calculated interaction of two genes in a sample group within an array experiment. This essentially involves a special application of the standard equation of linear discriminant analysis (LDA). The theory being that sample class discrimination can be more robustly calculated by using a two-gene method than by application of standard LDA. This method involves first calculating a "u" or "v" statistic for every combination of two genes in a given dataset with the u statistic representing the product of geneX x geneY (actually $Log_{10}geneX + Log_{10}geneY$) and the v statistic representing the ratio geneX/geneY (actually $log_{10}geneX - log_{10}geneY$), thus calculating both synergistic (u) and competitive (v) predictive interactions (SPIA and CPI respectively). These values, u and v, are then used under a LDA model where the slope of the separatrix (the curve marking the boundaries between different populations) is constrained to either 1 (SPIA) or -1 (CPIA). The ability of these applications of PIA to correctly discriminate each array into a sample class can then be measured statistically through straightforward sampling statistics by comparing class means using a t test to generate p values. This method has been shown to be more robust at class prediction than single gene LDA [318].

4.2.15 DAVID analysis for identification of functional themes

The Database for Annotation, Visualization and Integrated Discovery (DAVID) was utilized to look for functional themes enriched in lists of significantly differentially expressed genes found using the Affymetrix platform. DAVID is a web tool produced by the NIAID for this purpose (http://david.abcc.ncifcrf.gov/home.jsp). This tool uses

biologically validated gene interaction and pathway data found in several other online databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) and BioCarta. Lists of genes are uploaded onto the application's website, and are then searched for functional themes that are enriched in those lists. Statistical testing is performed using a modified Fisher's Exact test[319] to determine statistical significance. Pathways whose member genes are significantly enriched in the submitted gene list are then returned with a p value and links to the relevant database.

4.2.16 Quantitative real-time reverse transcriptase polymerase chain reaction

4.2.16.1 Reverse-transcription and PCR

For confirmation of significant expression differences between populations, quantitative real-time reverse trancriptase polymerase chain reaction (qRT-PCR) was used. Standard curves were generated for each gene by amplifying a 10-fold dilution series of total RNA extracted from a PHA stimulated pool of PBMC with each set of gene-specific primers. All standard curves used contained a minimum of 3 replicates over at least four orders of magnitude. All samples, including standards, were initially reverse-transcribed to generate cDNA using the Quantitect RT system (Qiagen, Mississauga Ontario). In brief, total RNA for both standards and samples were incubated with genomic DNA Wipeout Buffer for 2 minutes at 42^0C prior to cDNA generation. After this, a master mix including RT enzyme, dNTPs, buffer and random primers were added to all samples that were then incubated for 30 minutes at 42^0C. Random primers were used to increase reverse-transcription of 3' to improve transcript detection. For each RT reaction a no template control and a no RT control were included to ensure a lack of contamination of samples. No template controls were used in each subsequent qPCR reaction, no-RT controls were

evaluated using the protocol for amplification of 18s RNA. Newly generated cDNA were stored at -70^0C until use.

Quantitative PCR was then carried out using gene-specific primers and the Quantitect SYBR green PCR kit (Qiagen, Mississauga Ontario). The Quantitect system uses *in silico* evaluated gene-specific primers designed to minimize primer-dimerization during PCR reactions. Reaction mixtures included test sample, gene-specific primers and a master mix including dNTPs, SYBR green, HotStart Taq DNA polymerase, PCR buffer and RNase-free H_2O. All samples and standards were amplified for each target gene on the LightCycler version 1.0 (Roche, Laval Quebec) using the cycling conditions outlined in Table 2.

4.2.16.2 Relative quantification using 18s rRNA as a control

Quantification of transcript levels was performed using LightCycler Data Analysis (LCDA) and RelQuant software packages (Roche, Laval Quebec) using the calibrator normalized relative quantification with efficiency correction method. Standard curves were generated for each target gene as well as 18s RNA as a control gene. Standard curves were generated as described above and exported into RelQuant from LCDA. Coefficient files were then created in RelQuant by comparing standard curves from each target gene to that of the control gene. The purpose of each coefficient file is to correct for PCR efficiency differences that may occur as a result of template sequence differences on an individual gene basis. Each sample run consisted of the test samples, a no-template control and a calibrator sample. Sample data were then imported into

Table 2: Thermal cycling conditions used for qRT-PCR of all genes and samples

Stage	Temp (^{0}C)	Time	Ramp Rate	Detection	
Enzyme activation	95	15min	20^{0}C/sec	None	
Amplification	94	15sec	2^{0}C/sec	None	
	55	20sec	2^{0}C/sec	None	→ x30-45
	72	20sec	2^{0}C/sec	Single	cycles
Melt	95	5sec	20^{0}C/sec	None	
	40	30sec	20^{0}C/sec	None	
	94	0sec	0.1^{0}C/sec	Continuous	

RelQuant and relative quantities were calculated using the following equation: S^nRelative quantity $= (S_n Q_T / S_n Q_R)/(CalQ_T / CalQ_R)$ where S^n is any sample, Cal is the calibrator sample, Q_T is the test gene quantity calculated as $Q_T = Q_{T0} \times E_T^{CpT}$ (where Q_{T0} is the initial amount of test gene and E is the PCR efficiency) and Q_R is the reference gene quantity. Values are thus corrected for differing PCR efficiency using the previously generated coefficient files. The result of this is a normalized, efficiency-corrected ratio of target gene to reference gene for each sample that can be used for further analysis. Further details of these calculations can be found in the technical note "Overview of LightCycler Quantification Methods" (www.roche-applied-science.com/techresources).

4.2.16.3 Relative quantification using total input RNA normalization

As outlined in the results section, Affymetrix array results suggested that several "housekeeping" genes may in fact be differentially regulated between groups. This being the case, qRT-RT-PCR for confirmation of genes shown to be differentially expressed between groups by the Affymetrix arrays were normalized to amount of input RNA as described[320]. In brief, standard curves for each gene are created as described in the previous section. Samples were run as above and applied a relative quantity based on the standard curve for that gene using the LightCycler data analysis software. Relative quantities were then devided by amount of input RNA to express each sample's expression level as relative quantity per nanogram of input RNA.

4.2.17 Cytometric Bead Array

Plasma samples and supernatants isolated from PBMC cultures were used for quantification of levels of secreted cytokine and chemokines using two types of kits, the

Cytometric Bead Array (CBA) prefabricated kits, where beads and antibodies are pre-mixed, and CBA flex-set kits where individual antibodies were selected and mixed in the lab (both from BD Biosciences, Mississauga Ontario). Kits used included Human Th1/Th2 Kit II (IL-2, IL-4, IL-6, IL-10, TNF, IFNγ), Human Inflammation Kit (IL-1b, IL-6, IL-8, Il-10, IL-12p70, IFNγ) and Human Chemokine Kit (IL-8, RANTES, MIG, MCP-1 IP-10). Flex set mixtures included IL-1b, IL-2, Il-6, IL-10, IFNγ, TNF. The procedures for the prefabricated kits and the flex set system are essentially the same. Both methods include proteins standards that are reconstituted and serially diluted and run at the same time as all samples for generation of standard curves and quantification. These systems use beads of varying fluorescence intensity (for differentiation between analytes) coupled to antibodies specific for a particular cytokine or chemokine for primary binding, followed by addition of a secondary antibody specific for each cytokine that is labeled with a fluorescent molecule phycoerythrin (PE) read in a different channel for quantification. An equal volume (50ul) of bead mixture was added to each standard and sample (supernatant or plasma) and was incubated for 1 hour at room temperature in the dark. Secondary labeled antibody (50ul) was then added to the reaction and incubated for 2 hours in the dark at room temperature. Wash buffer was added and samples were then centrifuged at 1000 RPM for 5 minutes. Supernatant was discarded and samples were resuspended in wash buffer. All samples and standards were run on the BD FACScalibur (prefabricated kits) or the BD LSR II (Flex Set). Standard curves were generated and samples were quantified using either BD CBA add-in for Microsoft Excel (Prefabricated kits) or FCAP bead array software version 2.00 (Flex Set)(both from BD).

4.2.18 Mann-Whitney U test

To test for statistically significant differences between groups for both qRT-PCR and CBA results the Mann-Whitney U test was employed. This test is considered to be non-parametric in that the data are not assumed to be normally distributed, unlike a Student's T test. This test compares medians between two sample groups to determine whether or not they are significantly different and applies a p value. P values below 0.05 were considered to demonstrate a statistically significant difference. Tests for significance were performed using GraphPad Prism software version 4.00 for Mac (GraphPad Software, San Diego California).

Section 5.0: Results

5.1 Gene Expression Profiles of T cell Subsets Differ from the PBMC Population

5.1.1 Rationale

Previous genomic studies of the effects of HIV infection or antigen stimulation on cell function have focused on either cell lines or mixed cell populations such as PBMC[298]. The peripheral blood mononuclear cell population consists of several cell types, CD4+ and CD8+ T-lymphocytes (70%), B-lymphocytes (15%), natural killer cells (10%), monocytes (5%), and dendritic cells (<1%)[321] each expressing a unique set of genes. Thus, it is conceivable that large alterations (several fold) in genes expressed predominantly in an under represented cell type may be overlooked when examining the PBMC population. Direct comparison of gene expression differences between the PBMC population and T cell subsets within the same individual has been lacking in the literature. Such a study would provide direct evidence for the benefits of focusing on cellular subsets rather than the mixed cell population for future studies of immunologic function.

5.1.2 Hypothesis

- Within a given individual after antigenic stimulation, the gene expression profiles of the CD4+ and CD8+ T cell populations will differ from each other and from that of the mixed cell PBMC population.

5.1.3 Objectives

- To compare the gene expression profiles of the mixed cell PBMC population with that of the CD4+ and CD8+ T cell subsets in the context of both a healthy and HIV infected individual after antigenic stimulation.

- To determine, by fold change analysis, specific genes that show differential levels of expression in each of the cell subsets under study.

5.1.4 Study outline

Peripheral blood mononuclear cells were isolated from an asymptomatic, HIV-1 positive individual (CD4 count >500 cells/ul) and an HIV negative, age and gender-matched control individual. Cells were stimulated with either *C. albicans* (both individuals) or HIV p24 (HIV-1 positive subject only) for 24 hours. Untreated (media alone) cells were used as controls for gene expression analysis. Following stimulation, an aliquot containing the mixed cell PBMC population was removed and cells were washed and placed in cell lysis buffer for RNA isolation. A further two aliquots were then removed for cell subset purification. These aliquots were enriched for CD4+ or CD8+ T cells using a negative selection technique. Gene expression levels were measured using nylon immune microarrays. Quantified data files were processed as mentioned in the materials and methods section. Normalization and determination of fold change was carried out in the GeneSpring software package. Genes were considered to be significantly up or downregulated if their expression differed from the untreated condition by more than twofold. Table 3 summarizes the number of genes up or down regulated in each cell subset for both individuals after stimulation. Representative tables of genes shown to be up or down regulated are given in Tables 4-7.

Table 3: Number of genes shown to be up or down regulated* in a given individual after antigenic stimulation

		Cell Subset				
Sample #	HIV Status	PBMC	CD4	CD8	Direction	Stimulation
D003	Negative	19	17	27	Up	*C. albicans*
		34	27	42	Down	
BAH002	Positive	23	23	9	Up	*C. albicans*
		10	23	125	Down	
		40	67	30	Up	HIV p24
		11	3	19	Down	

* Genes were considered to be up or down regulated if their expression changed after stimulation by at least two-fold.

Table 4: Genes up-regulated in CD4+ T cells in an HIV negative individual after _C. albicans_ stimulation

Fold	Symbol	Description
81.27	EST	Unknown
38.09	RBBP5	Retinoblastoma-binding protein 5
34.51	NR2F	Nuclear receptor subfamily 2, group F, member 1
33.19	EST	Cardiac Muscle Myosin Beta-Actin
29.51	EST	IPL1-relatedkinase
18.28	NOTCH4	Notch homolog 4
4.27	SCYB14	Small inducible cytokine subfamily B 14
3.20	TFAR15	Apoptosis-related protein TFAR15
2.70	PTGDR	Prostaglandin D2 receptor
2.69	PAS8	Peroxisomal Protein 8
2.51	DNA-PKcs	DNA-dependent protein kinase catalytic subunit
2.36	EST	40 kDa protein kinase related to ERK2
2.35	MIF	Macrophage migration inhibitory factor
2.08	EST	Laminin
2.07	EST	Dihydro-pyrimidine-dehydrogenase
2.07	VCAM1	Vascular cell adhesion molecule 1
2.02	EPR-1	Effector cell protease receptor-1

EST=Expressed Sequence Tag

Table 5: Genes down-regulated in CD4+ T cells in an HIV negative individual after
C. albicans **stimulation**

Fold	Symbol	Description
-2.02	MAPK4	Mitogen-activated protein kinase 4
-2.04	CDC42	Cell division cycle 42
-2.08	ANNEXINV	Annexin V
-2.23	EST	Unknown
-2.25	TCF12	Transcription factor 12
-2.26	EST	G/T mismatch-specific thymine DNA glycosylase
-2.30	EST	Peroxisomal acyl-CoA oxidase
-2.33	ERCC2	Excision repair cross-complementing rodent repair deficiency, complementation group 2
-2.38	AP2	Adapter protein 2
-2.39	EST	Tyrosinase-related protein 1
-2.51	EST	Flavin containing monooxygenase 5
-2.70	EST	Zinc finger protein homologousto Zfp-36
-2.72	CASP5	Caspase 5
-3.05	E2F1	E2F transcription factor 1
-3.13	RB1	Retinoblastoma 1
-3.32	EST	Proto-oncogene p55 (c-fos)
-4.43	EST	Prostaglandin-endoperoxidesynthase2
-5.39	EST	Actin, alpha2
-5.79	EST	Aldehydedehydrogenase 1
-6.00	EST	DNA polymerase delta small subunit
-9.36	EST	Unknown
-11.08	EST	Unknown
-26.84	EST	Stearoyl-CoA desaturase
-64.13	ZFP37	Zinc-finger protein 37
-116.80	CDK7	Cyclin-dependent kinase 7
-134.20	EST	Unkown
-400.77	EST	Unknown

EST=Expressed Sequence Tag

Table 6: Genes up-regulated in CD8+ T cells in an HIV positive individual after HIV p24 stimulation

Fold	Symbol	Description
297.75	EST	Cardiac Muscle Myosin Beta-Actin
293.68	EST	Unknown
287.51	CHD1	Chromodomain helicase DNA binding protein 1
274.47	MYH1	Myosin Heavy Chain 1
260.51	PEA3	Polyomavirus enhancer activator 3
150.21	EST	Basic transcription factor2
128.64	GCA	Grancalcin
112.68	ERCC2	Excision repair cross-complementing rodent repair deficiency, complementation group 2
103.61	PDYN	Pro-dynorphin
80.32	CSF3	Colony stimulating factor 3
47.88	AMY1	Amylase alpha 1
46.13	CXCL2	Chemokine (C-X-C) ligand 2
14.87	N2RF1	Nuclear receptor subfamily 2, group F member 1
11.70	CENPA	Centromere protein-A
9.00	IL5RA	interleukin 5 receptor, alpha
8.85	ZFP37	Zinc finger protein 37
4.11	ANNEXINV	Annexin V
3.68	ITGA9	Integrin alpha 9
2.85	MADH3	Mothers against decapentaplegic homolog 3
2.80	AOAH	Acyloxyacyl hydrolase (neutrophil)
2.68	TRAP1	TNF receptor-associated protein 1
2.63	EST	Integrin alpha subunit
2.43	PTGDR	Prostaglandin D2 receptor
2.30	EST	unknown
2.23	EPR1	Effector cell protease receptor-1
2.16	HSPB3	Heat shock protein beta 3
2.11	HAS2	Hyaluronan synthase
2.10	EST	Unknown
2.07	EST	DNA repair protein RAD54 homolog
2.07	MET	Met proto-oncogene (hepatocyte growth factor receptor)

EST=Expressed Sequence Tag

Table 7: Genes down-regulated in CD8+ T cells in an HIV positive individual after HIV p24 stimulation

Fold	Symbol	Description
-2.01	MAPK7	Mitogen-activated protein kinase 7
-2.01	ARAF1	v-raf murine sarcoma viral oncogene homolog 1
-2.08	EST	Hemeoxygenase
-2.09	DNA-PKcs	DNA-dependent protein kinase catalytic subunit
-2.13	EST	Calponin
-2.17	EST	Unknown
-2.20	EST	Nitricoxide synthase 2A
-2.50	EST	Prostaglandin-endoperoxide synthase 2
-2.59	EST	Oncogene Tls/Chop, fusion activated
-2.80	EST	Unknown
-3.24	EST	Unknown
-3.88	EST	Retinoic acid receptor gamma1
-5.11	EST	Unknown
-6.06	EST	Bone morphogenetic protein 1
-6.30	E2F1	E2F transcription factor 1
-7.44	FAF1	Fas associated factor 1
-10.04	EST	Flavin containing monooxygenase 5
-26.02	EST	Fibromodulin
-425.57	EST	Unknown

EST=Expressed Sequence Tag

5.1.5 Antigen-specific changes in gene expression

Microarray analysis of different stimulated cell populations from the HIV infected or HIV uninfected individual showed the expression of several genes was changed at the twofold level when compared to the unstimulated condition. The gene lists generated contained those genes shown to be up-regulated or down-regulated by twofold. For each stimulation condition (*C. albicans* or p24) Venn diagrams were generated from gene lists to compare the similarity of the gene expression profiles between the cell subsets examined (PBMC, CD4+ T-lymphocytes, CD8+ T-lymphocytes) (Figure 3). The Venn diagrams show the largest proportion of changed genes to be unique to the individual cell population studied (red, green and dark blue areas in Figure 3). The profile of the HIV negative sample stimulated with *C. albicans*, for example, showed sixteen distinct genes were only upregulated in PBMC, two genes were similarly regulated in CD4+ T-lymphocytes and only one was common to all of the cell populations (Figure 3a). Interestingly, in all of the comparisons the PBMC and CD8+ T-lymphocyte populations show no (Figures 3a, 3c, 3d, 3f) or very few (Figures 3b, 3e) genes showing the same trend. This is likely due to the fact that CD8+ T-lymphocytes comprise a very small proportion of the PBMC population and that many genes showing expression changes in CD8+ T-lymphocytes would be missed when examining the total cell population.
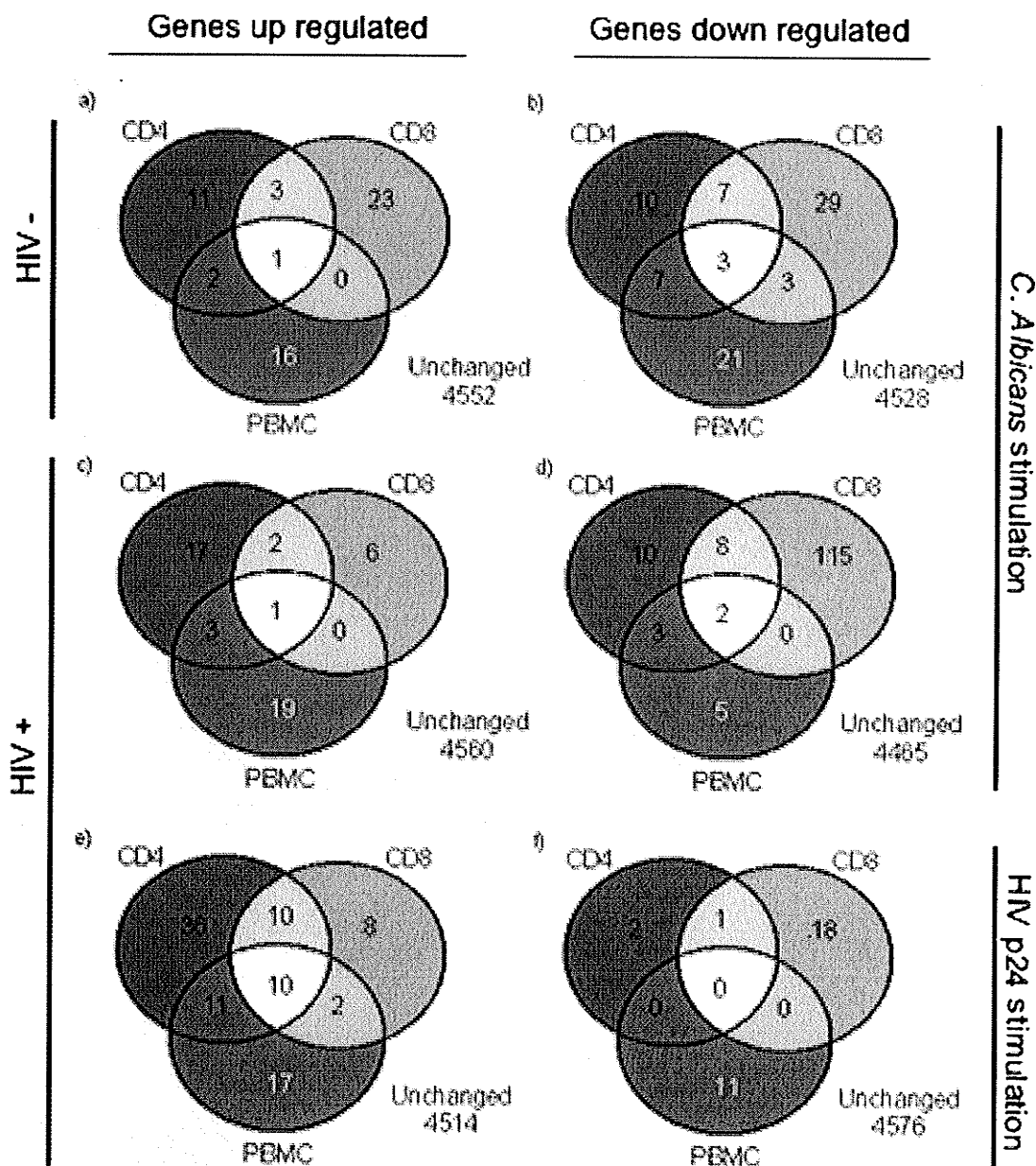
Figure 3: Venn diagrams of changed genes in single and mixed cell populations. Comparison of number of genes showing expression changes at the twofold level. a and b, HIV negative sample response to *C. albicans*, c and d HIV positive sample responses to *C. albicans* and e and f HIV positive response to p24. Diagrams a, c, e represent gene up-regulations, b,d,f represent gene down-regulations. Red, green and blue sections represent genes only changed in CD4+, CD8+ and PBMC populations respectively. Sections in yellow, purple and light blue represent genes sharing trends in two cell populations. Sections in white represent genes showing the same trend in all cell populations.

5.1.6 Comparison of gene expression values between cell types

To further highlight distinct gene expression profiles in the chosen cell populations, genes that were up-regulated in one cell population but not the others were selected. Comparison of fold change values across all cell types for the *C. albicans* stimulated conditions in the HIV uninfected individual and p24 responses in the HIV infected individual respectively (Figure 4) were carried out. Levels of macrophage migration inhibitory factor (MIF), tumor necrosis factor receptor superfamily member 5 (TNFRSF5), and monocyte chemoattractant protein-1 (MCP-1) in response to *C. albicans* stimulation in the HIV negative donor were examined. Likewise, interleukin 6 (IL-6), TGF β receptor associated protein -1 (TRAP-1) and insulin-like growth factor 2 (IGF2) levels in response to p24 stimulation in the HIV positive donor were investigated.

Genes up-regulated in one cell population showed either up-regulation below the twofold cutoff or even a trend towards down-regulation in the other two cell populations. In the *C. albicans* stimulated HIV negative sample (Figure 4a). MIF was 2.35 fold up-regulated in CD4+ cells yet showed a trend towards down-regulation in the CD8+ population (-1.7 fold). Furthermore, the resulting non-twofold change of MIF expression in the PBMC population (1.63 fold) is a result of the very different expression levels in the two distinct cell types (Figure 4a). With regards to the CD4+ cell population in the HIV positive sample, the demonstrated up-regulation of the pro-inflammatory cytokine IL-6 would have gone unnoticed in a study of either the CD8+ or the PBMC populations alone. These data underscore the importance of examining individual cell populations within the immune system in order to obtain a clear picture of immune function.

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a)
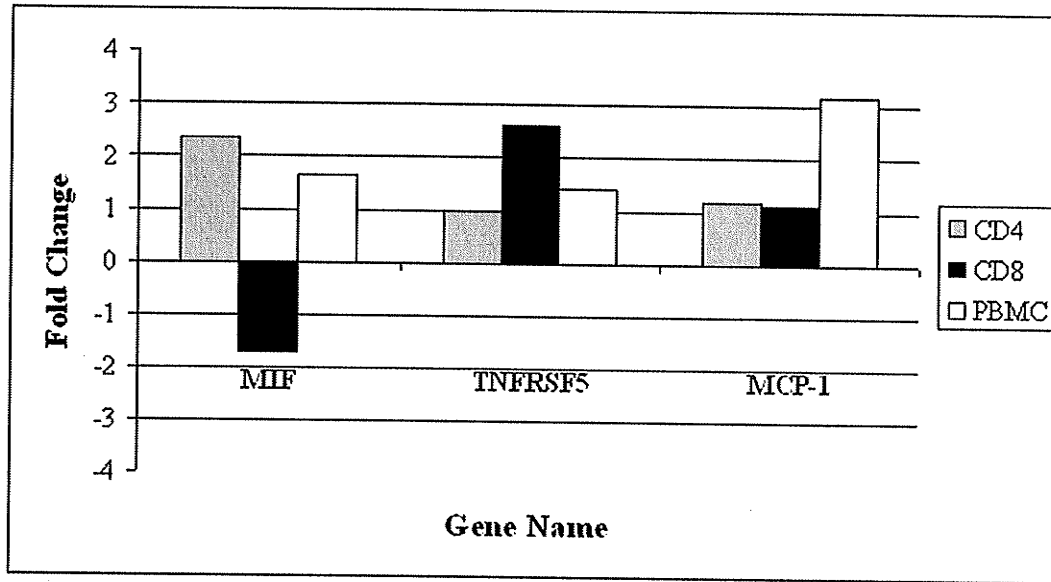
b)
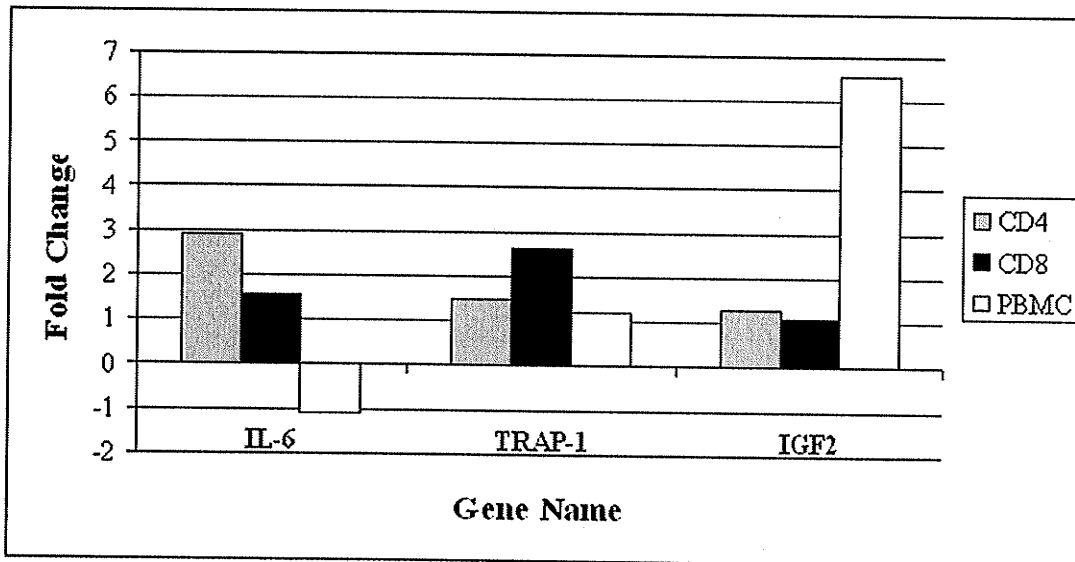
Figure 4: Fold change in gene expression in response to antigenic stimulation in single and mixed cell populations. Ratio values for selected genes show differing values in each cell population. Genes shown to have changed expression in one cell population do not meet the twofold criteria in the other two populations. Graph a) shows HIV negative sample responses to *C. albicans* stimulation with respect to expression of macrophage migration inhibitory factor (MIF), tumor necrosis factor receptor super family member 5 (TNFRSF5) and monocyte chemoattractant protein 1(MCP-1). Graph b) shows effects of p24 stimulation on expression of interleukin 6 (IL-6), TGF β receptor associated protein 1 (TRAP-1) and insulin-like growth factor 2 (IGF2) in the HIV positive sample.

5.1.7 Summary

At the time this work was carried out, the majority of microarray studies focused on either cell lines or PBMC populations. This was the first study to directly compare gene expression profiles of specific cell subsets to the mixed cell population in a given individual and resulted in a publication in Clinical and Diagnostic Laboratory Immunology[322]. Several studies since have endeavored to compare gene expression profiles of CD4+ and CD8+ T cells, as well as subsets of these cells, showing distinct transcriptional profiles depending on cell type [323, 305].

5.2 High Levels of Immune Dysfunction Seen by Gene Expression Analysis in Asymptomatic, HIV-1 Positive Individuals

5.2.1 Rationale

Our cohorts provide a unique opportunity to study HIV-1 positive (HIV+) individuals, infected for long periods of time prior to initiation of any anti-retroviral therapy. By conducting gene expression analysis of asymptomatic HIV-1 infected individuals with good CD4 counts (i.e. CD4 > 350) it will be possible to evaluate their CD4+ T cell function on a higher scale than is commonly carried out.

5.2.2 Hypotheses

- Relatively healthy (CD4 > 350) will show gene expression levels similar to those seen in uninfected individuals.

- Gene expression differences that are seen will confirm what is known about HIV-1 pathogenesis. Specifically we will see higher expression of genes involved in immune activation.

5.2.3 Objectives

- Determine levels of similarity of gene expression at baseline and in response to stimulation in HIV+ vs HIV-1 low-risk negative (HIV-LRN) women.

- Determine genes, and classes of genes that are differentially expressed in the two study populations.

5.2.4 Study Outline

PBMC were collected from a total of 10 HIV-1 positive (HIV+) and 10 HIV-1 low-risk negative (HIV-LRN) individuals from either the ML or MCH cohorts. All HIV+ women were antiretroviral naïve at the time of enrollment, and all individuals had CD4+ T cell counts greater than 350 cells/ul and were free of HIV-1-related disease. Baseline characteristic data for all HIV+ subjects can be found in Table 8.

Stimulations, cell separations and nylon immune microarray analyses were performed as outlined in the Methods section. Fold change and p values were calculated after RMA normalization and application of the customized correction function (for further detail see Sections 4.2.11.3 and 4.2.11.4). Lists of significantly differentially regulated genes ($p <$ 0.05) were created for data mining. Table 9 shows a summary of numbers of genes shown to be differentially expressed HIV+ individuals compared to HIV-LRN.

5.2.5 Correlation of gene expression data

Correlations of mean expression data were performed in order to visualize overall variation between stimulation conditions within a group, and between sample groups. Figure 5 shows correlation values within the HIV-LRN and HIV+ sample groups between stimulation conditions (Figures 5a,b) and between the HIV+ and HIV-LRN sample groups (Figure 5c). Stimulation with the mitogen PHA showed the greatest difference from the media alone condition in both groups. However, its correlation value in the HIV+ group (-0.03) is much lower than was seen in the HIV-LRN group (0.44) (Figures 5b and a respectively). Overall, each stimulation condition has a much lower

Table 8: Enrollment characteristics of HIV+ study subjects

Study Number	Years Positive	CD4	CD8
ML 2000	>1	606	6791
MCH 117535	>2	686	1480
MCH 114290	>3	930	2029
MCH 112735	>8	597	1071
ML 1772	>9	351	913
ML 1665	>10	615	1233
ML 1315	>12	387	1057
ML 1211	>12	621	2262
ML 713	>16	719	1087
ML 412	>18	530	1210

Note: All HIV+ study subjects were positive upon enrollment to their respective cohorts

Table 9: Summary of numbers of genes significantly differentially expressed between HIV+ and HIV-LRN groups

Stimulation	Genes Up	Genes Down	Total
Media	108	79	187
PHA	342	250	592
C. albicans	183	136	319
Flu peptide	107	106	213
HIV-1 p24	255	199	454

a)

HIV-LRN

	Media	PHA	C. albicans	Flu peptide	HIV p24
Media		0.44	0.48	0.49	0.47
PHA			0.27	0.37	0.29
C. albicans				0.46	0.51
Flu peptide					0.45
Hiv p24					

(HIV-LRN on left axis)

b)

HIV+

	Media	PHA	C. albicans	Flu peptide	HIV p24
Media		-0.03	0.14	0.09	-0.02
PHA			0.35	0.26	0.24
C. albicans				0.23	-0.02
Flu peptide					0.12
Hiv p24					

(HIV+ on left axis)

c)

HIV-LRN

	Media	PHA	C. albicans	Flu peptide	HIV p24
Media	0.21	0.28	0.12	0.19	0.11
PHA	-0.48	-0.27	-0.37	-0.39	-0.46
C. albicans	-0.09	0.10	-0.08	-0.02	-0.07
Flu peptide	-0.17	0.05	-0.19	-0.17	-0.16
Hiv p24	-0.25	-0.14	-0.32	-0.27	-0.33

(HIV+ on left axis)

Figure 5: Correlation of means of gene expression data of HIV-LRN and HIV+ individuals across all stimulation conditions. Correlation values were calculated comparing all stimulation conditions within the HIV-LRN group (a), HIV+ group (b) and between the HIV+ and HIV- groups (c). Red boxes indicate strong positive correlation (>0.40), pink boxes indicate weak positive correlations (</= 0.40), light blue boxes indicate weak negative correlations (-0.0 - -0.39), dark blue boxes indicate strong negative correlation (</= -0.40).

correlation value in the HIV+ group than is seen in the HIV-LRN group suggesting a larger degree of functional difference in the CD4+ T cells of HIV+ individuals after stimulation than was hypothesized. Interestingly, the p24 stimulation condition also showed a negative correlation when compared to the unstimulated (media alone) condition. This suggests a powerful recall response to this particular antigen.

When comparing the HIV+ to the HIV-LRN study subjects (Figure 5c) dramatic differences in gene expression can be observed. Overwhelmingly the comparisons are either only very weakly positive (i.e. less than 0.4) or negative. This is underscored by the large number of genes shown to be significantly differentially expressed between HIV+ and HIV-LRN individuals (Table 9). This observation suggests a large degree of immune dysfunction in HIV+ individuals, in absence of severe disease. Identifying genes that contribute to this observation may shed light onto previously unidentified areas of immune dysfunction caused by HIV-1 infection.

5.2.6 Unstimulated gene expression differences

It is well known that HIV infection increases levels of activation of T cells. This being the case, we hypothesized that the large gene expression differences identified by correlation of mean gene expression between HIV+ and HIV-LRN individuals may be in part due to genes involved in cellular activation. To address this, lists of significantly differentially expressed genes were scanned for the presence of genes involved in cellular immune response and activation. Table 10 shows selected genes both expressed at higher and lower levels in unstimulated CD4+ T cells of HIV+ compared to HIV-LRN women.

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Table 10: Selected genes showing significantly different unstimulated expression between HIV+ and HIV-LRN study groups

Symbol	p Value	Fold	Function	Description
ELF2	0.0019	1.95	Transcription factor	E74-like factor 2
STAT2	0.0002	1.19	Transcription factor	Signal transducer and activator of transcription 2
ISGF3G	0.048	1.11	Transcription factor	Interferon-stimulated transcription factor 3
NDRG	0.012	1.10	Signal transduction	N-myc downstream regulated
RAB36	0.030	1.09	Signal transduction	RAB36, member RAS oncogene family
SP1	**0.029**	**1.09**	**Transcription factor**	Sp1 transcription factor
RAB11B	0.002	1.08	Signal transduction	RAB11B, member RAS oncogene family
SH3BP2	0.029	1.08	Signal transduction	SH3-domain binding protein 2
TNFRSF11	0.029	1.06	Apoptosis	Tumor necrosis factor receptor subfamily, member 11
STAT5B	**0.030**	**-1.19**	**Signal transduction**	Signal transducer and activator of transcription 5b
CX3CR1	0.0086	-1.15	Cytokine/Chemokine/Receptor	Chemokine (C-X3-C) receptor 1
CCR5	**0.020**	**-1.10**	**Cytokine/Chemokine/Receptor**	**Chemokine (C-C motif) receptor 5**
IL1A	0.022	-1.09	Cytokine/Chemokine/Receptor	interleukin 1, alpha
IFNR1	0.037	-1.07	Cytokine/Chemokine/Receptor	Interferon gamma receptor 1

As expected, several genes involved in cellular activation and signaling are increased. Of particular importance, the transcription factor SP1, which has been implicated in HIV-1 replication was over expressed in HIV+ individuals. Interestingly, several of the genes expressed at a lower level by HIV+ women are receptors involved in cytokine/chemokine signaling, suggesting several previously unidentified targets for down-regulation by HIV-1. Possibly the most confusing finding is a relative lower expression level of STA5B in HIV+ women. This molecule has been shown to increase HIV-1 replication by binding the LTR[324]. However, it is possible that this effect may only be important in early infection.

5.2.7 Antigen-stimulated gene expression levels

Analysis of lists of genes expressed at a higher level in HIV+ women (Table 11) showed several genes involved in cellular activation and immune mediators induced after stimulation, particularly in PHA stimulated cells. Interestingly, in the HIV+ individuals, p24 stimulation resulted in relatively higher expression of the T cell receptor alpha chain, SHC1, CXCR4 and the activation marker CD69 suggesting specific induction of activation by p24, as well as upregulation of an HIV-1 coreceptor, CXCR4, which would likely result in higher levels of HIV-1 replication *in vivo*. As well, the higher expression of the alpha chains of both the IL-4 and IL-10 receptor suggest the induction of a type 2 immune response, possibly at the expense of a more protective, cell-mediated response.

Table 11: Selected genes showing significantly higher expression in HIV+ vs HIV-LRN study groups after antigenic stimulation

Stimulation	Symbol*	Fold	p Value	Function
PHA	TNFRSF10B	1.34	0.006	Apoptosis
	JUN	1.32	0.014	Transcription factor
	CXCR4	**1.26**	**0.020**	**Cytokine/Chemokine/Receptor**
	MAP2K4	1.23	0.015	Signal transduction
	MAPK7	1.21	0.009	Signal transduction
	E2F1	1.21	0.007	Transcription factor
	SHC1	**1.19**	**0.007**	**Signal transduction**
	MAP4K1	1.18	0.005	Signal transduction
	STAT4	1.17	0.004	Transcription factor
C. albicans	**BCL2**	**1.17**	**0.001**	**Anti-apoptosis**
	IL22R	1.15	0.018	Cytokine/Chemokine/Receptor
	IL10RA	**1.14**	**0.018**	**Cytokine/Chemokine/Receptor**
	CX3CR1	1.13	0.031	Cytokine/Chemokine/Receptor
	MAPK7	1.10	0.022	Signal transduction
	CD69	**1.08**	**0.018**	**Activation**
Flu peptide	IL9R	1.21	0.037	Cytokine/Chemokine/Receptor
	MAP2K4	1.15	0.041	Signal transduction
	TRA@	**1.12**	**0.005**	**T cell Signaling**
	BCL2	**1.12**	**0.006**	**Apoptosis**
	MAP4K1	1.11	0.022	Signal transduction
	MAPK7	1.11	0.025	Signal transduction
HIV p24	**STAT5B**	**1.34**	**0.034**	**Transcription factor**
	CXCR4	**1.27**	**0.020**	**Cytokine/Chemokine/Receptor**
	SHC1	**1.19**	**0.004**	**Signal transduction**
	CX3CR1	1.17	0.007	Cytokine/Chemokine/Receptor
	TRA@	**1.14**	**0.024**	**T cell Signaling**
	IL4RA	**1.13**	**0.001**	**Cytokine/Chemokine/Receptor**
	IL10RA	**1.12**	**0.017**	**Cytokine/Chemokine/Receptor**
	MAP4K1	1.11	0.002	Signal transduction
	STAT3	1.11	0.033	Transcription factor
	MAPK7	1.10	0.024	Signal transduction
	CD69	**1.09**	**0.002**	**Activation**

* An alphabetical listing of all gene symbols and descriptions used in this thesis can be found in Appendix B.

Lists of genes shown to be expressed at a lower level by HIV+ individuals after antigenic stimulation (Table 12) also show several interesting trends. Both STAT2 and CCR6 are under expressed in three of four stimulation conditions. As well, IRF2 is expressed at a lower level after stimulation by all 4 antigens. Whether these themes are evidence of specific immune pathways repressed by HIV-1 infection, would be interesting to further explore.

5.2.8 Summary

To our knowledge, this is the first study to demonstrate large overall differences in *ex vivo* immune gene expression in CD4+ T cells of asymptomatic HIV+ individuals compared to HIV-LRN women using genomics techniques. This finding does no support our hypothesis that asymptomatic HIV+ individuals would have relatively similar levels of gene expression compared to healthy controls. The negative correlations seen between mean gene expression values of HIV+ and HIV-LRN women after antigenic stimulation suggests not only different sets of genes activated in HIV-1 infected individuals, but possibly also inhibition of normal immunological processes.

Analysis of lists of differentially expressed genes showed predictably higher levels of immune activation in HIV+ individuals in unstimulated CD4+ T cells. Also, after antigenic stimulation we have identified specific immunological processes that may be dysregulated in HIV+ individuals. These findings have implications for treatment of HIV+ individuals and targeting of specific immune defects.

Table 12: Selected genes showing significantly lower expression in HIV+ vs HIV-LRN study groups after antigenic stimulation

Stimulation	Symbol*	Fold	p Value	Function
PHA	IL8RB	-2.10	0.001	Cytokine/Chemokine/Receptor
	IRF2	**-1.47**	**0.010**	**Cytokine/Chemokine/Receptor**
	IL2RB	-1.19	0.016	Cytokine/Chemokine/Receptor
	CCR6	**-1.11**	**0.024**	**Cytokine/Chemokine/Receptor**
	IL18	-1.09	0.024	Cytokine/Chemokine/Receptor
C. albicans	**IRF2**	**-1.17**	**0.018**	**Cytokine/Chemokine/Receptor**
	STAT2	**-1.15**	**0.004**	**Transcription factor**
	CCR6	**-1.12**	**0.005**	**Cytokine/Chemokine/Receptor**
	IL18	-1.11	0.015	Cytokine/Chemokine/Receptor
	IL2RB	-1.10	0.021	Cytokine/Chemokine/Receptor
Flu peptide	CASP1	-1.23	0.027	Apoptosis
	IRF2	**-1.13**	**0.034**	**Cytokine/Chemokine/Receptor**
	STAT2	**-1.13**	**0.012**	**Transcription factor**
	IL8	-1.13	0.019	Cytokine/Chemokine/Receptor
HIV p24	IL12A	-1.45	0.026	Cytokine/Chemokine/Receptor
	IRF2	**-1.39**	**0.043**	**Cytokine/Chemokine/Receptor**
	STAT2	**-1.20**	**0.010**	**Transcription factor**
	CCR6	**-1.19**	**0.001**	**Cytokine/Chemokine/Receptor**
	IL5	-1.15	0.007	Cytokine/Chemokine/Receptor
	IL2RB	-1.11	0.027	Cytokine/Chemokine/Receptor

* An alphabetical listing of all gene symbols and descriptions used in this thesis can be found in Appendix B.

5.3 Pilot Study Comparing Gene Expression in CD4+ T cells of HIV Resistant Commercial Sex-workers with HIV-1 low-risk Negative Individuals

5.3.1. Rationale

Several lines of evidence, outlined in the introduction, suggest both genetic and immunologic mechanisms mediating resistance to infection by HIV-1. Although many studies have investigated the contribution of CD8 lymphocytes to this phenomenon, fewer have addressed the role of CD4+ T cells. At the time this study was initiated there were no published studies addressing CD4+ T cell function in instances of reduced susceptibility to HIV using genomic techniques. Genes shown to be differentially-regulated in CD4+ T cells in HIV resistant commercial sex-workers, both at baseline and in response to stimulation may be directly involved in inhibition of viral infection or establishment of an antiviral immune response. With this in mind, we designed a small pilot study consisting of 5 individuals per study group to assess if gene expression differences could be detected between groups, and to determine what functional groups of genes and pathways could be associated with the HIV-1 resistance phenotype.

5.3.2 Hypotheses

- HIV resistant commercial sex-workers will show an inducible pattern of gene expression that differs from HIV negative women.
- Antigenic stimulation will impact specific families of genes in HIV-R individuals
- Baseline levels of gene expression will differ between the HIV resistant and HIV-LRN populations. Genes that are differentially expressed between the two populations will be considered biomarkers of resistance.

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5.3.3 Objectives

- Determine, on a small group of individuals, if a pattern of gene expression exists that can discriminate between HIV-R and HIV-LRN women.

- Determine sets of genes whose expression is significantly altered under a variety of stimulation conditions, in the HIV-R and HIV-LRN population.

- Compare baseline gene expression levels between HIV-R and HIV-LRN women to determine genes and pathways that may be involved in protection from HIV-1 infection

5.3.4 Study Outline

In order to assess if gene expression differences could be measured between HIV-1 resistant (HIV-R) women from the ML cohort and HIV-1 low-risk negative (HIV-LRN) women from the MCH cohort, an initial group of 5 HIV-R (mean age 49.4) and 5 HIV-LRN (mean age 33) were enrolled for study. Characteristics at enrollment for all study subjects can be found in Table 13.

Peripheral blood mononuclear cells were isolated and stimulated with media alone (baseline), PHA (a mitogen), *C. albicans*, flu peptide (recall antigens) or HIV p24 protein. After 24 hours of stimulation supernatants were collected and CD4+ T cells were enriched by negative selection. Gene expression, both at baseline and in response to antigenic stimulation, was measured using nylon immune microarrays. For this study, focused immune arrays, obtained from the National Institute on Aging through collaboration with Dr. Michael Mayne, were chosen as our initial hypotheses focused on immune function as the major factor contributing to the HIV-1 resistance phenotype.

Table 13: Enrollment characteristics of HIV-R and HIV-LRN study subjects

Study Number	HIV Status	Age	Years of Enrollment	CD4	CD8
ML 466	Resistant	51	17	1341	817
ML 767	Resistant	48	15	1175	569
ML 893	Resistant	59	15	1298	1321
ML 1498	Resistant	42	10	1137*	773*
ML 1803	Resistant	47	7	633	444
MCH 102749	Negative	33	15	857*	417*
MCH 112499	Negative	32	9	694	705
MCH 115150	Negative	41	6	967	254
MCH 117066	Negative	26	3	804	575
MCH 117436	Negative	33	2	1071	529

*CD4/CD8 counts were not available for these individuals at the time of enrollment in this study. The reported values were obtained at the earliest possible subsequent visit.

As well, due to their sensitivity, nylon arrays better suited our available sample quantity (maximum 1ug of total RNA) as at the time this study was conducted technologies for linear amplification of RNA for microarray analysis was untested and widely unavailable. Cluster and fold change analyses were employed to determine genes that characterize HIV-R individuals.

5.3.5 Data pre-processing and normalization

Data files generated by quantification of spot intensities on the nylon microarrays were imported into Microsoft Excel for pre-processing purposes. Duplicate spots for a given gene were compared for expression similarity within an array. Duplicates that varied by greater than 20% were eliminated from further analysis. The remaining values were then background subtracted and averaged. Normalization was carried out by the Z-score normalization method outlined in Section 4.2.11.1. Normalized data files were then used for both cluster and fold-change analysis.

5.3.6 HIV resistant women show an inducible pattern of gene expression

The HIV-1 resistance phenotype has been associated with the presence of HIV-specific CD4+ T cells in this and other cohorts. We sought to determine if, in this cohort, this was associated with differential function of CD4+ T cells within our resistant women or simply evidence of previous exposure to HIV-1. In order to address this, a non-biased cluster analysis of genes and arrays was performed. This technique sorts both genes and arrays based on their similarity of expression. Lists of gene expression levels after antigenic stimulation were generated for each of the samples outlined in Table 13 by comparing each antigen-stimulated sample (PHA, *C. albicans*, flu peptide, HIV-1 p24) to

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its media control. Complete linkage, hierarchical clustering of both genes and arrays was performed using an Euclidean distance metric. Gene lists containing fold change values of either all genes, filtered to remove only those genes that were present in less than 80% of the datasets (Figure 6), or on lists filtered to remove any gene that did not show a twofold change (either up or down) in at least 1 dataset (Figure 7) were used for this analysis. Clustering was performed using Cluster version 3.0 (University of Tokyo) and heat maps were visualized using TreeView version 1.1.0 (Saldanha Bioinformatics 2004). For both the complete set of genes and those filtered to removed genes not showing a twofold change in any dataset clustering of resistant individuals is seen in both the *C. albicans* and flu peptide stimulated conditions (Figures 6a, 6c, 7a, 7c). Interestingly, the HIV-R women did not show evidence of clustering after HIV p24 stimulation, suggesting that an inducible pattern of gene expression may exist that characterizes CD4+ T cell function in HIV-R women that may not be HIV-specific.

5.3.7 Antigen-stimulated gene expression differences

To address the issues of which genes and pathways might play a role in mediating HIV resistance in this cohort; antigen-stimulated gene expression changes were investigated. Normalized expression values for each gene in the array were averaged for all individuals based on HIV status (HIV-R or HIV-LRN). Fold change values were then calculated

a) *C. albicans*

b) PHA

4337 genes

c) Flu peptide

4336 genes

d) HIV-1 p24

4312 genes

3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00

4347 genes

■ HIV-1 Resistant
▨ HIV-1 Low risk negative
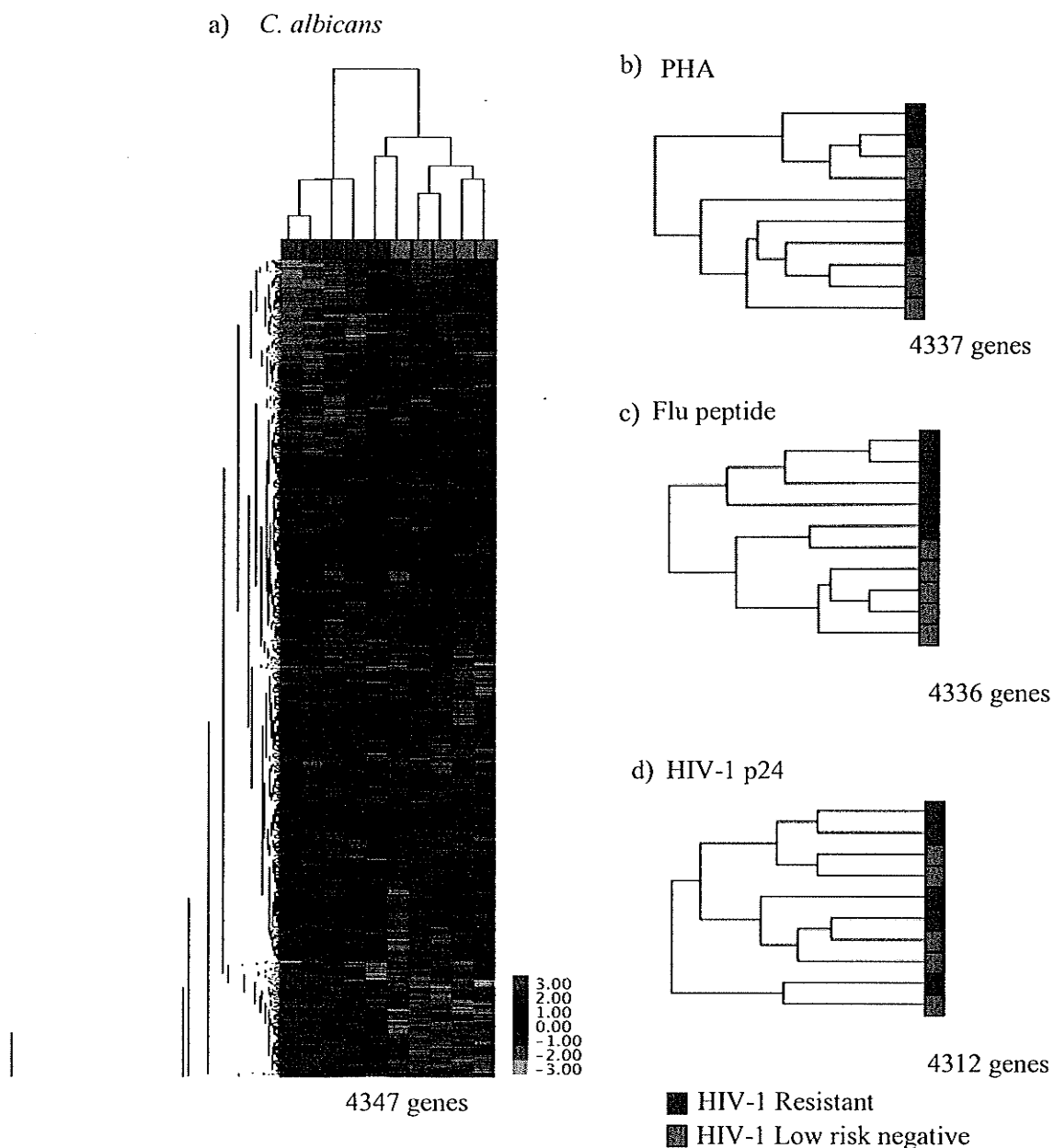
Figure 6: Complete linkage hierarchical clustering based on expression of all genes after antigenic stimulation. Heat map of genes up-regulated (red) or down-regulated (green) after *C. albicans* stimulation is shown in a) with a single gene appearing in each row and all genes in a given array contained within a column. Dendograms alone based on expression changes of genes after PHA (b), flu peptide (c) or HIV p24 (d) stimulation are shown. The distance of the arms extending from the nodes is relative to relatedness of expression patterns. The number of genes passing the filter is given below the respective figure. Blue squares represent HIV-1 resistant individuals, red squares represent HIV-1 low-risk negatives. Clustering of arrays based on expression of all genes shows similar expression profiles of 4 of 5 HIV-R samples after *C. albicans* (a) and flu peptide (c) stimulation.

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a) *C. albicans*

b) PHA

1157 genes

c) Flu peptide

1206 genes

d) HIV-1 p24

1181 genes

1233 genes

3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00

■ HIV-1 Resistant
▨ HIV-1 Low risk negative
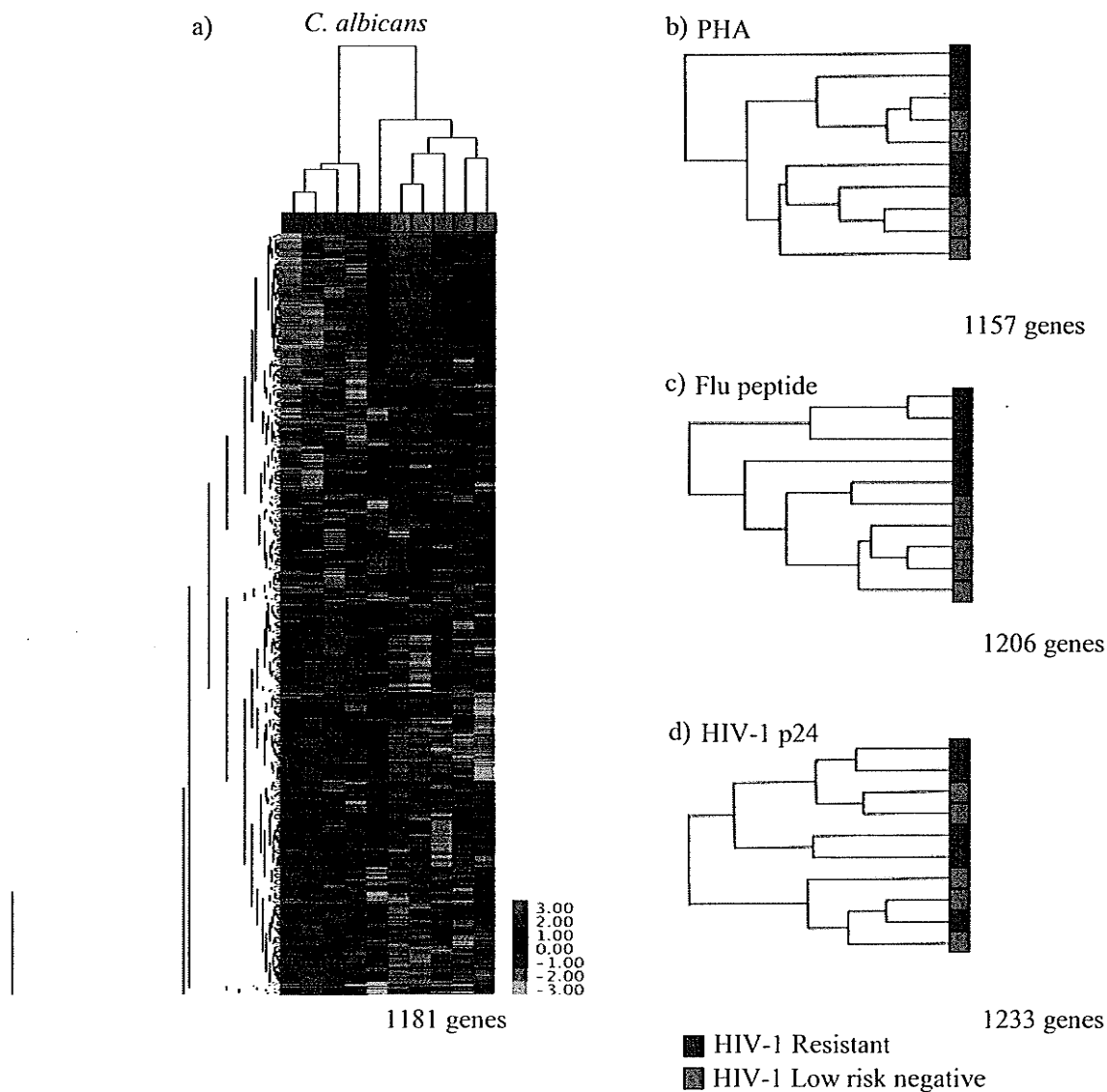
Figure 7: Complete linkage hierarchical clustering based on expression of genes shown to be twofold changed in at least 1 individual after antigenic stimulation. Heat map of genes up-regulated (red) or down-regulated (green) after *C. albicans* stimulation is shown in a) with a single gene appearing in each row and all genes in a given array contained within a column. Dendograms alone based on expression changes of genes after PHA (b), flu peptide (c) or HIV p24 (d) stimulation are shown. The distance of the arms extending from the nodes is relative to relatedness of expression patterns. The number of genes passing the filter is given below the respective figure. Blue squares represent HIV-1 resistant individuals, red squares represent HIV-1 low-risk negatives. Clustering of arrays based on expression of changed genes shows similar expression profiles of 4 of 5 HIV-R samples after *C. albicans* (a) and 3 of 5 after flu peptide (c) stimulation.

based on change in intensity of a given gene after stimulation (PHA, *C. albicans*, flu peptide or p24) compared to the baseline (media alone) condition. Genes were considered to be significantly up or down regulated if they showed a p value of less than 0.05. Genes significantly changed after stimulation in both the HIV-R and HIV-LRN study individuals were excluded from the list to compile a list of genes uniquely regulated by the HIV-R population. A summary of numbers of genes significantly up or down regulated is given in Table 14.

5.3.7.1 HIV-R individuals show lower expression of specific cytokines, chemokines and transcription factors after antigenic stimulation

Analysis of lists of genes shown to be uniquely up or down regulated in HIV-R women after stimulation showed a trend towards down-regulation of specific cytokines, chemokines and transcription factors in one or more stimulation condition (Table 15). Several members of the CC chemokine family (CCL2, CCL11, CCL22, MIP-1β, MCP-3) and cytokines involved in inflammation (IL-6, IL-8) show a greater than two-fold down regulation with the largest proportion of these being down regulated after *C. albicans* stimulation. Of particular interest, the chemokine MIP-1β, and the transcription factors SP2 and SP3 are down regulated in either 3 (MIP-1β and SP2) or all 4 (SP3) stimulation conditions. Both MIP-1β and SP3 have been shown to have anti-HIV properties[183, 325]. However it is possible that the observed down regulation may reflect a high level of expression prior to stimulation instead of a specific down regulation.

Table 14: Summary of number of genes uniquely up or down regulated after stimulation in CD4+ T cells of HIV-R women

Stimulation	Genes Up	Genes Down	Total
PHA	49	61	110
C. albicans	122	180	302
Flu peptide	51	62	113
HIV p24	96	81	177

Table 15: Chemokine and transcription factor genes are down regulated in HIV-R women after stimulation

			Fold		
Symbol*	**Class**	**PHA**	*C. albicans*	**Flu peptide**	**HIV p24**
CCL2	**Cytokine/Chemokine**	**NS**	**-2.09**	**NS**	**NS**
CCL11	**Cytokine/Chemokine**	**-2.08**	**NS**	**NS**	**-3.14**
CCL22	**Cytokine/Chemokine**	**NS**	**-2.70**	**NS**	**NS**
IL1R1	Cytokine/Chemokine	NS	-2.35	NS	NS
IL6	**Cytokine/Chemokine**	**NS**	**-2.01**	**NS**	**NS**
IL8	**Cytokine/Chemokine**	**NS**	**-2.29**	**-2.14**	**NS**
IL11	Cytokine/Chemokine	NS	-3.08	NS	NS
IRF1	Cytokine/Chemokine	NS	2.08	NS	NS
IRF2	Cytokine/Chemokine	NS	NS	NS	-2.59
MIP1B	**Cytokine/Chemokine**	**-2.03**	**-2.38**	**-2.17**	**NS**
MCP3	**Cytokine/Chemokine**	**-2.18**	**NS**	**NS**	**-2.49**
SP2	**Transcription Factor**	**-2.12**	**-2.20**	**-2.28**	**NS**
SP3	**Transcription Factor**	**-3.09**	**-3.76**	**-2.91**	**-2.33**
STAT5B	Transcription Factor	-2.84	NS	NS	NS
TCF3	Transcription Factor	NS	-2.29	NS	-2.92
TCF7	Transcription Factor	NS	-2.12	NS	NS
TCF8	Transcription Factor	NS	-3.16	-3.06	NS

NS = Not significantly changed

* An alphabetical listing of all gene symbols and descriptions used in this thesis can be found in Appendix B.

5.3.8 Unstimulated gene expression differences

Since exposure to HIV-1, and thus resistance to infection, likely occurs at a variety of different time points and under various degrees of immune stimulation, it is important to understand how CD4+ T cells of HIV-R women are functioning at baseline as well as in response to antigenic stimulation. To address this issue, gene expression values of CD4+ T cells of the HIV-R individuals at baseline (media alone) were compared to those of the HIV-LRN population. As above, unstimulated gene expression values were averaged for each gene in the resistant population and compared to averaged values in the HIV-LRN population. Fold changes were calculated by comparing sample class mean intensity values (HIV-R divided by HIV-LRN) and were considered to be differentially expressed if, after comparison, they had a p value of less than 0.05.

5.3.8.1 HIV-R women show higher expression of specific genes implicated in blocking HIV-1 replication

List of genes differentially expressed between HIV-R and HIV-LRN individuals were analysed in order to identify both individual genes and functional categories of genes that may play a role in resistance to HIV-1 infection. Table 16 outlines genes selected from that list of genes expressed at a higher level by HIV-R women compared to HIV-LRN. Notably, both MIP-1β and SP3 are expressed at higher levels in the HIV-R population (3.56 fold, p=0.0004 and 2.71 fold, p=0.0067 respectively) at baseline. As these transcripts were also differentially regulated after antigenic stimulation, a possible role for these genes in mediating HIV-1 resistance is suggested. Furthermore, a higher level of

Table 16: Selected genes expressed at a higher level in HIV-R vs HIV-LRN individuals at baseline

Symbol	Fold	p Value	Function	Description
AATK	5.00	<0.0001	Apoptosis	apoptosis-associated tyrosine kinase
CASP10	1.99	0.047	Apoptosis	caspase 10
SRC	2.90	0.0037	Cell cycle regulation	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
HRB	3.11	0.0019	Protein transport	HIV-1 Rev binding protein
MAP3K1	2.86	0.0043	Signal transduction	.mitogen-activated protein kinase kinase kinase 1
MAP3K5	2.66	0.0079	Signal transduction	mitogen-activated protein kinase kinase kinase 5
TRAF2	2.24	0.025	Signal transduction	TNF receptor-associated factor 2
MAPK10	2.14	0.032	Signal transduction	mitogen-activating protein kinase 10
MAP3K8	2.09	0.036	Signal transduction	mitogen-activated protein kinase kinase kinase 1
TCF8	5.60	<0.0001	Transcription factor	transcription factor 8
STAT5B	3.92	<0.0001	Transcription factor	signal transducer and activator of transcription 5b
SP3	**3.56**	**0.0004**	**Transcription factor**	**Sp3 transcription factor**
TCF3	2.40	0.016	Transcription factor	transcription factor 3
SP2	2.28	0.022	Transcription factor	Sp2 transcription factor
TCF7	2.04	0.042	Transcription factor	transcription factor 7
RBBP1	3.19	0.0014	Transcription inhibition	retinoblastoma-binding protein 1
CD59	3.92	<0.0001	Complement	CD59 molecule, complement regulatory protein
IFNGR1	6.94	<0.0001	Cytokine/Chemokine/Receptor	interferon gamma receptor 1
IL1R1	3.03	0.0024	Cytokine/Chemokine/Receptor	interleukin 1 receptor 1
IL11	2.78	0.0055	Cytokine/Chemokine/Receptor	interleukin 11
MIP1B	**2.71**	**0.0067**	**Cytokine/Chemokine/Receptor**	**macrophage inflammatory protein-1beta**
IL10	2.69	0.0071	Cytokine/Chemokine/Receptor	interleukin 10
MCP3	2.19	0.028	Cytokine/Chemokine/Receptor	monocyte chemoattractant protein 3
IL1R2	2.13	0.033	Cytokine/Chemokine/Receptor	interleukin 1 receptor 2
LIFR	1.99	0.047	Cytokine/Chemokine/Receptor	leukemia inhibitory factor receptor
DEFA6	**4.65**	**<0.0001**	**Innate immunity**	**defensin, alpha 6, paneth cell-specific**

expression of defensin alpha 6 (DEFA6, 4.65 fold, p<0.0001) is also of interest as

defensins have previously been shown to limit HIV-1 replication[**188**].

5.3.8.2 HIV-R women show lower expression of genes involved in cell cycle regulation,

cytokine signaling and T cell activation

Analysis of genes expressed at a lower level by HIV-R compared to HIV-LRN

individuals, showed a large number of genes involved in T cell activation, signal

transduction and cell division (Table 17). In particular, lower expression of CD69 (-3.90

fold, p<0.0001), STAT1 (-3.65 fold, p=0.0003), SHC1 (-6.03 fold, p<0.0001), RAF1 (-

2.05 fold, p=0.04), JUN (-2.45 fold, p=0.014) and IL-2 (-2.72 fold, p=0.0065) are

indicative of lower levels of T cell activation and signaling in the HIV-R population.

5.3.8.3 Pathway analysis reveals lower baseline expression of several genes involved in T

cell activation and cytokine signaling in HIV-R individuals

Although describing individual genes that function differentially between HIV-R and

HIV-LRN individuals is obviously important, possibly more important is the discovery of

several differentially regulated genes that act in the same biological pathway. In order to

gain further insight into pathways that may play a role in mediating resistance to HIV-1

infection, lists of genes expressed either significantly higher or lower in the HIV-R

population compared to the HIV-LRN population were imported into the Bioresource for

array genes software (BioRag, www.biorag.org). For this analysis genes are grouped

together based on known functional interactions using gene accession numbers. This

analysis

Table 17: Selected genes expressed at a lower level at baseline in HIV-R vs HIV-LRN individuals

Symbol	Fold	p Value	Function	Description
CD69	**-3.90**	**<0.0001**	**Activation**	**CD69 antigen (p60, early T-cell activation antigen)**
CDC2	-3.10	0.0019	Cell cycle regulation	Cell division cycle 2
CDK8	-2.44	0.015	Cell cycle regulation	Cyclin-dependent kinase 8
CDKN3	-2.16	0.031	Cell cycle regulation	cyclin-dependent kinase inhibitor 3
CDKN1A	-2.10	0.036	Cell cycle regulation	Cyclin-dependent kinase inhibitor 1A
CDK9	-2.01	0.044	Cell cycle regulation	Cyclin-dependent kinase 9
SHC	**-6.03**	**<0.0001**	**Signal transduction**	**SHC (Src homology 2 domain containing) transforming protein 1**
STAT1	**-3.65**	**0.0003**	**Signal transduction**	**Signal transducer and activator of transcription 1**
MAP3K8	-3.65	0.0003	Signal transduction	Mitogen-activated protein kinase kinase kinase 1
MAPK1	-2.93	0.0034	Signal transduction	Mitogen-activating protein kinase 1
MAPK3	-2.58	0.010	Signal transduction	Mitogen-activating protein kinase 3
TRAF3	-2.45	0.014	Signal transduction	TNF receptor-associated factor 3
MAPK7	-2.24	0.025	Signal transduction	Mitogen-activated protein kinase 7
MAP3K14	-2.13	0.034	Signal transduction	Mitogen-activated protein kinase kinase kinase 14
RAB3A	-2.11	0.035	Signal transduction	RAB3A, member RAS oncogene family
TRAF4	-2.09	0.037	Signal transduction	TNF receptor-associated factor 4
RAF1	**-2.05**	**0.040**	**Signal transduction**	**v-raf-1 murine leukemia viral oncogene homolog 1**
MAP3K7	-2.03	0.043	Signal transduction	Mitogen-activated protein kinase kinase kinase 7
E2F5	-2.49	0.013	Transcription factor	E2F transcription factor 5
JUN	**-2.45**	**0.014**	**Transcription factor**	**v-jun avian sarcoma virus 17 oncogene homolog**
AP2S1	-2.44	0.015	Transcription factor	Adaptor-related protein complex 2, sigma 1 subunit
ATF3	-2.28	0.023	Transcription factor	activating transcription factor 3
ATF5	-2.27	0.023	Transcription factor	activating transcription factor 5
IL2	**-2.72**	**0.0065**	**Cytokine/Chemokine**	**interleukin 2**
IL10RB	-2.66	0.0079	Cytokine/Chemokine	interleukin 10 receptor, beta
IL16	-2.38	0.017	Cytokine/Chemokine	interleukin 16 (lymphocyte chemoattractant factor)
IL1B	-2.16	0.031	Cytokine/Chemokine	interleukin 1, beta
CXCL11	-2.15	0.031	Cytokine/Chemokine	Chemokine (C-X-C) ligand 11
IFNGR2	-2.02	0.044	Cytokine/Chemokine	Interferon gamma receptor 2
IRF6	-3.85	<0.0001	Innate immunity	interferon regulatory factor 6
IRF1	-2.76	0.0058	Innate immunity	interferon regulatory factor 1

revealed that three signaling molecules expressed at lower levels by HIV-R women, specifically SHC-1, RAF-1 and JUN, make up a portion of both the T cell receptor signaling pathway and the gp130 signaling pathway involved in signaling by several cytokines including IL-2 (Figure 8).

5.3.9 HIV-R women show lower production of proinflammatory cytokines ex vivo

To determine whether the noted lower levels of genes involved in T cell receptor and cytokine signaling were reflected in the levels of soluble markers, Cytometric Bead Array assays (BD Biosciences, Mississauga Ontario) were employed to measure the levels of secreted cytokines. Matched supernatants from unstimulated PBMC cultures from HIV-R (N=5) and HIV-LRN (N=4, insufficient supernatant was collected from one individual in this group) women were assayed for levels of IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IP-10, MCP-1, MIG, RANTES and TNF. Levels of certain cytokines in all patients were either above (IL-8, MCP-1) or below (IFNγ, IL-2, IL-4, IL-5, IL-12p70, MIG) the limit of detection (data not shown). HIV-R women showed significantly lower levels of secretion of IL-6 (p=0.001), IL-1β (p=0.015) and TNF (p=0.015) and a trend towards lower secretion of IL-10 (p=0.064). No differences were seen in the expression levels of IP-10 and RANTES (Figure 9).

5.3.10 Summary

Initial microarray analysis of gene expression differences between HIV-R and HIV- LRN individuals, both at baseline and in response to stimulation, show specific genes and pathways associating with the HIV-R phenotype. Based on individual gene expression changes after stimulation, cluster analysis showed an inducible pattern of gene expression

a)
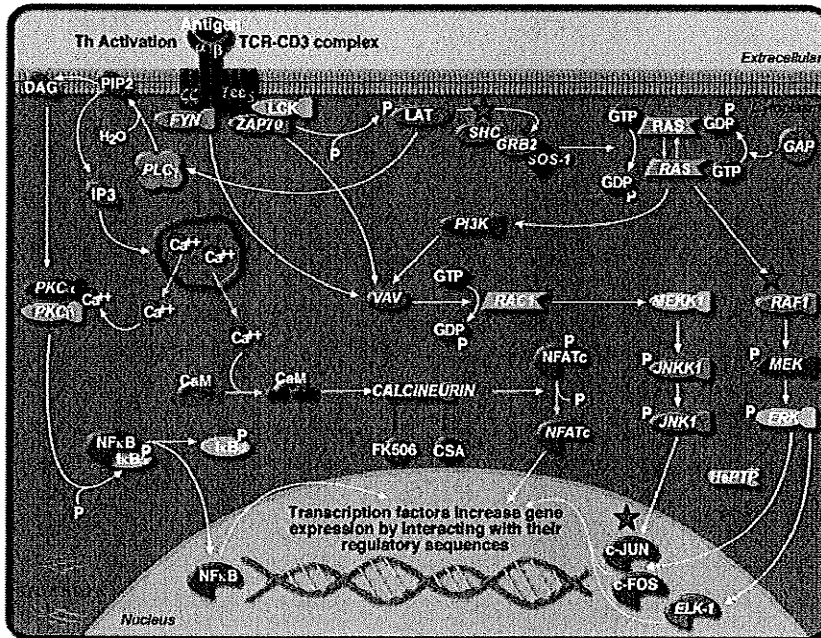
b)
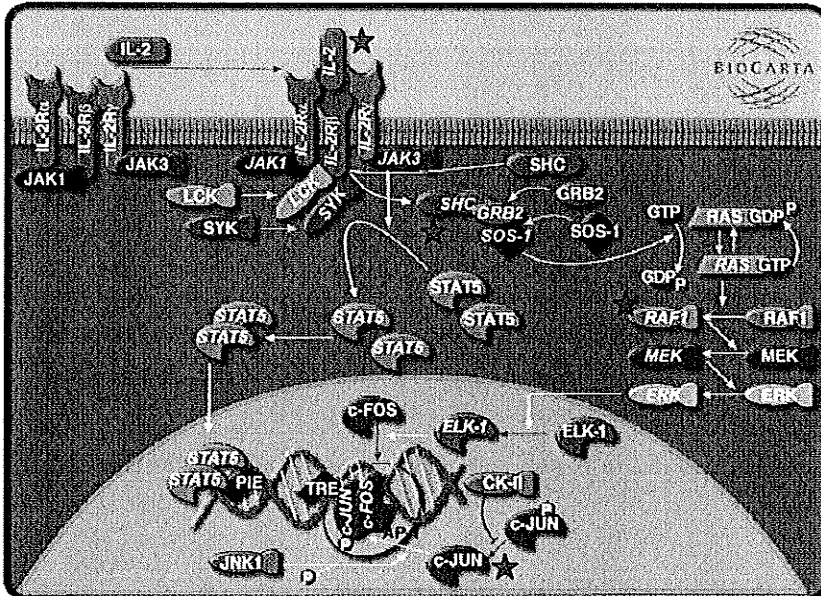
Figure 8: Genes expressed at a lower level by HIV-R women are involved in T cell receptor activation and cytokine signaling pathways. The signal transduction molecules SHC1 (Src homology 2 domain-containing transforming protein 1), RAF1 (v-raf-1 murine leukemia viral oncogene homolog 1) and c-Jun (jun oncogene) are all involved in both T cell activation (a) and cytokine signaling, an example pathway for interleukin 2 is shown (b). The location of these molecules in the respective pathways are marked by green stars. Diagrams are reproduced from www.BioCarta.com (permission obtained March 2007).
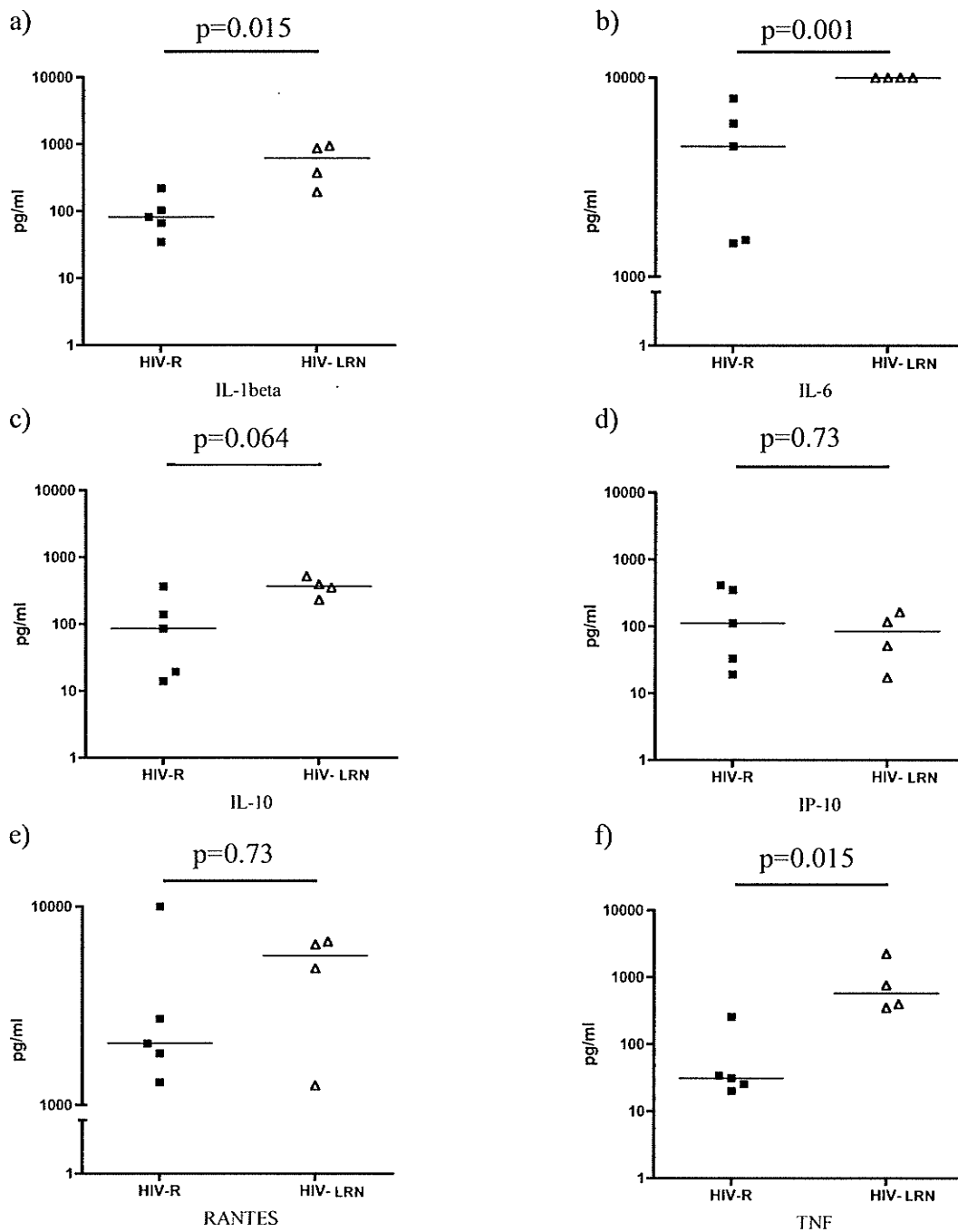
Figure 9: Unstimulated cytokine expression in cell culture supernatants of HIV-1 resistant (HIV-R) and HIV-1 low-risk negative (HIV-LRN) individuals. Expression levels (pg/ml) of (a) IL-1β (p=0.015), (b) IL-6 (p=0.001), (c) IL-10 (p=0.064), (d) IP-10 (p=0.73), (e) RANTES (p=0.73) and (f) TNF (p=0.015) by unstimulated PBMC of HIV-R (black squares, N=5) and HIV- (open triangles, N=4). Each point indicates a single individual, bars represent median expression values. p Values were calculated by Mann-Whitney U test. NS = not significant.

associated with the HIV-R phenotype after both *C. albicans* and flu peptide stimulation, suggesting that a specific set of genes can be determined that are associated with HIV-1 resistance. Analysis of genes shown to be significantly changed after stimulation showed specific transcription factors and chemokines were up-regulated after stimulation with several antigens. Unstimulated gene expression differences between HIV-R and HIV-LRN women showed a specific set of genes differentially expressed between the two populations. Genes expressed at a higher level in the HIV-R population included SP3, previously shown to suppress transcription of the HIV-1 LTR and MIP-1β, shown to block HIV-1 interaction with the CCR5 co-receptor. These genes were also differentially regulated after stimulation in the HIV-R population but not the HIV-LRN individuals, suggesting possible sites for genetic differences between the poulations. Gene and pathway analysis showed a group of genes involved in T cell activation and cytokine signaling to be expressed at a lower level in HIV-R women. The finding of lower levels of secreted proinflammatory cytokines in HIV-R women supports the gene expression data and indicates the possibility of lower levels of immune activation in the HIV-R population.

Overall these data support the hypotheses that A) HIV-R individuals have a distinct pattern of gene expression, B) antigenic stimulation impacts specific families of genes in HIV-R individuals and C) that baseline gene expression differs between the two study populations. Interestingly, a possible role for both higher expression of genes known to suppress the HIV-1 life cycle and a lower expression of genes involved in T cell activation in the HIV-R women is demonstrated at baseline. Expansion of the dataset to include more individuals would strengthen these findings.

5.4 HIV-1 Resistant Commercial Sex-workers show lower Expression of Genes Involved in Immune Activation and Soluble Immune Mediators Ex *Vivo* Compared to HIV-1 Low-risk Negative Individuals

5.4.1 Rationale

Analysis of an initially small group of HIV-R and HIV-LRN individuals showed a relative increased expression of genes known to inhibit steps of the HIV-1 life cycle and decreased expression of genes involved in T cell receptor and cytokine signaling in CD4+ T cells in the HIV-R population. This was mirrored by lower production of proinflammatory cytokines *ex vivo*. Expansion of this sample set will improve statistical confidence and allow for higher order analyses of gene interactions and pathways involved in mediating resistance to infection by HIV-1.

5.4.2 Hypotheses

- Previously observed stimulation-induced patterns of gene expression seen by cluster analysis in a small group of HIV-R women will be confirmed in a larger study.

- Unstimulated levels of gene expression in HIV-R women will show evidence of higher relative expression of genes known to inhibit steps in the HIV-1 life cycle and lower expression of genes involved in T cell activation.

- HIV-R women will show lower levels of secretion of proinflammatory cytokines both at baseline and after stimulation.

- Single gene and gene interaction analyses will allow for discrimination of individuals into phenotypic categories based on HIV susceptibility.

5.4.3 Objectives

- Determine genes differentially expressed after stimulation and at baseline in a larger sample of HIV-R and HIV-LRN women.

- Perform cluster analysis based on antigen-stimulated gene expression values to determine if clustering of HIV-R samples can be observed.

- Examine lists of differentially expressed genes for evidence of increased expression of genes implicated in blocking HIV-1 replication and decreased expression of genes involved in activation.

- Correlate gene expression changes with production of cytokines *ex vivo*.

5.4.4 Study Outline

Peripheral blood mononuclear cells were collected and handled as in section 5.3. A total of 17 HIV-R (mean age 42.7) and 10 HIV-LRN (mean age 32.6) women were included in this expanded study. Baseline characteristics for all women can be found in Table 18. For this larger study, stimulation, cell separation and microarray analysis was performed in the same manner as the pilot study (section 5.3). Cluster, fold change and predictive interaction analyses were carried out to determine genes and gene interactions that characterize HIV-1 resistant individuals. Cytokine levels were measured in cell culture supernatants to confirm previous results.

5.4.5 Cluster analysis of the larger sample set does not show a pattern of gene expression associated with HIV-1 resistance following stimulation

In the previous section it was shown that HIV-R individuals clustered together based on gene expression changes after stimulation with both *C. albicans* and flu peptide,

Table 18: Enrollment characteristics of HIV-R and HIV-LRN study subjects

Study Number	HIV Status	Years of Enrollment	CD4	CD8
ML 466	Resistant	17	1341	817
ML 556	Resistant	17	1247	352
ML 767	Resistant	15	1175	569
ML 881	Resistant	15	1265	426
ML 887	Resistant	15	1230	676
ML 893	Resistant	15	1298	1321
ML 1072	Resistant	13	503	820
ML 1260	Resistant	12	997	1766
ML 1430	Resistant	11	1259	399
ML 1482	Resistant	10	1064	585
ML 1498	Resistant	10	1137*	773*
ML 1643	Resistant	9	1256	613
ML 1796	Resistant	7	902	502
ML 1803	Resistant	7	633	444
ML 1952	Resistant	3	929	483
ML 1960	Resistant	3	1002	883
ML 1997	Resistant	3	1510	634
MCH 102749	Negative	15	857*	417*
MCH 105452	Negative	14	1301	543
MCH 107058	Negative	13	1144	392
MCH 111757	Negative	10	997*	628*
MCH 112499	Negative	9	694	705
MCH 114277	Negative	7	890	633
MCH 115150	Negative	6	967	254
MCH 116641	Negative	3	1310	985
MCH 117066	Negative	3	804	575
MCH 117436	Negative	2	1071	529

*CD4/CD8 counts were not available for these individuals at the time of enrollment in this study. The reported values were obtained at the earliest possible subsequent visit.

suggesting that CD4+ T cells of HIV-R individuals behave differently from HIV-LRN individuals after stimulation with non-HIV antigens. We sought to extend this observation on a larger dataset (see Table 18). As previously, complete linkage hierarchical clustering was performed on gene lists containing all genes, filtered to remove genes present in less than 80% of all samples (Figure 10) or on lists including only those genes showing a twofold change (either up or down) in at least one individual (Figure 11). Clustering was performed on both genes and arrays as outlined in section 5.3.5.

Cluster analysis based on gene expression after stimulation showed limited clustering of HIV-R individuals for both the complete list of genes and gene lists including only genes showing a twofold change in at least one individual. Although in almost all stimulation conditions clusters containing either only HIV-R or a high proportion of HIV-R individuals exist (black boxes in Figures 10 and 11), no clear pattern seemed to distinguish between the two populations in every case using this technique. This suggests that a more focused approach may be necessary to determine a subset of genes that may better classify HIV-R individuals.

5.4.6 Data analysis of combined datasets

During analysis of array data acquired earlier in this study (Section 5.3) in combination with newly acquired data (an additional 12 HIV-R and 5 HIV-LRN individuals) it was necessary to correct for batch effect. This is a common concern when dealing with microarray data acquired at different time points and was corrected for using Partek (Partek Inc., St. Louis Missouri) software's batch effect removal algorithm. During this

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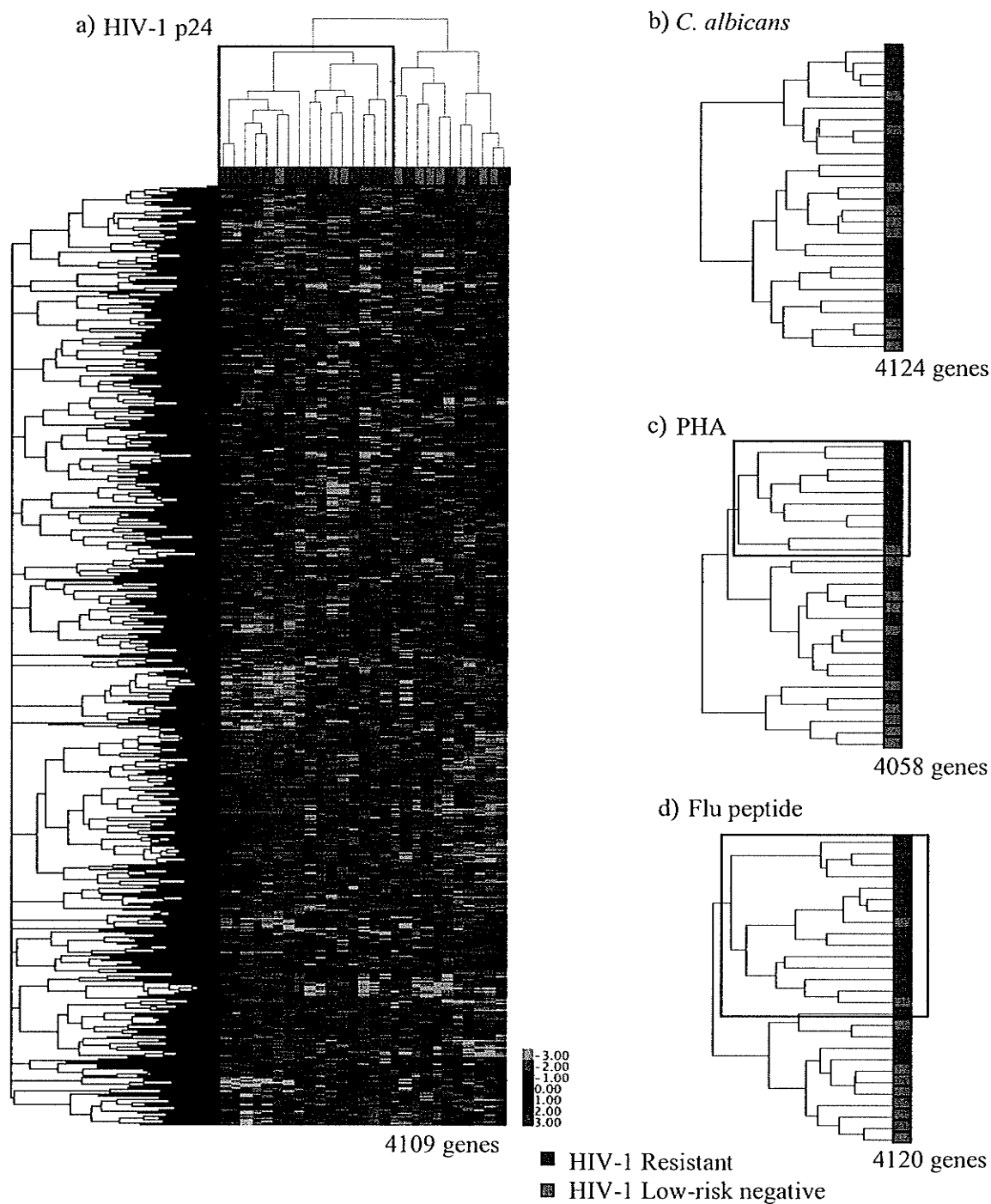
Figure 10: Complete linkage hierarchical clustering of all genes after antigenic stimulation. Heat map and dendogram of genes and arrays after HIV-1 p24 stimulation (a), and dendograms of arrays only after *C. albicans* (b), PHA (c) and flu peptide (d) stimulation show only limited clustering of HIV-R individuals. Black boxes (a, c and d only) indicate nodes containing high proportions of HIV-R individuals. Numbers of genes on which clustering of arrays is based is given below. Blue squares represent HIV-1 resistant individuals, red squares represent HIV-1 low-risk negatives.

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a) HIV-1 p24

b) *C. albicans*

1892 genes

c) PHA

1827 genes

d) Flu peptide

```
-3.00
-2.00
-1.00
0.00
1.00
2.00
3.00
```

2023 genes ▣ HIV-1 Resistant 1813 genes
▣ HIV-1 Low-risk negative
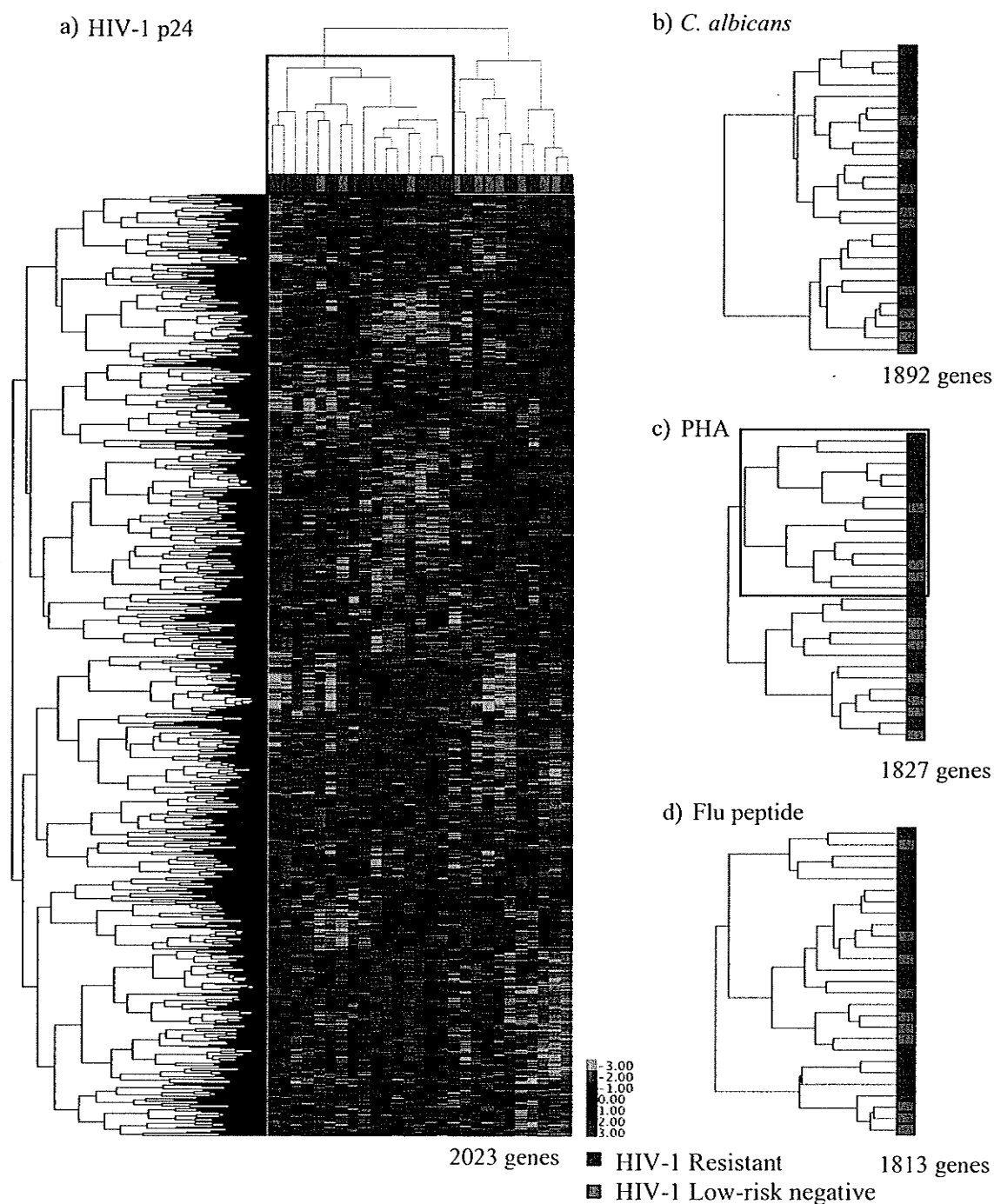
Figure 11: Complete linkage hierarchical clustering based on expression of genes showing a twofold or greater change after antigenic stimulation. Heat map and dendogram showing assortment of genes and arrays after HIV-1 p24 stimulation (a), and dendograms of arrays only after *C. albicans* (b), PHA (c) and flu peptide (d) stimulation show only limited clustering of HIV-R individuals. Black boxes (a and c only) indicate nodes containing high proportions of HIV-R individuals. Numbers of genes on which clustering of arrays is based is given below. Blue squares represent HIV-1 resistant individuals, red squares represent HIV-1 low-risk negatives.

process, artifactual, platform-specific background signal originating from the nylon array platform was identified. As this background signal could interfere with detection of gene expression changes it was necessary to develop custom normalizations methods for each individual array in the study to remove this effect. This method, developed by Biosystemix Ltd. (Sydenham Ont.), involved empirical mode decomposition analysis (EMD)[316] of each individual array followed by calculation of a moving average of the non-biological variation across all genes on a given array to generate a correction function specific to that array. The so-called correction function was then subtracted from the original data, resulting in a dataset corrected for the platform-specific background signal.

To ensure that this process did not, in itself, introduce artificial gene expression changes, genes were considered to be differentially expressed between populations if they achieved significance (p<0.05) when comparisons were made both before and after application of the correction function. P values were calculated for both datasets by ANOVA, fold changes were determined by comparing gene expression averages between classes.

5.4.7 Sources of variation analysis demonstrates large biological variation between groups

After correction for batch effect, a two-way ANOVA was run over all genes on all arrays (i.e. inclusive of all stimulation conditions) to determine which biological variables contributed to the gene expression differences seen between the HIV-R and HIV-LRN populations, and to what degree. All biological and systematic variables were assigned to

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each array and a sources of variation analysis was carried out in Partek software (Partek Inc., St. Louis Missouri). The average mean square for each variable in the ANOVA model denotes the contribution of each variable to the variance within the model. Average mean squares were then plotted to visualize the contribution of both biological and systematic variables (Figure 12). Most of the variation occurred due to two biological factors, patient identification (i.e. any given individual varies from any other individual) and HIV-1 status (i.e. HIV-R vs HIV-LRN). This suggests that beyond individual variation, a significant amount of variation in gene expression is class specific, implying a group of genes exists that can be used to classify HIV-R individuals. Interestingly, stimulation factor showed a much lower contribution to variation than was expected. It is possible that this is due to the fact that the differences in gene expression seen between groups are not dependant on stimulation.

5.4.8 Correlations of gene expression

Meaningful visualization of entire microarray datasets can be a significant challenge. One method by which to compare groups of arrays, in this case either between phenotypic groups or between stimulation conditions is to calculate correlation values between mean expression levels of all genes over all arrays. This allows for easy visualization of the amount of similarity of any given comparison.

Sources of Variation
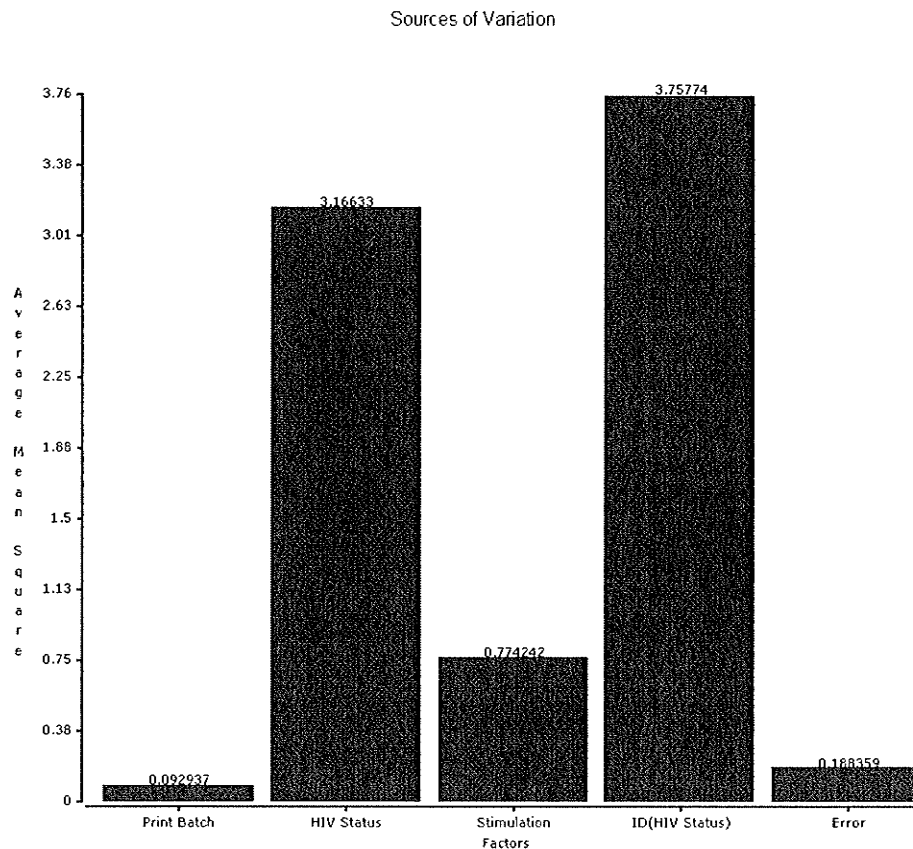
Figure 12: Source of variation analysis shows the majority of variation occurs due to individual variation (ID) and HIV status. A two-way ANOVA was performed using Partek software (Partek Inc., St. Louis, Missouri) to determine which biological variables contributed to the overall gene expression differences observed. The average mean square for each variable in the ANOVA model denotes the contribution of each variable to the variance within the model. The error term denotes the variation not accounted for in the model. Most of the variation occurred due to patient ID (i.e. a single given individual) or HIV status (HIV-R vs HIV-LRN). Print batch is shown as an example of systematic variation after batch effect correction.

In order to visualize the impact of each stimulation condition, as well as the similarity or difference between the HIV-R and HIV-LRN groups, correlation of mean expression values were calculated between stimulation conditions within a particular group (e.g. HIV-R media gene expression vs HIV-R PHA gene expression, HIV-LRN *C. albicans* gene expression vs HIV-LRN p24 gene expression etc.) and between groups (e.g. HIV-R PHA vs HIV-LRN PHA etc.) seen in Figures 13 and 14 respectively.

As seen in Figure 13, the PHA stimulated condition is generally the least similar stimulation condition showing the lowest correlation value in almost all comparisons (pink boxes in Figure 13). This is expected as the mitogen PHA is the strongest stimulation condition used relative to the others. As well, the HIV-1 p24 stimulation condition has a larger effect on the HIV-R population as compared to the HIV-LRN populations (r=0.40 vs r=0.47) possibly due to the HIV-R individuals mounting a recall response to this protein, as has been demonstrated in previous studies in our group[268].

Furthermore, comparisons between groups (Figure 14) show the lowest correlation values between the PHA stimulation condition and all other stimulation conditions. As PHA is a mitogen it is possible that this low correlation between the HIV-R and HIV-LRN groups is indicative of differential regulation of gene expression in CD4+ T cells in instances of heightened immune activation. Interestingly the relatively low correlation (r=0.44) between the unstimulated (media alone) conditions suggests a subset of genes may be differentially expressed between HIV-R and HIV-LRN individuals in the absence of

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a)

HIV-LRN

	Media	PHA	C. albicans	Flu peptide	Hiv p24
Media		0.44	0.48	0.49	0.47
PHA			0.27	0.37	0.29
C. albicans				0.46	0.51
Flu peptide					0.45
Hiv p24					

(row labels under "HIV-LRN")

b)

HIV-R

	Media	PHA	C. albicans	Flu peptide	Hiv p24
Media		0.41	0.54	0.41	0.40
PHA			0.34	0.43	0.23
C. albicans				0.53	0.53
Flu peptide					0.44
Hiv p24					

(row labels under "HIV-R")

Figure 13: Correlation matrix of means of gene expression data across all stimulation conditions within groups. Correlation values were calculated comparing all stimulation conditions within both the HIV-LRN (a) and HIV-R (b) groups. Red boxes indicate strong correlation values (>/= 0.40), pink boxes indicate weak positive correlations (</= 0.40).

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	HIV-LRN				
	Media	PHA	*C. albicans*	Flu peptide	Hiv p24
Media	0.44	0.36	0.41	0.46	0.47
PHA	0.35	0.35	0.24	0.31	0.28
C. albicans	0.52	0.37	0.52	0.57	0.47
Flu peptide	0.52	0.39	0.35	0.48	0.39
Hiv p24	0.51	0.34	0.46	0.45	0.42

(Row group label: HIV-R)

Figure 14: Correlation matrix of means of gene expression data across all stimulation conditions between groups. Correlation values were calculated comparing all stimulation conditions between the HIV-LRN and HIV-R groups. Red boxes indicate strong correlation values ($>/= 0.40$), pink boxes indicate weak positive correlations ($</= 0.40$).

stimulation. Determining the various genes that contribute to the differences in each individual stimulation condition between groups will be essential to defining the HIV resistant phenotype.

5.4.9 Significant expression analysis of combined dataset

5.4.9.1 Antigen-stimulated gene expression differences

As was done previously (Section 5.3.7), gene expression levels after antigenic stimulation were compared between the HIV-R and HIV-LRN groups. In comparison to the previous analysis, the larger sample set resulted in a smaller number of genes that were significantly different between groups in each stimulation condition (Table 19). Interestingly, the HIV-1 p24 stimulation condition showed the largest number of genes differentially expressed between the two groups, likely demonstrating response to previous exposure to HIV-1 in the HIV-R group.

Analysis of lists of genes shown to be differentially expressed after antigenic stimulation showed very little similarity between antigen conditions (Table 20). Genes shown to be affected similarly in several stimulation conditions previously (Table 15) did not remain significant once the larger dataset was analysed. However, the HIV-1 p24 stimulation condition showed that HIV-R women had significantly lower expression of the T cell receptor (TCR) alpha and delta chains as well as higher expression of the transcription factor SP3. As mentioned previously (Section 5.3.7.1) SP3 has been shown to have an inhibitory effect on the early stages of HIV-1 replication[325]. As well, the lower levels of expression of the TCR alpha chain may be evidence of a lower capacity for activation

Table 19: Summary of numbers of genes significantly differentially expressed in HIV-R vs HIV- individuals after antigenic stimulation

Stimulation	Genes Up	Genes Down	Total
PHA	12	21	33
C. albicans	32	22	54
Flu peptide	11	22	33
HIV-1 p24	67	28	95

Table 20: Selected genes shown to be differentially expressed in HIV-R vs HIV-LRN individuals after antigenic stimulation

Stimulation	Symbol	Fold	p Value	Description
PHA	TFAP4	-1.09	0.022	Transcription factor AP-4
	MAP2K3	-1.07	0.0065	Mitogen-activated protein kinase kinase 3
	E2F4	-1.06	0.045	E2F transcription factor 4
C. albicans	RAF1	1.45	0.042	Murine leukemia viral oncogene homolog 1
	PTPRC	1.43	0.0006	Protein tyrosine phosphatase, receptor type C
	TEAD1	1.14	0.033	TEA domain family member 1
	MAP2K4	1.12	0.037	Mitogen-activated protein kinase kinase 4
	HLA-C	1.10	0.027	Major histocompatibility complex, class I, C
	CLK1	1.10	0.033	CDC-like kinase 1
	STAT2	1.09	0.025	Signal transducer and activator of transcription 2
	CCR6	1.07	0.030	Chemokine (C-C motif) receptor 6
	IGFBP2	1.05	0.045	Insulin-like growth factor binding protein 2
	MAP4K2	-1.63	0.006	Mitogen-activating protein kinase kinase kinase kinase 2
	BTF3	-1.06	0.007	Basic transcription factor 3
Flu peptide	TRD	1.495	0.045	T cell receptor delta locus
	TNF	1.111	0.0061	Tumor necrosis factor
	NFATC4	1.098	0.026	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4
	CCR5	-1.061	0.015	Chemokine (C-C motif) receptor 5
	E2F4	-1.043	0.009	E2F transcription factor 4
	BTF3	-1.041	0.039	Basic transcription factor 3
HIV-1 p24	ERF	1.14	0.0071	Ets2 repressor factor
	NFKBIE	1.12	0.043	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon
	GTF2H2	1.11	0.0048	General transcription factor IIH polypeptide II
	MAP3K1	1.10	0.010	Mitogen-activated protein kinase kinase kinase 1
	IL5	1.10	0.007	Interleukin 5
	SP3	**1.09**	**0.0004**	**Sp3 transcription factor**
	SH3BP2	1.07	0.009	SH3-domain binding protein 2
	RAB6A	1.06	0.015	RAB6A, member RAS oncogene family
	RAP1A	1.05	0.039	RAP1A, member of RAS oncogene family
	IGFBP6	-1.08	0.028	Insulin-like growth factor binding protein 6
	TRA@	-1.12	0.015	T cell receptor alpha
	FGFR3	-1.21	0.045	Fibroblast growth factor receptor 3
	TRD@	-1.82	0.0009	T cell receptor delta

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in the HIV-R population. As this effect seems to be HIV-specific, it is possible that HIV-R individuals respond in a specific manner to HIV-1, resulting in reduced susceptibility. However, it is also possible that this is an artifact of previous exposure to HIV-1.

5.4.9.2 Unstimulated gene expression differences

As mentioned previously (Section 5.3.8) likely the most important comparison, in terms of defining genes involved in HIV-1 resistance, is of unstimulated (baseline) gene expression levels between HIV-R and HIV-LRN individuals. Thus, this comparison was also run on the larger dataset. Selection for genes that showed a p value of <0.05 resulted in a list of 48 genes shown to be differentially expressed in the HIV-R population (29 expressed at a higher level, 19 lower). Table 21 shows the list of all genes expressed at a higher level in the HIV-R population including several expressed sequence tags (ESTs), which are transcripts known to be expressed but whose function has yet to be determined. Interestingly, and in agreement with the earlier hypothesis of lower levels of activation in the HIV-R population, an inhibitor of cell growth CDKN2A and an inhibitor of NF-κB NFKBIE were expressed at a higher level in the HIV-R population.

Analysis of genes expressed at a lower level in HIV-R women also suggests lower levels of CD4+ T cell activation (Table 22). The genes PCNA, ADORA2B, AP2S1, MAPKAPK3 and the NF family transcription factor NFIC are all involved in the processes of cellular activation and transcription. It is also worth mentioning that the most highly significant gene expressed at a lower level in the HIV-R population, CAV1, has been shown to be involved in endocytic uptake and productive infection by HIV-1 in CD4 T cell lines[326]. Taken together these results strengthen the hypothesis that lower

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Table 21: Genes expressed at a higher level in unstimulated CD4+ T cells of HIV-R commercial sex-workers compared to HIV-LRN women

Symbol	Fold	p Value	Description
EST*	1.53	0.044	EST*
FLJ22004	1.50	0.031	Hypothetical protein FLJ22004
OSR1	1.41	0.030	Oxidative-stress responsive 1
LIFR	1.38	0.005	Leukemia inhibitory factor receptor
CDKN2A	**1.37**	**0.009**	**Cyclin-dependent kinase inhibitor 2A**
GRM3	1.34	0.008	Glutamate receptor metabotropic 3
EST*	1.25	0.023	EST*
EST*	1.21	0.037	EST*
EST*	1.18	0.031	EST*
GAL	1.17	0.021	Galanin
NFKBIE	**1.15**	**0.041**	**Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon**
BIRC1	1.14	0.001	Baculoviral IAP repeat-containing 1
TST	1.14	0.002	Thiosulfate sulfurtransferase
STAT2	1.12	0.005	Signal transducer and activator of transcription 2
EST*	1.12	0.005	EST*
EST*	1.11	0.013	EST*
EST*	1.10	0.035	EST*
PPP2R3	1.10	0.014	Protein phosphatase 2 (formerly 2A), regulatory subunit B"
BRCA1	1.09	0.031	Breast cancer 1
CCR6	1.09	0.001	Chemokine (C-C motif) receptor 6
PSTPIP1	1.08	0.049	Proline-serine-threonine phosphatase interacting protein 1
HSPA5	1.08	0.021	Heat shock 70kD protein 5
APAF1	1.07	0.041	Apoptotic protease activating factor
TXK	1.07	0.013	TXK tyrosine kinase
NDRG1	1.05	0.012	N-myc downstream regulated
HLA-DPB1	1.05	0.026	Major histocompatibility complex, class II, DP beta 1
FLJ13639	1.05	0.025	Hypothetical protein FLJ13639
VAMP4	1.05	0.019	Vesicle-associated membrane protein 4
EST*	1.05	0.037	EST*
HSPB3	1.05	0.038	Heat shock 23kD protein 3

*EST = Expressed Sequence Tag

Table 22: Genes expressed at a lower level in unstimulated CD4+ T cells of HIV-R commercial sex-workers compared to HIV- women

Symbol	Fold	p Value	Description
CAV1	**-1.76**	**0.015**	**Caveolin 1**
EST*	-1.45	0.032	EST*
ITPR3	-1.10	0.014	Inositol 1,4,5-triphosphate receptor, type 3
SEMA3B	-1.09	0.043	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B
FLJ11904	-1.09	0.035	Hypothetical protein FLJ11904
MMP7	-1.09	0.046	Matrix metalloproteinase 7 (matrilysin, uterine)
PCNA	**-1.09**	**0.021**	**Proliferating cell nuclear antigen**
NFIC	**-1.07**	**0.016**	**Nuclear factor I/C (CCAAT-binding transcription factor)**
ALDH1	-1.07	0.028	Aldehyde dehydrogenase 1
SELE	-1.07	0.023	Selectin E
USP9X	-1.07	0.007	Ubiquitin specific protease 9 X
ADORA2B	**-1.06**	**0.010**	**Adenosine A2b receptor**
AP2S1	**-1.06**	**0.029**	**Adaptor-related protein complex 2, sigma 1 subunit**
KCNMA1	-1.06	0.044	Calcium-activated potassium channel
GRIK1	-1.05	0.022	Glutamate receptor, ionotropic, kainate 1
SYNGR1	-1.05	0.022	Synaptogyrin 1
MYBP-H	-1.05	0.044	Myosin binding protein H mRNA
NTF3	-1.05	0.022	Neurotrophin 3
MAPKAPK3	**-1.05**	**0.019**	**Mitogen-activated protein kinase-activated protein kinase 3**

*EST = Expressed Sequence Tag

levels of activation of CD4+ T cells is associated with HIV-1 resistance and possibly demonstrates that the under-expression of a specific gene involved in the HIV-1 life cycle, such as CAV1, may contribute to this resistance.

5.4.10 Quantitative real-time PCR

In order to attempt to confirm gene expression differences between the HIV-R and HIV-LRN populations, gene-specific primers were designed for JUN (identified in initial analysis, Section 5.3.8), NFIC, BIRC1, CCR6, STAT2, NFKBIE, AP2S1, PSTPIP1, PCNA and CAV1 using the Qiagen GeneGlobe system. This system uses a proprietary algorithm to design primers optimized to work using the SYBR Green detection system. We chose to focus on genes that were differentially expressed in the unstimulated condition as these represent the most likely candidates for mediators of resistance to infection. Expression levels of each gene for 16 HIV-R and 9 HIV-LRN were measured using the SYBR Green detection method on the LightCycler (Roche Applied Sciences, Laval Quebec). Gene expression intensity for each gene in each individual was normalized to levels of 18s rRNA in that same sample and relative quantification was performed as described in Materials and Methods. Group comparisons were made using the Mann-Whitney U test, fold change and p values observed in both the microarray and qRT-PCR analyses are given in Table 23. Of the 9 genes selected for confirmation, CAV1 could not be reliably detected in the remaining RNA samples, likely due to low abundance of transcript and thus could not be analysed. Both the transcription factors JUN and NFIC showed significant differences between the HIV-R and HIV-LRN groups by qRT-PCR (p=0.014 and 0.029 respectively) and, as shown by array analysis both were expressed at a lower level in the HIV-R population. The remaining genes selected either

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Table 23: qPCR confirmation of gene expression values found to be significant by array analysis

Symbol*	Array		qPCR		
	Fold	p Value	Fold	p Value	Mean Cp**
NFKBIE	1.15	0.041	-2.91	0.013	32.27
JUN	-2.45	0.014	-1.53	0.014	24.57
NFIC	-1.07	0.016	-2.60	0.029	28.51
PSTPIP1	1.08	0.049	-1.68	0.15	28.52
AP2S1	-1.06	0.029	-1.24	0.47	34.40
STAT2	1.12	0.005	-1.63	0.61	28.48
BIRC1	1.14	0.001	-1.61	0.62	35.67
PCNA	-1.09	0.021	-1.08	0.93	30.76
CCR6	1.09	0.001	1.00	0.98	36.22

* An alphabetical listing of all gene symbols and descriptions used in this thesis can be found in Appendix B.

**Cp = Crossing point

did not achieve significance or, as in the case of NFKBIE, showed the opposite trend from that seen in array analysis. Interestingly, JUN and NFIC showed two of the highest raw expression levels in the qRT-PCR assay (as measured by mean crossing point or Cp) suggesting that the limited success in confirming the expression of the remaining genes may be in part due to the limited sensitivity of the assay.

5.4.11 Predictive interaction analysis

As mentioned perviously, gene expression differences in unstimulated CD4+ T cells between the HIV-R and HIV-LRN populations are of substantial interest. Therefore, we sought to determine if samples could be classified as either HIV-R or HIV-LRN based on gene expression data. It has been previously shown that analysis of either synergistic or competitive interaction between gene pairs, a process known as predictive interaction analysis (PIA), can improve predictive accuracy in human disease over single gene analysis[318]. As such, we sought to determine whether measuring gene pair interactions in the unstimulated condition could improve our ability to discriminate between the HIV-R and the HIV-LRN populations. This was performed by first calculating either a synergistic (u) or competitive (v) statistic (as outlined in Section 4.2.14) for every possible gene pair from the list of genes shown to be significantly differentially expressed between our study populations in at least one stimulation condition. As this type of modeling requires intense computer processing power and custom design it was carried out by Biosystemix Ltd (Sydenham Ontario). Following calculation of the gene pair interaction value a gene pair interaction p value is calculated using a standard t test assuming equal variance, in order to assess and rank the class separation performance of a particular gene pair. As this analysis dramatically increases the number of statistical

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tests being performed we set a stricter p value cutoff of p<0.001 in order for a particular interaction to be considered significant. This resulted in the determination of 38 gene pair interactions that we considered significant (Table 24). In all cases the interaction p value greatly exceeded the single gene p value for either member of the gene pair. In several cases, genes that were not seen to be differentially regulated in unstimulated CD4+ T cells of HIV-R women became significant when their interaction with a second gene was considered. Figure 15 demonstrates how PIA analysis improves class discrimination over single gene analysis with a particularly interesting gene pair. The individual expression of the adenosine A2b receptor (ADORA2B) or Proline-serine-threonine phosphatase interacting protein 1 (PSTPIP1), only partially discriminate between HIV-R and HIV-LRN individuals (Figure 15 a and b), whereas when their interaction is considered, the class discrimination is much improved (Figure 15c). This is reflected by the improvement in their statistical significance as the interaction p value, 0.00009 is more than two logs greater than either individual p value. This interaction is also particularly interesting from a functional perspective as ADORA2B, under-expressed in HIV-R, has been shown to be involved in cellular activation and induction of cytokine expression[327] whereas PSTPIP1, also known as CD2BP1, has been shown to be a negative regulator of T cell activation[328]. These data further support the hypothesis that lower T cell activation may play a role in mediating HIV-1 resistance.

5.4.12 Predictive interaction network analysis

Single gene, as well as two-gene interaction analysis is obviously an over-simplification of what is likely a complex multi-parameter trait. Thus, it is interesting to consider building larger networks of genes based on gene expression data. This can be

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Table 24: Unstimulated gene interactions that strongly classify HIV-R

Gene 1	Gene 2	G1 p Value	G2 p Vaule	Interaction p Value	Interaction type G1	G2
TST	TNFSF4	0.002	NS	0.000015	+	+
TST	ACVR2B	0.002	NS	0.000044	+	-
ADORA2B	PSTPIP1	0.01	0.05	0.000090	-	+
SEMA3B	ITGAL	0.04	NS	0.00016	-	+
PSTPIP1	FLJ13639	0.05	0.03	0.00017	+	+
MKLP1	CAV1	NS	0.02	0.00020	-	-
NFIC	NTF3	0.02	0.02	0.00024	-	-
ACVR2B	DDX1	NS	NS	0.00024	-	-
BIRC3	PSTPIP1	NS	0.05	0.00025	-	+
LIFR	MDM2	0.005	NS	0.00025	+	+
LIFR	GPSM3	0.005	NS	0.00026	+	+
LIFR	PTGER2	0.005	NS	0.00029	+	+
ARHGAP1	DDX1	NS	NS	0.00030	+	-
EPHA1	ITPR3	NS	0.01	0.00032	+	-
NRN1	ITPR3	NS	0.01	0.00035	+	-
NFIC	BIRC3	0.02	NS	0.00039	-	-
CD59	NFIC	NS	0.02	0.00040	-	-
GPSM3	PGY3	NS	NS	0.00042	+	+
EGF	AP2S1	NS	0.03	0.00043	+	-
SNAP23	PSTPIP1	NS	0.05	0.00044	+	+
NTF3	VAMP4	0.02	0.02	0.00050	-	+
CDKN2A	CAV1	0.009	0.02	0.00051	+	-
CDKN2A	OSR1	0.009	0.03	0.00061	+	+
SYNGR1	INPPL1	0.02	NS	0.00063	-	+
ARHGAP1	BIRC3	NS	NS	0.00063	+	-
PPP2R3	ACVR2B	0.002	NS	0.00071	+	-
VDAC3	ACVR2B	NS	NS	0.00076	-	-
NFIC	FLJ13639	0.02	0.03	0.00078	-	+
TUBA1	OSR1	NS	0.03	0.00079	-	+
ARHGAP1	IGFBP6	NS	NS	0.00083	+	-
PRKCL2	PSTPIP1	NS	0.05	0.00085	+	+
FLJ22004	CAV1	0.03	0.02	0.00086	+	-
ALDH1	NFIC	0.03	0.02	0.00087	-	-
PIN1	OSR1	NS	0.03	0.00089	-	+
NRN1	NTF3	NS	0.02	0.00091	+	-
PTGER2	PGY3	NS	NS	0.00095	+	+
NFIC	DDX1	0.02	NS	0.00095	-	-
IGFBP2	TRAF4	NS	NS	0.001	-	-

Note: NS=Not Significant

* An alphabetical listing of all gene symbols and descriptions used in this thesis can be found in Appendix B.
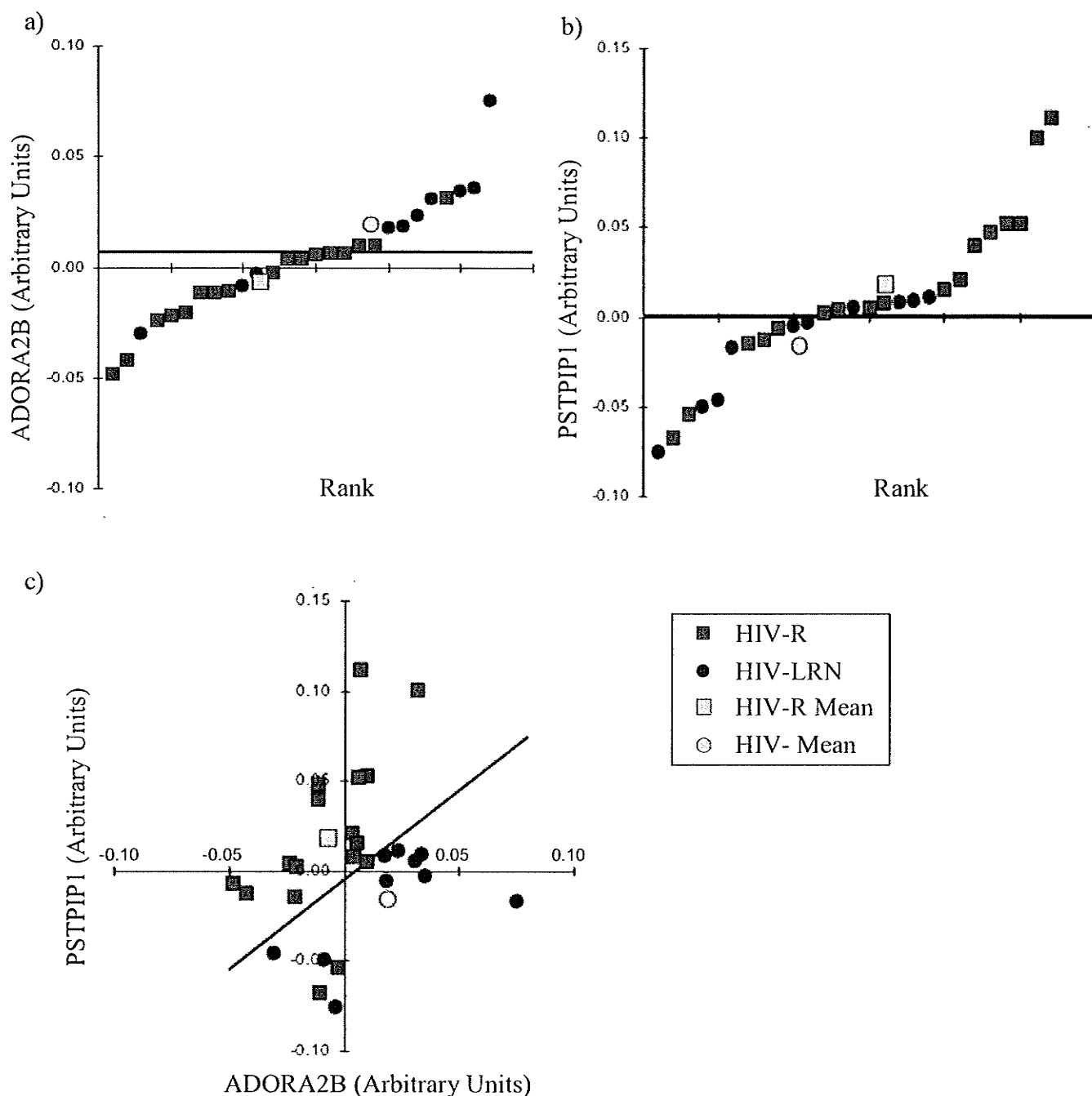
Figure 15: PIA analysis improves discrimination between HIV-R and HIV-LRN groups. LDA-based scatterplots of unstimulated gene expression data of (a) adenosine receptor A2B (ADORA2B), (b) proline-serine-threonine phosphatase interacting protein 1 (PSTPIP1) and (c) the ADORA2B/PSTPIP1 gene pair after competitive PIA shows improved class discrimination in the gene pair analysis. Black lines indicate the LDA separatrices for the given gene or gene pair analysis.

accomplished using the information provided by PIA. As seen in Table 24, several genes form significant interactions with more than one other gene. By combining this information, several gene networks can be created that may provide a larger picture of cellular processes and functions that may contribute to the HIV-1 resistant phenotype. Figure 16 shows the largest such network that can be built from the PIA data generated from the unstimulated gene expression analysis comparing HIV-R to HIV-LRN women. Interestingly, NFIC, a multi-functional transcription factor, is seen at the middle of this network, interacting with several genes both synergistically and competitively. As well, several genes involved in activation and inhibition of activation appear in this network, further supporting the hypothesis that HIV-R in this cohort associates with reduced levels of activation. It would be interesting to test this gene expression pattern both in a larger subset of HIV-R women as well as in newly enrolled CSW in order to better determine its predictive ability, however, this would require the establishment of custom assays and a larger sample set than was feasible in this study.

5.4.13 HIV-R women show lower levels of proinflammatory cytokines ex vivo

In the pilot study, significantly lower levels of proinflammatory cytokine production were noted in unstimulated cell culture supernatants of PBMC of HIV-R women compared to HIV-LRN women (Section 5.3.9). As microarray results in the above section support the lower levels of immune activity, we sought to determine if the same trends would be seen in the larger sample set and if these differences would be maintained after stimulation. Cell culture supernatants from PBMC in media alone (baseline) or stimulated with PHA, *C. albicans*, flu peptide or HIV-1 p24 from HIV-R (N=13) or HIV-LRN (N=12) women
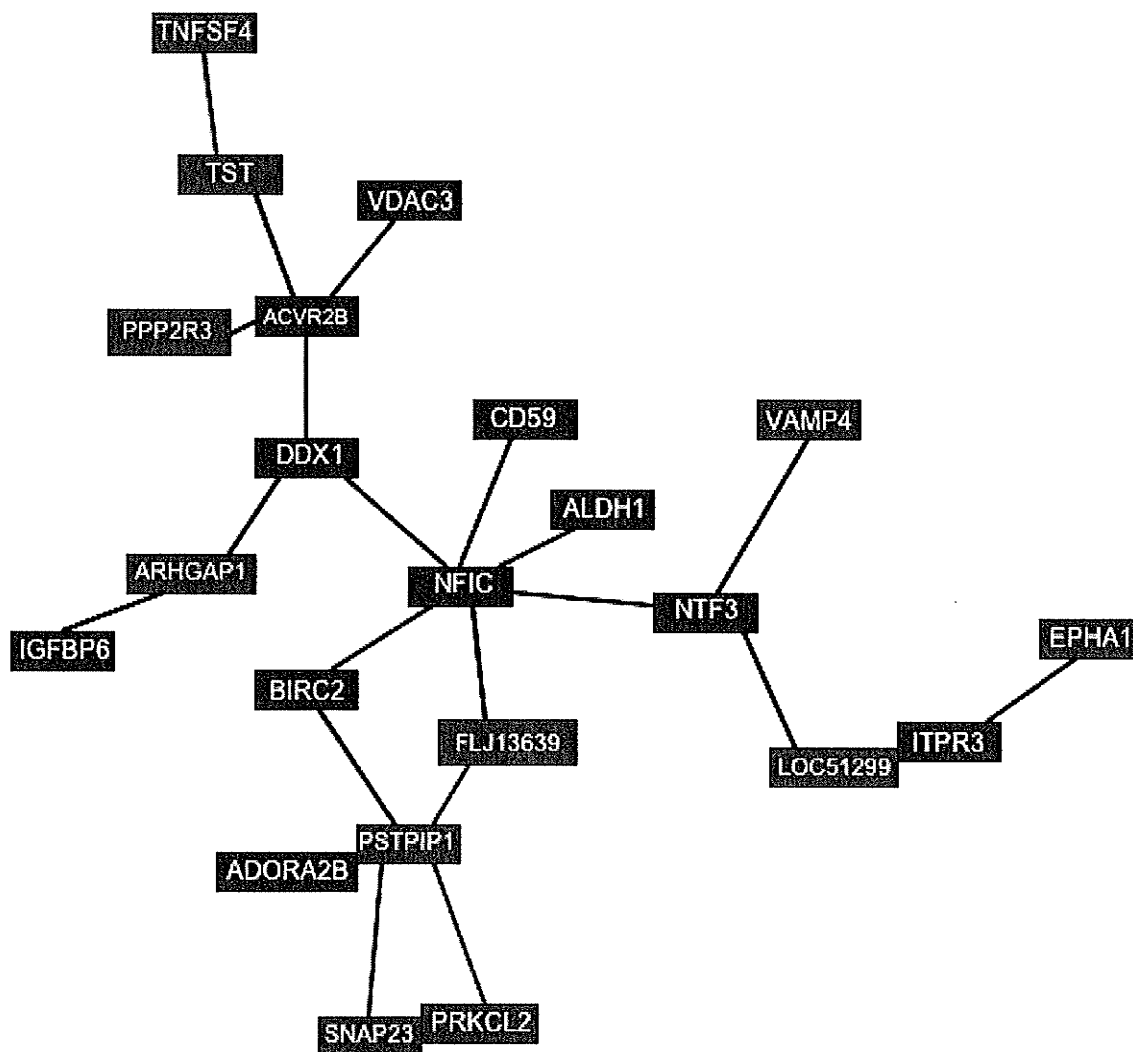
Figure 16: Predictive interaction analysis gene interaction network. Genes shown to interact by PIA form networks when compiled. Each gene in the network is signified by its own box. Red boxes indicate higher expression in HIV-R vs HIV-LRN, blue boxes indicate lower expression in HIV-R vs HIV-LRN. Red lines indicate positive synergistic interaction, blue lines indicate negative synergistic interactions, black lines indicate competitive interaction. Interaction p values are given in Table 24. An alphabetical listing of all gene symbols and descriptions used in this thesis can be found in Appendix B.

were assayed for levels of IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IP-10, MCP-1, MIG, RANTES, and TNF. As well, in order to determine if these changes were reflected *in vivo*, plasma samples from HIV-R (N=11) and HIV-LRN (N=8) were also analysed for cytokine/chemokine levels. In agreement with previous data, significantly lower levels of production of IL-1β (p=0.007), IL-6 (p=0.026) and TNF (p=0.001) were observed. As well, levels of IL-10, which trended towards lower production in the HIV-R population previously, were significantly different in the larger sample set (p=0.028) (Figure 17). In all cases these differences were not maintained after stimulation (Table 25), suggesting that HIV-R women may mount a normal immune response despite these lower baseline secretion levels. Interestingly, in the case of IL-1β, where detectable levels could be measured in plasma, HIV-R women showed lower circulating levels of this cytokine, although this trend was not seen in any stimulated condition (Figure 18).

5.4.14 Summary

Expansion of the original pilot study to include more samples demonstrated similar trends under certain conditions. Cluster analysis failed to confirm earlier observation of an inducible pattern of gene expression that characterized HIV-1 resistance. Gene expression analysis of antigen stimulated CD4+ T cells also did not confirm the previously observed associations with resistance. However, the upregulation of SP3 and the downregulation of the TCR alpha and delta chains after HIV-1 p24 stimulation in the HIV-R population support a model of resistance that includes both lower T cell activation and a specific anti-HIV environment.
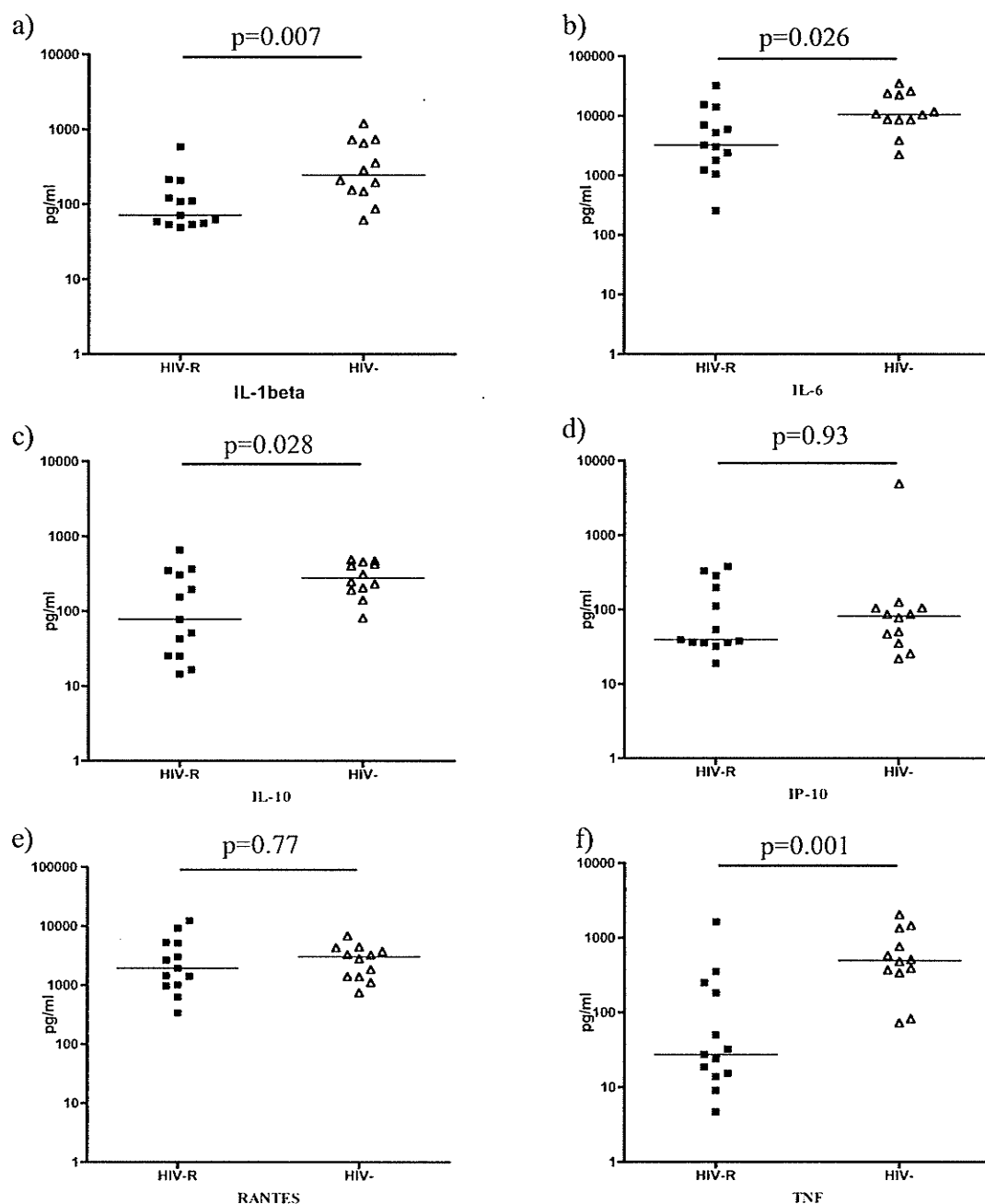
Figure 17: Unstimulated cytokine/chemokine production in cell culture supernatants of HIV-1 resistant (HIV-R) and HIV-1 low-risk negatives (HIV-) individuals. Expression levels (pg/ml) of (a) IL-1β (p=0.007), (b) IL-6 (p=0.026), (c) IL-10 (p=0.028), (d) IP-10 (0.93), (e) RANTES (p=0.77) and (f) TNF (p=0.001) by unstimulated PBMC of HIV-R (black squares, N=13) and HIV- (open triangles, N=12). Each point indicates a single individual, bars represent median expression values. p Values were calculated by Mann-Whitney U test. NS = not significant.

Table 25: Summary of median cytokine production (pg/ml) HIV-R vs HIV-LRN

Plasma	HIV-R	HIV-LRN	p Value		Media Alone	HIV-R	HIV-LRN	p Value
IFN-γ	27.5	22.8	0.62		IFN-γ	BD	BD	NA
IL-1β	*47.5*	*74.6*	*0.03*		IL-1β	*71.5*	*246.1*	*0.007*
IL-2	BD	BD	NA		IL-2	BD	BD	NA
IL-4	BD	BD	NA		IL-4	BD	BD	NA
IL-5	BD	BD	NA		IL-5	BD	BD	NA
IL-6	BD	BD	NA		IL-6	*3218.4*	*10489.1*	*0.026*
IL-8	22.7	18.9	0.84		IL-8	AD	AD	NA
IL-10	BD	BD	NA		IL-10	*78.0*	*279.5*	*0.028*
IL-12p70	BD	BD	NA		IL-12p70	BD	BD	NA
IP-10	829.2	845.2	0.77		IP-10	39.5	81.7	0.93
MCP-1	43.6	57.5	0.14		MCP-1	10082.8	7839.7	0.13
MIG	428.8	474.7	0.71		MIG	BD	BD	NA
RANTES	29382.0	35327.5	0.77		RANTES	1925.9	3011.6	0.76
TNF	BD	BD	NA		TNF	*27.3*	*494.3*	*0.001*

PHA	HIV-R	HIV-LRN	p Value		C. albicans	HIV-R	HIV-LRN	p Value
IFN-γ	1500.9	6044.0	0.082		IFN-γ	45.4	51.1	0.21
IL-1β	342.1	864.7	0.31		IL-1β	534.2	768.9	0.37
IL-2	195.8	570.6	0.76		IL-2	BD	BD	NA
IL-4	115.6	209.7	0.46		IL-4	BD	BD	NA
IL-5	32.4	130.1	0.28		IL-5	BD	BD	NA
IL-6	13803.0	22196.5	0.22		IL-6	16858.0	20940.0	0.58
IL-8	57390.0	57412.8	0.93		IL-8	68030.0	64122.2	0.74
IL-10	722.6	847.5	0.97		IL-10	642.2	504.5	0.80
IL-12p70	17.9	59.3	0.12		IL-12p70	BD	BD	NA
IP-10	4127.1	9648.6	0.31		IP-10	42.2	63.7	0.11
MCP-1	*12398.0*	*5378.6*	*0.044*		MCP-1	5146.7	4246.4	0.49
MIG	1588.4	2923.2	0.13		MIG	BD	BD	NA
RANTES	3970.2	4907.3	0.89		RANTES	2233.5	3258.0	0.91
TNF	733.0	2780.9	0.16		TNF	587.1	1380.4	0.30

Flu peptide	HIV-R	HIV-LRN	p Value		HIV p24	HIV-R	HIV-LRN	p Value
IFN-γ	37.1	37.6	0.14		IFN-γ	37.1	37.1	0.45
IL-1β	116.3	361.0	0.32		IL-1β	875.0	1152.5	0.27
IL-2	BD	BD	NA		IL-2	BD	BD	NA
IL-4	BD	BD	NA		IL-4	BD	BD	NA
IL-5	BD	BD	NA		IL-5	BD	BD	NA
IL-6	4686.7	15525.5	0.40		IL-6	20753.0	27478.0	0.20
IL-8	32636.0	57659.7	0.76		IL-8	82226.0	78733.9	0.97
IL-10	284.8	305.5	0.53		IL-10	832.8	867.5	0.43
IL-12p70	BD	BD	NA		IL-12p70	BD	BD	NA
IP-10	44.3	64.7	0.84		IP-10	36.6	82.5	0.70
MCP-1	8735.0	4564.5	0.12		MCP-1	5022.3	2939.3	0.24
MIG	BD	BD	NA		MIG	BD	BD	NA
RANTES	2409.3	2282.5	0.97		RANTES	2331.5	4287.3	0.68
TNF	66.4	758.6	0.20		TNF	668.4	2110.8	0.07

BD = Below Detection   AD = Above Detection   NA = Not Applicable
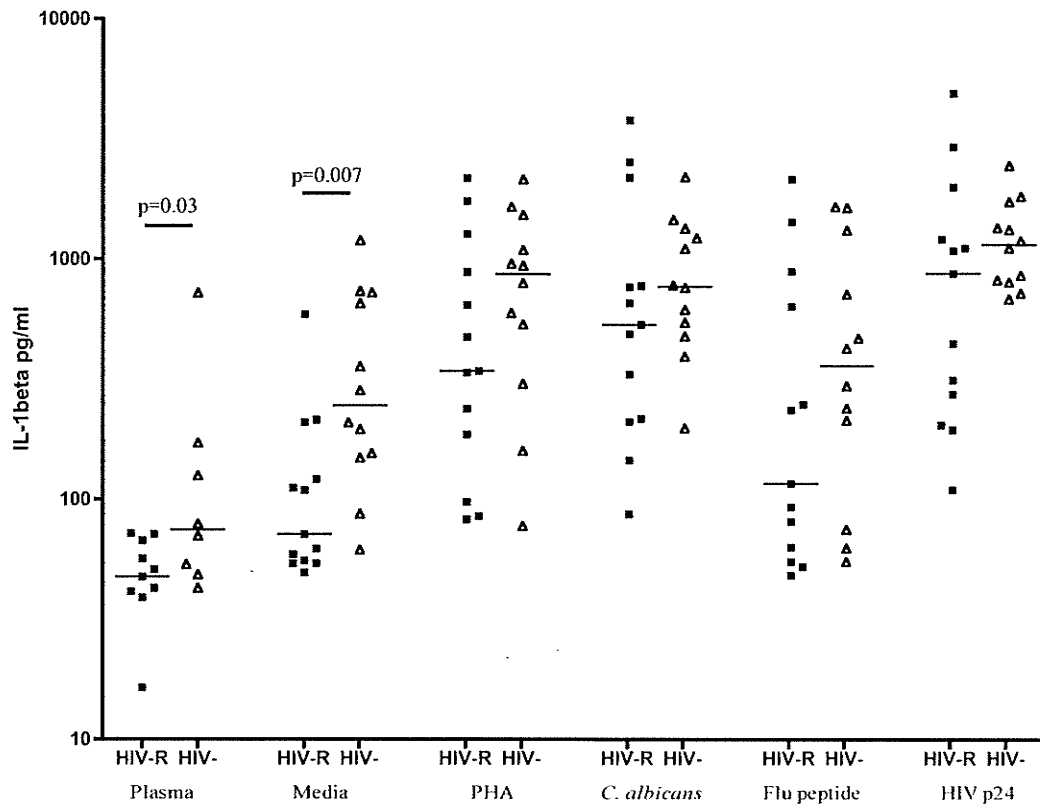
Figure 18: IL-1β production by PBMC in cell culture supernatants of HIV-1 resistant (HIV-R) and HIV-1 low-risk negative (HIV-) individuals at baseline and in response to stimulation. Expression levels (pg/ml) of IL-1β in plasma (p=0.03) and by PBMC after 24 hours of cell culture in the presence of media alone (p=0.007) or after stimulation with PHA, *C. albicans*, flu peptide, or HIV-1 p24 (all p>0.05) of HIV-R (black squares, N=13) and HIV- (black triangles, N=12). Each point indicates a single individual, bars represent median expression values. p Values were calculated by Mann-Whitney U test. Comparisons not labeled had p>0.05.

Unstimulated gene expression analysis supported the hypothesis of lower activation in HIV-R women. Several genes involved in activation of transcription were downregulated in the larger group analysis as well inhibitors of activation were upregulated. Predictive interaction analysis revealed several gene pairs that performed better at discriminating between HIV-R and HIV-LRN than single gene analysis. These gene pairs were then combined to form a larger gene network that could be useful in prospective studies in determining which individuals may go on to meet the HIV-1 resistance criteria. Perhaps the most intriguing finding in this section is the confirmation of lower baseline expression of proinflammatory cytokines in cell culture supernatants, and in the case of IL-1β, plasma. These data further support the hypothesis that lower levels of immune activation may partially explain HIV-1 resistance in this cohort.

5.5 Lower Levels of Global Gene Expression in HIV-1 Resistant Individuals Compared to High-risk HIV-1 Negative Individuals: Impact on Immune Activation and HIV-1 Replication

5.5.1 Rationale

Thus far, the studies involving HIV-1 resistant commercial sex-workers presented here have used HIV-1 uninfected non-commercial-sex-workers (i.e. female non-sex-workers attending an antenatal clinic in the same district of Nairobi) as a control group. Although these control women are matched generally for age and socio-demographic factors (i.e. many social and environmental factors are controlled for) the criticism could be made that sex-work itself, a significant environmental exposure not previously controlled for, is accountable for many of the observed differences. With this in mind a separate genomic study was designed to compare function of CD4+ T cells in HIV-1 resistant women (HIV-R) to that of HIV-1 negative commercial sex-workers that do not meet our definition of resistance, so called HIV-1 high-risk negatives (HIV-HRN).

As the major findings presented to this point indicate a potential role for lowered immune activation and lower expression of genes involved in the HIV-1 life cycle in HIV-R individuals at baseline, this study focused only on unstimulated CD4+ T cells.

5.5.2 Hypotheses

- CD4+ T cells of HIV-1 resistant commercial sex-workers will express a distinct set of genes at baseline compared to HIV-1 high-risk negatives.

- HIV-1 resistant women will express lower levels of genes involved in cellular activation and host genes involved in HIV-1 replication compared to high-risk HIV-1 negative women.

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5.5.3 Objectives

- Using the latest genomics technologies compare baseline gene expression in CD4+ T cells between HIV-1 resistant women and high-risk HIV-1 negative women.

- Determine genes that show significant differential expression between HIV-1 resistant and HIV-1 high-risk negative individuals.

- Determine if the differentially expressed genes implicate biological processes and pathways that may mediate resistance to HIV-1 infection.

5.5.4 Study outline

To address the above mentioned hypotheses, PBMC samples were collected from nine HIV-1 resistant (HIV-R) commercial sex-workers (mean age 46) and nine HIV-1 high-risk negative (HIV-HRN) commercial sex-workers (mean age 32.3). The HIV-HRN women are characterized as not meeting the definition of resistance outlined in Section 4.2.1.2 (i.e. all HIV-HRN individuals have less than the required 3 years of follow-up). Both groups of women are enrollees in the Pumwani commercial sex-workers cohort. This sample collection occurred 3 years later time point than the samples used in Sections 5.2, 5.3 and 5.4. Study subject characteristics at the time of sample collection are outlined in Table 26.

Table 26: Enrollment characteristics of HIV-R and HIV-HRN study subjects

Study Number	HIV Status	Age	Years of Enrollment	CD4	CD8
ML 767	Resistant	51	18	1123	855
ML 893	Resistant	62	18	1040	1179
ML 1260	Resistant	46	15	927	927
ML 1643	Resistant	47	12	1156	563
ML 1700	Resistant	47	12	1413	414
ML 1747	Resistant	54	11	1117	627
ML 1928	Resistant	39	5	902	322
ML 1938	Resistant	32	5	884	451
ML 1969	Resistant	36	5	864	838
ML 2034	Negative	33	2	1159	947
ML 2040	Negative	41	2	674	467
ML 2053	Negative	27	2	1012	559
ML 2140	Negative	37	2	1432	1103
ML 2200	Negative	24	2	1209	708
ML 2231	Negative	24	2	1065	813
ML 2236	Negative	41	2	1454	872
ML 2243	Negative	39	2	1489	854
ML 2282	Negative	25	1	2056	946

Peripheral blood mononuclear cells were collected from all individuals and incubated for 24 hours in the absence of stimulation (i.e. media alone). After incubation, CD4+ T cells were enriched by negative selection and total cellular RNA was isolated. RNA quantity and quality was then assessed using the Agilent Bioanalyser 2100. Gene expression levels were then quantified using Affymetrix GeneChip U133 Plus 2.0 arrays following a two-cycle RNA amplification procedure (see Materials and Methods for details). As GeneChip technologies require significant infrastructure; and individual results are greatly impacted by procedural variation, these types of experiments are best carried out in laboratories with the necessary robotics and fluidics in place. To this end, the sample amplification and array hybridization were carried out at The Center for Applied Genomics (TCAG, Toronto Ontario). Cluster, fold change, statistical testing and pathway analyses were all carried out to determine genes and pathways that may mediate resistance to HIV-1 infection.

5.5.5 Data normalization and determination of significant genes

Quantified data files were imported into ArrayAssist software for normalization and statistical testing. Data were log transformed and normalization was performed using the PLIER and MAS5 algorithms as outlined in the Methods section. After application of the MAS5 algorithm, individual spots that were not above the signal to noise threshold in more than 75% of the arrays (i.e. spots must be assigned a 'Pass' in at least 14 of 18 arrays) were discarded from further analysis. Fold difference in gene expression was determined by comparing group mean expression levels for each spot on the array. Statistical significance was assessed using a one-way ANOVA. Numbers of genes differentially expressed at a given p value cutoff are given in Table 27. Lists of

Table 27: Number of genes differentially expressed at various p value cutoffs at baseline in CD4+ T cells of HIV-R vs HIV-LRN individuals

p Value	Genes Up	Genes Down	Total
<0.0001	0	24	24
<0.001	8	124	132
<0.01	50	681	731
>0.05	224	2024	2248

significant genes were exported to Microsoft Excel for manual inspection. Cluster

analysis and visualization was performed using Cluster and Treeview software and

pathway analysis was performed using the DAVID web tool (see Materials and Methods

for details).

5.5.6 Cluster analysis shows global downregulation of genes that characterize HIV-R

individuals

In order to better visualize gene expression differences between HIV-R and HIV-HRN

individuals, and to assess the ability of a given set of genes to discriminate between

sample classes, hierarchical clustering was performed. Gene lists were filtered for genes

showing significant differential expression between HIV-R and HIV-HRN individuals

($p<0.001$). Figure 19 shows the heat map and gene and array dendograms.

Not surprisingly this analysis shows perfect class discrimination between HIV-R and

HIV-LRN individuals. Importantly, the obvious trend that can be used to discriminate

between classes is towards lower overall expression of genes (green boxes). This is

underscored by the large proportion of differentially expressed genes that are expressed at

a lower level in HIV-R individuals at all p value cutoffs (Table 27). This striking

observation is of obvious importance as not only does it support the hypothesis of lower

immune activation in HIV-R individuals, but implies a level of immune quiescence

associates with resistance to infection, a finding not previously described. Determining

which genes and pathways contribute to this observation should further support this

hypothesis.
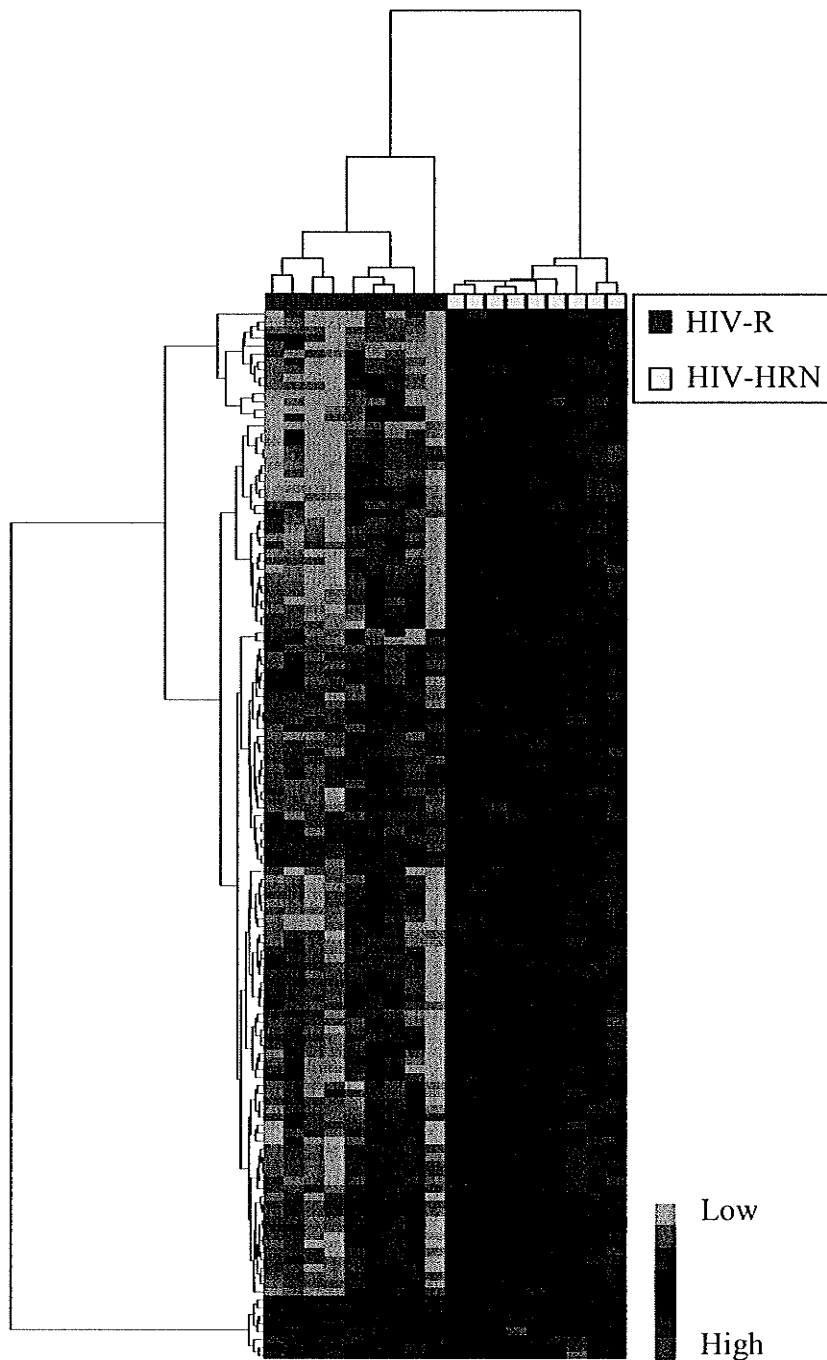
Figure 19: Lower general gene expression defines an HIV-1 resistant pattern of expression. Hierarchical complete linkage clustering of HIV-1 resistant (blue squares) and HIV-1 high-risk negative (yellow squares) individuals shows that class discrimination associates with lower gene expression in HIV-1 resistant women. Clustering based on 132 genes showing significantly different expression between groups (p < 0.001). Of these, 124 genes are expressed at a lower level by HIV-1 resistant women compared to controls

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5.5.7 HIV-R women show lower levels of expression of host genes involved in the HIV-1 life cycle

For initial analysis, a broad p value cutoff of $p<0.05$ was used as an initial filter in order to not unduly discount interesting biological observations. This resulted in a list of 2,248 genes, of which 224 were expressed at a higher level and 2,024 were expressed at a lower level in HIV-R individuals. Manual inspection of this gene list revealed several host genes previously known to be involved in HIV-1 replication were expressed at a lower level in HIV-R women. Gene names with fold changes and p values are listed in Table 28. Of particular interest is the RNA trafficking molecule Staufen (STAU) as a total of 4 spots on the GeneChip specific for STAU independently demonstrated significant differential expression between the two groups (Figure 20). Staufen has been shown to be involved in transport of the HIV-1 genome to the cell surface and is required for proper packaging into the virion. In fact, STAU has been shown to be incorporated into the mature virion and reduction of its expression using siRNA has been shown to dramatically reduce HIV-1 replication *in vitro*[329]. As well, a second gene involved in viral assembly CyclophilinA[330], the HIV-1 entry coreceptor CXCR4[60] and several genes involved in HIV-1 gene transcription, PP1[331], NF-κB (formed by heterodimers of NFKB1 and RELA)[332] and KIF22[333] and a gene recently shown to be involved in virological synapse formation, ZAP70, important for cell to cell transmission of HIV-1[334], were all expressed at lower levels in HIV-R individuals. This suggests that the global quiescence observed may impact on the ability of HIV-1 to replicate once inside the cells of an HIV-R individual, likely contributing to resistance.

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Table 28: Host genes known to be involved in HIV-1 replication are expressed at a lower level in HIV-R compared to HIV-HRN individuals

Symbol	p Value	Fold	Description
PPP1CA (PP1)	0.00001	-2.19	protein phosphatase 1, catalytic subunit, alpha isoform
STAU	0.00002	-1.67	staufen, RNA binding protein (Drosophila)
KIF22	0.00005	-1.88	kinesin family member 22
RELA	0.0009	-1.40	v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, (p65)
CYPA	0.009	-1.43	peptidylprolyl isomerase A (cyclophilin A)
NFKB1	0.01	-1.71	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p50)
CXCR4	0.04	-1.76	chemokine (C-X-C motif) receptor 4
ZAP70	0.05	-2.38	zeta-chain (TCR) associated protein kinase 70kDa
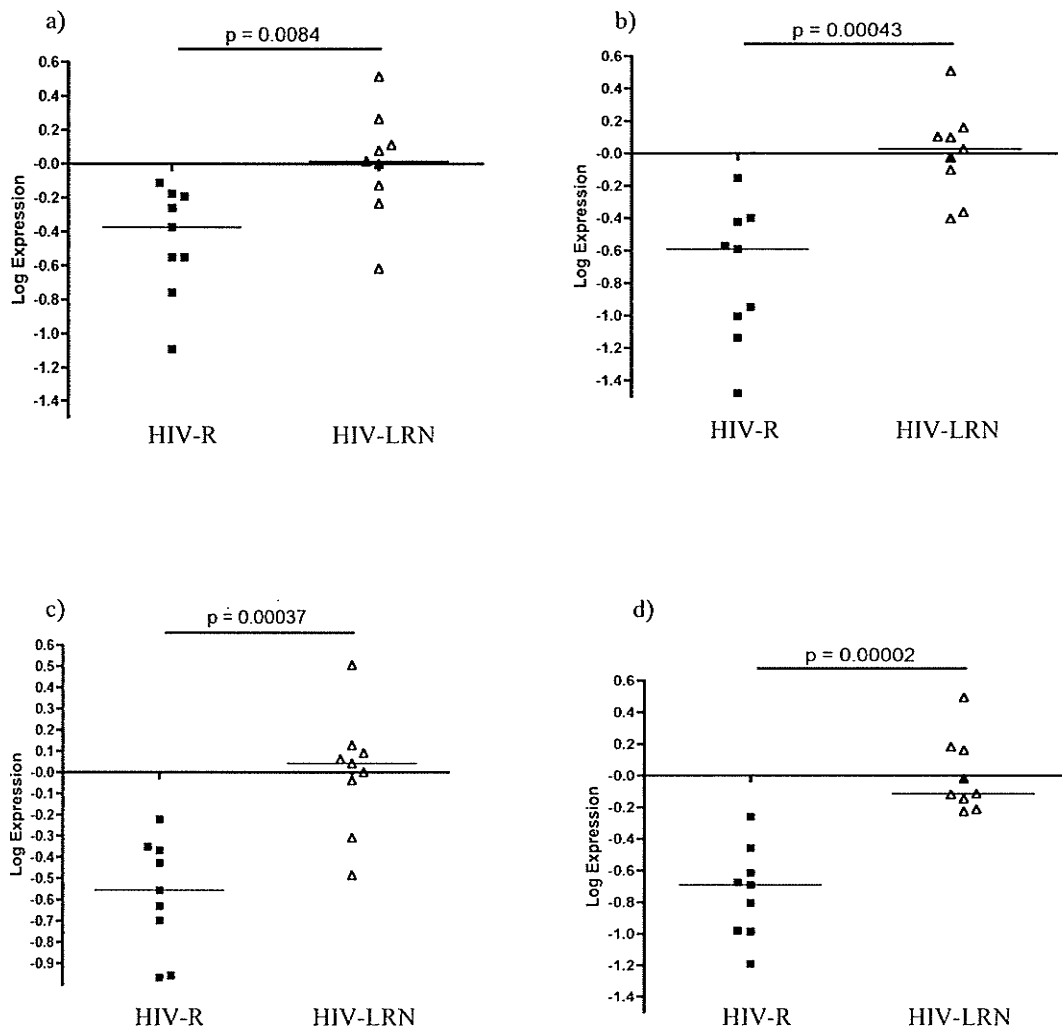
Figure 20: Staufen-1 is expressed at a lower level at baseline in CD4+ T cells of HIV-R women compared to HIV-LRN. Log expression of Staufen-1 from 4 separate spots (a-d) on the Affymetrix GeneChip U133 Plus 2.0. Raw expression values have been normalized using the PLIER algorithm and median centered around the HIV-LRN group. Black squares repsrent HIV-R individuals, open triangles represent HIV-LRN individuals. All data were were adjusted such that the mean value for the control group (HIV-LRN) would be zero. Black lines within each grouping indicate group medians. P values were calculated using 1-way ANOVA.

5.5.8 HIV-R women show lower levels of expression of genes involved in immune activation

The list of genes significantly differentially expressed between HIV-R and HIV-HRN individuals was manually scanned for genes and functional categories that may play a role in mediating protection. This analysis identified several interesting genes and functional categories to be expressed at a lower level in HIV-R women (Table 29). The major functional themes arising were cytokine and chemokines and their recpetors, human leukocyte antigen molecules, signal transduction, T cell receptor signaling and transcription initiation. All genes were expressed at a lower level by HIV-R women further lending support to the lowered immune activation hypothesis. Of particular interest is the lowered expression of CCR7 and HLA-DRα, proteins known to be expressed on acticvated T cells, the HIV-1 coreceptor CXCR4, and the T cell receptor alpha and beta chains as well as the NF-κB subunits NFKB1 and RELA. The lowered expression of all of these genes both demonstrate the lower activation state of CD4+ T cells in HIV-R women and, in the case of CXCR4 and NF-κB, suggest a reduced ability to support HIV-1 replication if infected. Non-biased pathway analysis, used to determine if the differential expression gene list is enriched for genes in a particular pathway, would greatly enhance these results and possibly identify new candidate genes and pathways that mediate resistance.

Table 29: Selected genes expressed at a lower level in HIV-R individuals

Symbol	Fold	p Value	Class	Description
IL23A	-2.1	0.002	Cytokine/Chemokine	interleukin 23, alpha subunit p19
IL16	-1.8	0.004	Cytokine/Chemokine	interleukin 16 (lymphocyte chemoattractant factor)
IL27RA	-1.5	0.01	Cytokine/Chemokine	interleukin 27 receptor, alpha
IL10RB	-1.7	0.01	Cytokine/Chemokine	interleukin 10 receptor, beta
CXCR3	-2.0	0.02	Cytokine/Chemokine	chemokine (C-X-C motif) receptor 3
CCR7	**-2.1**	**0.04**	**Cytokine/Chemokine**	**chemokine (C-C motif) receptor 7**
CXCR4	**-1.8**	**0.04**	**Cytokine/Chemokine**	**chemokine (C-X-C motif) receptor 4**
HLA-DQB1	-2.2	0.005	HLA	major histocompatibility complex, class II, DQ beta 1
HLA-G	-1.9	0.01	HLA	HLA-G histocompatibility antigen, class I, G
HLA-DRB1	-2.7	0.03	HLA	major histocompatibility complex, class II, DR beta 1
HLA-DRB5	-2.1	0.04	HLA	Major histocompatibility complex, class II, DR beta 3
HLA-DRA	**-2.9**	**0.04**	**HLA**	**major histocompatibility complex, class II, DR alpha**
HLA-B	-1.6	0.05	HLA	major histocompatibility complex, class I, B
MAPK9	-2.0	0.0004	Signal Transduction	mitogen-activated protein kinase 9
RAF1	-2.0	0.003	Signal Transduction	v-raf-1 murine leukemia viral oncogene homolog 1
MAP3K7IP2	-1.7	0.003	Signal Transduction	mitogen-activated protein kinase kinase kinase 7 interacting protein 2
MAP2K7	-1.4	0.006	Signal Transduction	mitogen-activated protein kinase kinase 7
RAB2	-1.5	0.008	Signal Transduction	RAB2, member RAS oncogene family
MAP3K7	-1.9	0.03	Signal Transduction	mitogen-activated protein kinase kinase kinase 7
JAK1	-1.5	0.03	Signal Transduction	Janus kinase 1 (a protein tyrosine kinase)
TRA@	-1.9	0.0002	T Cell Signaling	T cell receptor alpha locus
TRBC1	-1.8	0.008	T Cell Signaling	T cell receptor beta constant 1
CD3D	-1.4	0.02	T Cell Signaling	CD3D antigen, delta polypeptide (TiT3 complex)
LAT	-1.6	0.02	T Cell Signaling	linker for activation of T cells
ZAP70	-2.4	0.05	T Cell Signaling	zeta-chain (TCR) associated protein kinase 70kDa
POLR2E	-1.9	0.00009	Transcription	polymerase (RNA) II polypeptide E, 25kDa
TAF9	-1.7	0.0007	Transcription	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 32kDa
RELA	**-1.4**	**0.001**	**Transcription**	**v-rel nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65**
TCEB2	-1.7	0.004	Transcription	transcription elongation factor B (SIII), polypeptide 2 (18kDa, elongin B)
TCEB3	-1.8	0.004	Transcription	Transcription elongation factor B (SIII), polypeptide 3 (110kDa, elongin A)
TCEA2	-2.9	0.005	Transcription	transcription elongation factor A (SII), 2
NFKB1	**-1.7**	**0.01**	**Transcription**	**nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p50)**
NFRKB	-1.2	0.02	Transcription	nuclear factor related to kappaB binding protein

5.5.9 qRT-PCR confirmation of expression differences

In order to confirm expression differences found by GeneChip analysis, qRT-PCR was run using the SYBR green detection method on the Lightcycler for the genes STAU, PP1, NFKB1, RELA, LAT1, LCP2, HLA-DR and CXCR4 all shown to be expressed at a lower level by HIV-R individuals and the gene POLA which showed no significant difference between groups. As gene expression analysis showed a global trend towards lower gene expression in HIV-R women it was reasoned that standard housekeeping genes, generally genes involved in metabolism or the cytoskelelton, may not be an appropriate control for this analysis. In fact the commonly used housekeeping gene, GAPDH, was actually expressed at a significantly lower level in HIV-R women compared to HIV-LRN (-1.75 fold, p=0.006). To this end, rather than normalize to any single gene, qRT-PCR data were normalized to amount of input total RNA. Results of thes analyses are listed in Table 29 with representative dot plots in Figure 21. Of the 8 genes chosen for confirmation, 7 showed significantly (p<0.05) lower expression by both GeneChip and qRT-PCR, while CXCR4 trended towards significance (p=0.06). As well, the gene POLA showed no significant difference in expression by either GeneChip analysis or qRT-PCR. For GeneChip data, fold change and p value were calculated as mentioned. For qRT-PCR analysis fold changes were calculated by dividing mean relative expression values of HIV-R samples by HIV-LRN samples, and p values were calculated by Mann-Whitney U test. These data suggest a good ability to confirm expression level differences seen on GeneChip analysis by qRT-PCR.

Table 30: qRT-PCR confirmation of genes shown to be expressed at a lower level by HIV-R compared to HIV-LRN individuals by GeneChip analysis

Symbol*	Fold qRT	p qRT	Fold Array	p Array
RELA	-2.41	0.0008	-1.40	0.0009
NFKB1	-1.70	0.001	-1.71	0.01
LCP2	-1.97	0.008	-1.43	0.0003
STAU	-1.63	0.008	-1.67	0.00002
HLADR	-4.11	0.01	-2.89	0.04
LAT1	-2.06	0.01	-1.62	0.02
PPP1CA	-1.62	0.01	-2.19	0.00001
CXCR4	-1.64	0.06	-1.76	0.04
POLA	-1.14	0.43	-2.06	0.99

* An alphabetical listing of all gene symbols and descriptions used in this thesis can be found in Appendix B.
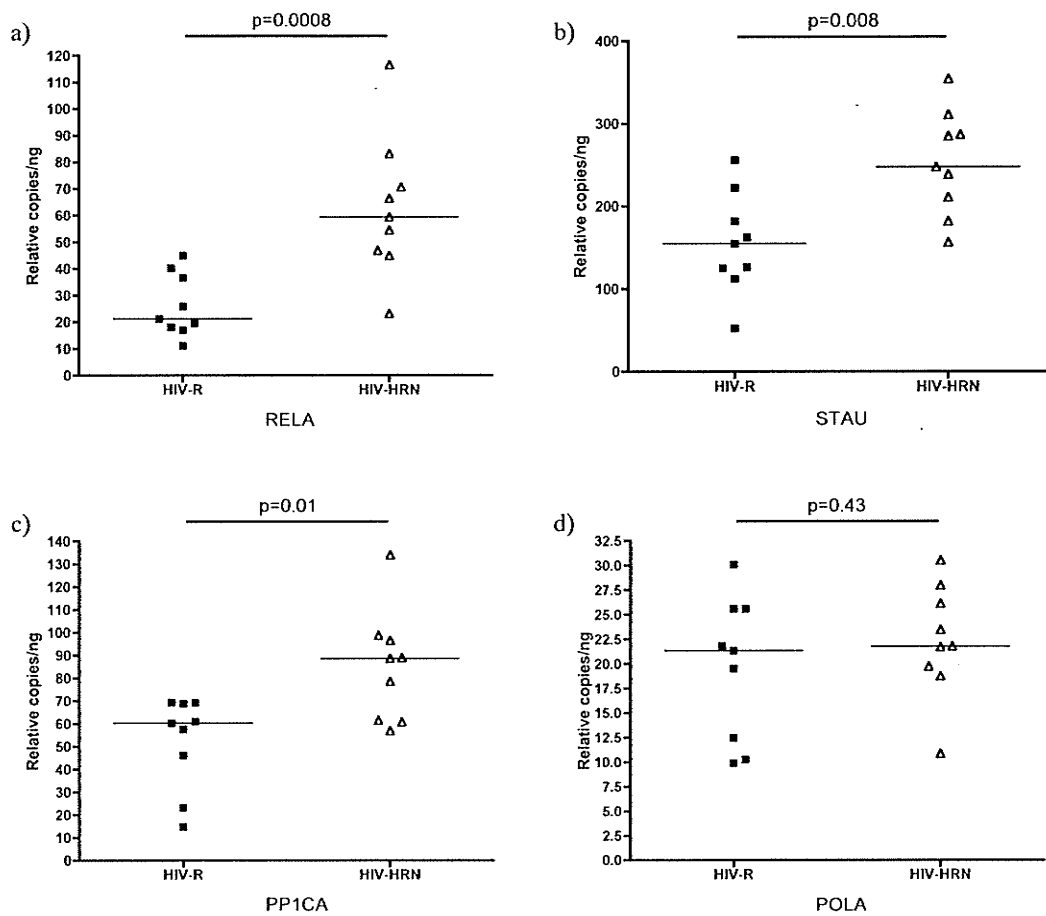
Figure 21: Representative dot plots of confirmatory qRT-PCR data. Dot plots show relative gene expression (expressed as relative copies/ng) as measured by qRT-PCR for the genes RELA (a), STAU (b), PP1CA (c) and POLA (d). Squares indicate HIV-R individuals, open triangles represent HIV-HRN individuals, black bars indicate group medians. qRT-PCR confirms array findings of significantly lower expression of RELA (p=0.0008), STAU (p=0.008) and PP1CA (p=0.01) by HIV-R women. POLA a showed no difference by either array analysis or qRT-PCR (Table 30).

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5.5.10 Pathway analysis supports lower levels of activation associates with HIV-1 resistance

Although single gene analyses as performed above are informative, functional annotations allow for unbiased determination of genes and pathways that may be involved in a particular biological process. Functional enrichment for particular pathways can be assessed statistically for a given gene list using the Database for Visualization Annotation and Integrated Discovery (DAVID) functional annotation web tool (http://david.abcc.ncifcrf.gov/home.jsp). In order to determine specific pathways that may be involved in mediating protection, the list of genes shown to be differentially expressed by HIV-R compared to HIV-HRN individuals was entered into the DAVID web tool. This tool uses separate databases containing information on genes known to be involved in complex pathways (such as BIOCARTA and the Kyoto Encyclopedia of Genes and Genomes) to identify functional themes in the provided gene list, and applies a modified Fisher's Exact test to determine statistical significance[319]. Table 31 lists the pathways whose members were statistically over-represented in the provided gene list.

Several pathways involved in metabolism and immune signaling were identified. Perhaps most interestingly, in terms of statistical significance, is the oxidative phosphorylation pathway (Figure 22 and Table 32). Of the genes considered for this analysis, 34 were involved in this process, all but one of these were expressed at a lower level by HIV-R individuals. As this is a well characterized pathway involved in cellular metabolism and ATP synthesis, the overall lowered expression of genes involved in this

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Table 31: Pathways significantly associated with HIV-1 resistance by DAVID functional annotation analysis

Pathway	# Genes	p Value	Database
Proteasome	*17*	*<0.00001*	*KEGG*
Oxidative phosphorylation	*34*	*<0.0001*	*KEGG*
Ribosome	38	0.003	KEGG
T cell receptor signaling	*11*	*0.01*	*BIOCARTA*
Ceramide signaling	8	0.01	BIOCARTA
SUMOylation	4	0.01	BIOCARTA
Fas signaling	13	0.02	BIOCARTA
Protein export	6	0.02	KEGG
ER associated degradation	6	0.02	BIOCARTA
Aminoacyl-tRNA synthetases	9	0.02	KEGG
CO2 fixation	6	0.02	KEGG
Influence of Ras and Rho proteins on G1 to S transition	8	0.03	BIOCARTA
SNARE interactions in vesicular transport	9	0.03	KEGG
MAPK signaling	17	0.06	BIOCARTA
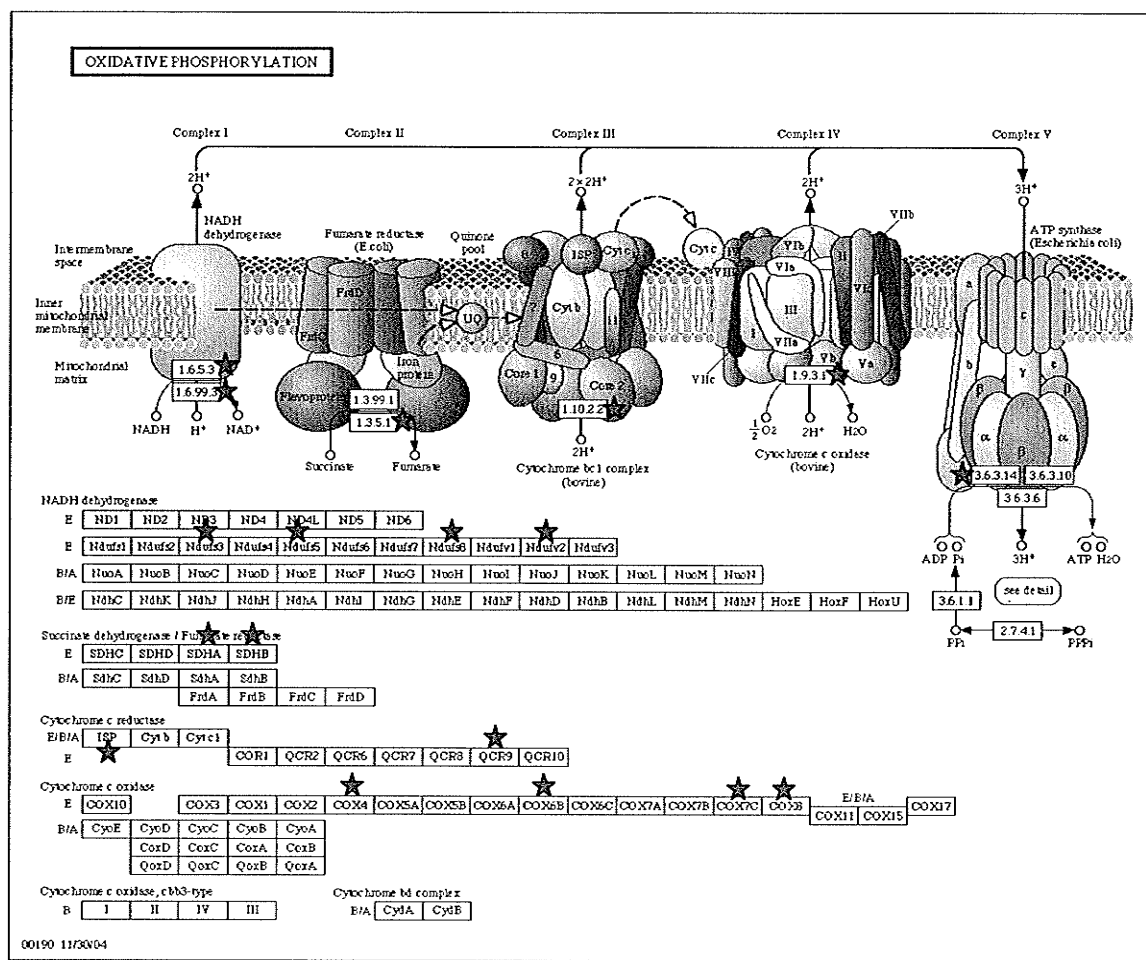
Figure 22: Diagram of the oxidative phosphorylation pathway. As determined by DAVID functional annotation analysis several genes involved in oxidative phosphorylation are differentially expressed by HIV-R compared to HIV-HRN individuals. Oxidative phosphorylation diagram reproduced from the Kyoto Encyclopedia of Genes and Genomes (freely available to academic users). Red stars indicate differentially expressed genes. Gene descriptions, fold changes and p values are listed in Table 32.

Table 32: Genes differentially expressed in HIV-R vs HIV-HRN individuals involved in oxidative phosphorylation identified by DAVID analysis

Symbol	p Value	Fold	Description
NDUFB7	0.0001	-2.6	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa
ATP6V1F	0.0003	-1.7	ATPase, H+ transporting, lysosomal 14kDa, V1 subunit F
NDUFS3	0.001	-2.0	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase)
NDUFA13	0.002	-2.1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13
NDUFS8	0.003	-1.5	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductase)
ATP5L	0.003	-1.6	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit g
ATP6V0D2	0.003	1.3	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d isoform 2
NDUFB9	0.004	-1.7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9, 22kDa
ATP6V1G1	0.007	-1.5	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G isoform 1
SDHB	0.007	-2.1	succinate dehydrogenase complex, subunit B, iron sulfur (Ip)
ATP5B	0.009	-1.6	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide
COX4I1	0.009	-1.4	cytochrome c oxidase subunit IV isoform 1 /// cytochrome c oxidase subunit IV isoform 1
NDUFV2	0.01	-1.4	NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa
COX7C	0.01	-1.5	cytochrome c oxidase subunit VIIc
ATP6AP1	0.01	-1.7	ATPase, H+ transporting, lysosomal accessory protein 1
ATP6V0E	0.01	-1.8	ATPase, H+ transporting, lysosomal 9kDa, V0 subunit e
ATP6V1H	0.01	-1.8	ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H
ATP5J	0.01	-1.5	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F6
COX8A	0.02	-1.4	cytochrome c oxidase subunit 8A (ubiquitous)
ATP5O	0.02	-1.8	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit (oligomycin sensitivity conferring protein)
COX6B1	0.02	-2.0	cytochrome c oxidase subunit VIb polypeptide 1 (ubiquitous)
UQCRFS1	0.02	-1.5	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1
NDUFA8	0.03	-2.0	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8, 19kDa
ATP5G2	0.03	-2.8	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 2
ATP6V1D	0.03	-1.4	ATPase, H+ transporting, lysosomal 34kDa, V1 subunit D
ATP6V1E1	0.04	-1.4	ATPase, H+ transporting, lysosomal 31kDa, V1 subunit E isoform 1
ATP6V1B2	0.04	-1.4	ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B, isoform 2
NDUFS5	0.04	-1.4	NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15kDa (NADH-coenzyme Q reductase)
SDHA	0.05	-1.4	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
NDUFC1	0.05	-1.4	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1, 6kDa
NDUFA12	0.05	-1.4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12

process would be expected from cells at a lower level of activation. Also of interest is the

lowered expression of genes involved in proteasome formation (Figure 23 and Table 33).

The proteasome is an intracellular polyprotein involved in proteolysis and antigen

processing, however it also plays a role in both HIV-1 degradation upon infection and

proper cleavage of Gag polyproteins[335]. It is possible that lower expression of genes

involved in proteasome formation would limit proper assembly of HIV-1 virions thus

limiting infection.

Finally and perhaps most interestingly, is the lowered expression of several genes

involved in the T cell receptor signaling pathway (Figure 24 and Table 34). This lowered

expression is indicative of lower levels of CD4+ T cell activation in HIV-R women.

Taken together, these data strongly support the hypothesis of lowered levels of activation

in HIV-R women and demonstrate several, previously un-reported pathways that may

play a role in mediating protection against infection.

5.5.11 Summary

Gene expression analysis of CD4+ T cell function in HIV-R individuals in comparison to

HIV-1 negative, non-resistant, commercial sex-workers supports the hypothesis that

lower levels of T cell activation plays a role in establishing the HIV-1 resistant

phenotype. Previous studies have outlined both the importance of T cell activation in the

intial stages of HIV-1 infection and have shown some association of lower levels of cell

surface markers indicative of immune activation with reduced susceptibility to HIV-1

infection. However, this study probes much deeper into this phenomenon, outlining
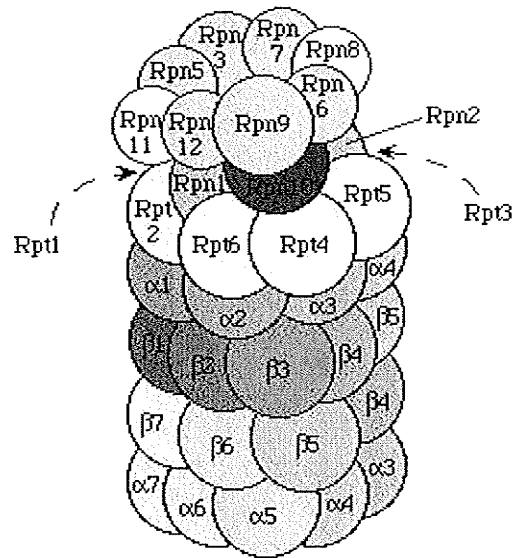
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Figure 23: Diagram of the proteasome. As determined by DAVID functional annotation analysis several subunits of the proteasome are expressed at a lower level in HIV-R compared to HIV-HRN individuals. Proteasome diagram reproduced from the Kyoto Encyclopedia of Genes and Genomes (freely available to academic users). Red stars indicate differentially expressed genes. Gene descriptions, fold changes and p values are listed in Table 33.

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Table 33: Genes differentially expressed in HIV-R vs HIV-HRN individuals involved in proteasome formation identified by DAVID analysis

Symbol	p Value	Fold	Description
PSMD8	0.0002	-2.0	proteasome (prosome, macropain) 26S subunit, non-ATPase, 8
PSMC3	0.0007	-2.3	proteasome (prosome, macropain) 26S subunit, ATPase, 3
PSMA4	0.001	-1.9	proteasome (prosome, macropain) subunit, alpha type, 4
PSMB4	0.002	-1.8	proteasome (prosome, macropain) subunit, beta type, 4
PSMD6	0.003	-1.9	proteasome (prosome, macropain) 26S subunit, non-ATPase, 6
PSMD11	0.004	-1.8	proteasome (prosome, macropain) 26S subunit, non-ATPase, 11
PSMD1	0.005	-1.4	proteasome (prosome, macropain) 26S subunit, non-ATPase, 1
PSMD4	0.01	-1.7	proteasome (prosome, macropain) 26S subunit, non-ATPase, 4
PSMB1	0.01	-1.6	proteasome (prosome, macropain) subunit, beta type, 1
PSMD3	0.01	-1.7	proteasome (prosome, macropain) 26S subunit, non-ATPase, 3
PSMC4	0.01	-1.7	proteasome (prosome, macropain) 26S subunit, ATPase, 4
PSMC2	0.01	-1.9	proteasome (prosome, macropain) 26S subunit, ATPase, 2
PSMA3	0.02	-1.7	proteasome (prosome, macropain) subunit, alpha type, 3
PSMA1	0.03	-1.7	proteasome (prosome, macropain) subunit, alpha type, 1
PSMA2	0.03	-1.5	proteasome (prosome, macropain) subunit, alpha type, 2
PSMA6	0.03	-1.3	proteasome (prosome, macropain) subunit, alpha type, 6
PSMD2	0.05	-1.6	proteasome (prosome, macropain) 26S subunit, non-ATPase, 2

**Figure 24:** Diagram of the T cell receptor signaling pathway. As determined by DAVID functional annotation analysis several genes involved in T cell signaling are expressed at a lower level in HIV-R compared to HIV-HRN individuals. T cell receptor signaling pathway diagram reproduced from BIOCARTA (permission obtained November 2007). Red stars indicate differentially expressed genes. Gene descriptions, fold changes and p values are listed in Table 34.

Table 34: Genes differentially expressed in HIV-R vs HIV-HRN individuals involved in T cell receptor signaling identified by DAVID analysis

Symbol	Fold	p Value	Description
TRA@	-1.9	0.0002	T cell receptor alpha locus
RELA	-1.4	0.0009	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p65)
FYB	-2.4	0.002	FYN binding protein (FYB-120/130)
HRAS (RAS)	-1.5	0.005	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
NFKB1	-1.7	0.01	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p50)
CD3D	-1.4	0.02	CD3D antigen, delta polypeptide (TiT3 complex)
PTPN6 (HePTP)	-1.6	0.02	protein tyrosine phosphatase, non-receptor type 6
LAT	-1.6	0.02	linker for activation of T cells
RHOA (RAF1)	-1.4	0.03	ras homolog gene family, member A
ZAP70	-2.4	0.05	zeta-chain (TCR) associated protein kinase 70kDa

several genes whose lowered expression may significantly impact the ability of HIV-1 to replicate in the host cell. Manipulation of the specific genes and pathways outlined in this section may prove to be a fruitful target for novel therapies and may aid in educating vaccine design.

Section 6.0: Discussion

The HIV-1 pandemic is one of the greatest global health crises the world has ever known. Although many strides have been taken in terms of treatment and prevention, the prevalence of HIV-1 infection continues to grow, and all attempts to date to create a preventative vaccine have failed. Understanding naturally occurring protection against infection (like Jenner's milkmaids) is the best hope for discovery of such a vaccine. The advent of large-scale gene expression monitoring has allowed for the study of cellular function in instances of disease and reduced susceptibility to infection in unparalleled detail. The work described in this thesis has focused on using genomic technologies to explore CD4+ T cell function in HIV-1 infection and resistance. The overall hypotheses of the research presented here are as follows:

- Within a given individual, after antigenic stimulation, the gene expression profiles of the CD4+ and CD8+ T cell populations will differ from each other and from that of the mixed cell PBMC population.

- HIV-1 infected individuals with intermediate to high CD4 counts (CD4 > 350) will show gene expression levels similar to those seen in uninfected individuals. The gene expression differences that are seen will confirm what is known about HIV-1 pathogenesis. Specifically, higher expression of genes involved in immune activation will be observed.

- CD4+ T cells of HIV-1 resistant (HIV-R) commercial sex-workers function differently, both at baseline and in response to stimulation, than do those of HIV-

1 low-risk negative (HIV-LRN) non-resistant, and HIV-1 high-risk negative

(HIV-HRN) non-resistant women. These differences can be measured at the level

of gene expression.

- The resistance phenotype will correlate with a general under-expression of genes involved in T cell activation and genes known to contribute to HIV-1 replication and/or an over-expression of inhibitors of activation and viral replication compared to HIV-LRN and HIV-HRN women.

- This functional hypo-activation state will correlate with an under-production of soluble mediators of the immune response measured in supernatants of PBMC culture.

The initial stages of these studies were among the first to demonstrate the need to

examine specific cell subsets individually, rather than as a mixed cell population, in order

to better link observed gene expression differences with cell subtype. In the context of

HIV-1 infection, our study has demonstrated a level of immune dysfunction not

previously realized and confirmed previous findings implicating immune activation in

HIV disease. As well, this is the first study to describe in detail the differential function

of CD4+ T cells in HIV-1 resistant individuals implicating several novel biomarkers of

resistance, lowered levels of immune activity and a pattern of gene expression associated

with HIV-1 resistance. These results should serve to supplement the body of knowledge

of immune cell function in HIV-1 infection as well as inform further studies on treatment

and prevention of HIV-1 infection.

6.1 Gene Expression Profiles of T cell Subsets Differ from the PBMC Population

Previously, microarray studies of immune cell function, particularly in the field of HIV-1 research, had focused on either the effects of infection or antigenic stimulation in cell lines or in mixed cell populations (reviewed in Giri, 2006[298]). Thus, in studies using mixed cell populations, there was a disconnect between the observed gene expression changes and the cell types responsible for those changes. It had been argued that cell type could be inferred from the gene expression profiles, but no study had directly addressed that assumption. Therefore, prior to initiation of our cohort gene expression studies, we asked if the transcriptional profiles of the CD4+ or CD8+ T cell population were accurately reflected in the PBMC population.

In section 5.1, the intrasubject variation in antigen-specific gene expression profiles of T-lymphocyte subsets were compared to that of the PBMC population. For antigen-specific responses, we looked at a very disease specific response, p24 stimulation, and at a ubiquitous antigen, *C. albicans*. The p24 response was studied for an HIV-infected individual, whereas the *C. albicans* response was assessed for both the HIV-positive subject and a normal, HIV-negative donor. It was hypothesized that within a given individual, after antigenic stimulation, the gene expression profiles of the CD4+ and CD8+ T cell populations would differ from each other and from that of the mixed cell PBMC population. No intersubject comparisons were performed, as the sample size was not appropriate for such an analysis.

In comparing the expression profiles of CD4+ T lymphocytes, CD8+ T lymphocytes, and the PBMC populations, we saw tremendous variation within the same individual with regard to antigen-stimulated changes in gene expression. For example, in response to *C. albicans*, the HIV-negative sample showed 136 genes with changed expression in at least one cell population, with only 26 genes showing the same trend in at least 2 cell populations and only 4 genes showing the same trend in all three of the populations studied (Figures 3a and b). These data underscore the point that tremendously different pictures of global gene expression are seen depending on the cell populations examined.

When specific genes (MIF, TNFRSF5, MCP-1, IL-6, TRAP-1, and IGF2) were compared, we observed expression changes that were unique to each cell type (Figure 4). Specifically, the up-regulation in the HIV-positive donor of the expression of IL-6, a key proinflammatory cytokine known to show altered expression in HIV infection[336], after p24 stimulation (Figure 4b) was observed only in CD4+ T lymphocytes. This interesting observation would not have been made if only the mixed population had been studied. Other examples of changes in T cells that are not reflected in PBMC are provided by MIF expression in CD4+ lymphocytes and TNFRSF2 and TRAP-1 expression in CD8+ lymphocytes from the normal and HIV-infected donor respectively (Figure 4).

Although our primary observation in this study is that the gene expression of PBMC does not reflect that of the T-cell subsets, the study of PBMC is not without value. Indeed, if the alteration in gene expression in a cell subset is large enough, it may be detected even if expression of that gene is not altered in the T-cell population. The increased expression of MCP-1, a CC chemokine involved in monocyte trafficking[337], in response to *C.*

albicans in the HIV-negative individual (Fig. 4a) is an example. Large changes in MCP-1 expression were observed in the PBMC population but not in either CD4+ or CD8+ T lymphocytes. MCP-1 has been shown to affect the cytokine profiles of T cells and other facets of adaptive immunity[338], as well as inducing inflammatory responses to influenza A virus[339]. Although the change in MCP-1 expression was large enough to be detectable in the mixed cell population, it is impossible to determine which of the cell types known to secrete MCP-1[340, 339] is responsible for this change.

Since the publication of this study[322], several other studies have gone on to look at cell-type specific functions in HIV-1 infection. Most notably, Hyrcza et. al[305] showed overall distinct transcriptional profiles of CD4+ and CD8+ T lymphocytes in acute and chronically HIV-1 infected individuals compared to aviremic or HIV-1 negative controls. Although this group does report similar expression of some classes of genes in the CD4+ and CD8+ populations of acute and chronically infected individuals, specifically the interferon stimulated genes, the bulk of differentially regulated genes were unique to each cell type, particularly in the CD8+ compartment.

Although different array platforms and the larger number of samples tested in the study by Hyrcza et. al. make direct comparison difficult, the overall message of specific cell types expressing unique sets of genes remains clear. This being the case, we feel genomic analysis is most informative when a specific cell subset is studied. Thus, for all further studies outlined in this thesis we chose to focus on only the CD4+ T cell population due to their obvious importance in HIV-1 infection and, as shown previously by our lab[268], their pivotal role in HIV-1 resistance.

6.2 High Level of Immune Dysfunction is seen by Gene Expression Analysis in Asymptomatic, HIV-1 Positive Individuals

Loss of CD4+ T lymphocytes is seen as the hallmark of HIV disease progression. Several factors are thought to play a role in this process including viral load, rate of T cell loss and immune activation. Several functional immunologic investigations have been carried out comparing individuals at all stages of HIV-1 disease progression. It is known that throughout the course of HIV-1 infection, and in the absence of treatment, levels of immune activation may remain high (reviewed in Douek D, 2003[341]). However, despite these elevated levels of immune activation, individuals who maintain relatively high levels of CD4+ T cells are capable of mounting reasonably normal immune responses. These observations lead to the question, to what extent does HIV-1 infection impact CD4+ T cell function prior to dramatic losses of CD4+ T cell numbers and progression to AIDS? Microarray analysis allows this question to be addressed in detail, by monitoring expression of several thousands of genes simultaneously. As well, the cohorts under study by this group and previously described in this thesis provide an unique opportunity to study CD4+ T cell function in the relatively early stages of HIV disease (CD4 > 350) prior to the initiation of antiretroviral therapy.

For this study it was hypothesized that HIV-1 infected individuals with relatively high CD4+ T cell counts would demonstrate only minor differences in CD4+ T cell function, as measured by gene expression, when compared to HIV-1 low-risk negative individuals. Initial total gene expression analysis, in the form of correlations of gene expression (Section 5.2.5) showed this not to be the case (Figure 5b and c). Correlation of mean gene

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expression values showed much larger changes in gene expression after antigenic stimulation in HIV+ individuals compared to HIV-LRN individuals as measured by the correlation of each stimulation condition to that of the media alone condition (Figure 5b and a respectively). Furthermore, correlations of mean gene expression between HIV+ and HIV-LRN individuals showed much lower values than those seen when comparisons were made between HIV-LRN and HIV-R individuals, often showing negative correlation values (Figure 5c compared to Figure 14). These data suggest a level of differential CD4+ T cell function in asymptomatic HIV+ individuals that is much greater than was originally hypothesized.

It was further hypothesized that the differences in gene expression that did exist would primarily be seen in genes involved in CD4+ T cell activation. Analysis of lists of genes expressed at a lower level in HIV+ individuals compared to HIV-LRN women at baseline (Section 5.2.6) showed several interesting trends (Table 10). First, as expected several transcription factors, signal transduction molecules and genes involved in apoptosis were expressed at a higher level in HIV+ individuals. This is in line with several other genomic studies reporting increased activation in cell populations that are either HIV-1 infected or that have been exposed to HIV-1 proteins[303, 302, 304, 300, 301]. Particularly, the higher expression of the transcription factor SP-1, a gene that has been shown to be up-regulated by HIV-1 Tat protein and to be involved in LTR activation[325, 55], is evidence of the induction of a cellular state permissive for HIV-1 replication.

Interestingly, expression of CCR5 is lower in HIV+ individuals, as this receptor is of obvious importance to HIV-1 replication this is a curious finding. However, whether this

is an actual down-regulation or simply a reflection of a lower proportion of cells expressing CCR5 among the CD4+ T cell population is unknown. Also, the lower expression of STAT5B is also unexpected as this molecule has been shown to be involved in HIV-1 replication and thus would be expected to be expressed at a higher level by HIV-1 infected individuals. However, this may be a kinetic issue as the impact of STAT5B on HIV-1 replication may be greatest during the early events of infection[324].

Gene expression levels in HIV+ individuals after antigen stimulation may provide insight into levels of immune dysfunction not seen in unstimulated cells. This issue was investigated in Section 5.2.7. Of particular interest to this study are those genes expressed at either a higher or lower level in HIV+ individuals after stimulation with HIV-1 p24 (Tables 11 and 12), as this best models what may be going on during infection in terms of a consistent recall response to HIV-1 proteins. Interestingly, both the TCR alpha locus and SHC1, two genes involved in T cell receptor signaling, are expressed at a higher level in HIV+ individuals (Table 11). This, combined with the higher expression of CD69 (Table 11), suggests a heightened activation of CD4+ T cells after p24 stimulation, a condition that would favor virus production. Furthermore, the type of immune response generated after p24 stimulation is of interest. Both the IL-4 and IL-10 receptors are expressed at a higher level in HIV+ individuals (Table 11) while the IL-2 and IL-12 receptors are expressed at a lower level (Table 12). This suggests both an activation of a Th2 type of immunity (IL-4) and an inhibition of a Th1 type immunity (IL-10), which may be favorable for the virus, as humoral immunity is less effective at controlling HIV infection than is cell-mediated immunity.

Overall, the data presented here demonstrate a large amount of immune dysfunction in HIV+ individuals prior to the onset of HIV disease. However, one of the main drawbacks of this study is the lack of available data on viral load in this population. Two other genomic studies exist that examine T cell function in HIV-1 infection, both showing large numbers of differentially expressed genes in cases of heightened viremia, even in the presence of a normal CD4+ T cell count[**304, 305**]. Further studies in these cohorts including virological details would be of interest to confirm findings by these other groups.

6.3 HIV-1 Resistant CSW Show Reduced Levels of Expression of Key Immune Mediators Ex *Vivo*

6.3.1 Perspective on HIV-1 resistance

The idea that not all individuals that are continuously exposed to HIV-1 will necessarily become infected is now well accepted. Several real-world models of reduced susceptibility to HIV-1 infection have been demonstrated (reviewed in Kulkarni 2003[**22**]). Among these, populations of highly exposed individuals, such as commercial sex-workers living in areas of high HIV-1 prevalence, stand out as the best model to inform vaccine design as it is supposed that they resist infection despite extensive exposure to a heterogenous population of virus. Determining precise mechanisms that mediate protection in these populations is crucial to establishing better methods to prevent infection in the general population.

As outlined in the introduction, several studies have been conducted into both genetic and immunologic mechanisms of reduced susceptibility to HIV-1 infection. Genetic factors including co-receptor mutation, HLA alleles, KIR alleles and immune gene polymorphism have all been associated with resistance to HIV-1 infection in one or more cohort[247, 232, 282, 283, 286, 287, 288, 290, 291, 292]. As well, HIV-1 specific CTL, mucosal IgA, circulating CD4+ T cells and a variety of innate immune factors have been shown to correlate with the resistance phenotype[268, 259, 24, 256, 264, 265, 262, 251, 250, 252, 237, 232, 254, 255, 257, 258, 260, 261, 263, 28, 233, 269]. However, to date the only proven mechanism mediating HIV-1 resistance is the delta32 CCR5 mutation that prevents infection by interrupting the earliest stage of infection. Since this mutation is not present in all resistant populations the search to describe other mechanisms of resistance, be they genetic or immunologic, is ongoing.

In very broad terms, resistance to HIV-1 infection may be due to either adaptive immune function, allowing the virus to be cleared prior to the establishment of persistent infection or to low levels of cellular susceptibility, in the form of either inhibition of some stage in the viral life cycle or the presence of some innate antiviral factor. In the first instance, infection would have to progress to an early stage, allowing for antigenic processing and presentation to an adaptive immune system that responds more capably to clear infected cells than occurs in the susceptible population. The presence of circulating HIV-1 specific CTL and CD4+ T cells may be seen as evidence for this type of resistance. However, these HIV-1 specific responses are only seen in a proportion of HIV-1 resistant people and are transient even in these individuals. In the second case, the cells of HIV-1 resistant individuals would necessarily function differently than HIV-1 susceptible

individuals by either increased production of some innate antiviral factor(s) or decreased levels of some factor(s) required by the virus for productive replication.

As both of these mechanisms require some amount of differential function at the cellular level, either innately or in response to stimulation, between HIV-R and HIV-1 negative (susceptible) individuals, we chose to analyse cell function as multi-parametrically as possible. To date, only one population-based genomic study of cell function in the context of reduced susceptibility to HIV-1 has been published[24]. This study focused on a discordant couple population and studied activated T cell function. The major finding of this study was the discovery of higher production levels of several innate immune factors, most importantly IL-22. This study both underscores the utility of genomics in identifying factors potentially mediating resistance to infection and the necessity to examine subsets of cells in such studies. However, as with all studies of HIV-1 exposed-uninfected populations these results are possibly population-specific and as such, similar studies in other populations are essential to either confirm these results in a distinct population or define other mechanisms mediating protection.

6.3.2 The role of CD4+ T cells in HIV-1 resistance

As outlined in Section 6.1, microarray studies are most informative when conducted on a specific cell subset. In this study we chose to examine the role of CD4+ T cell function in the context of reduced susceptibility to HIV-1 infection. This population is of particular interest for several reasons including; the proven association of HIV-specific CD4+ T cell responses with resistance in this cohort[268], the crucial role of CD4+ T cells in directing the adaptive immune response, and because they are the primary cellular target of HIV-1

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infection, thus allowing for the simultaneous evaluation of immune function and the possibility of reduced cellular susceptibility to HIV-1.

In addition to the possible role of HIV-1 specific CD4+ T cells in mediating protection, a small number of studies have investigated differing levels of immune activation in various cohorts of HIV-1 exposed seronegative individuals. Koning et al demonstrated a lower percentage of activated CD4+ T cells as measured by expression of the cellular markers HLA-DR, CD38 and CD70 and a lower number of proliferating CD4+ T cells, as measured by the marker Ki67, in a cohort of sexually-exposed seronegative men compared to susceptible individuals[271]. As well, Begaud et al showed lower levels of HLA-DR+ CD4+ cells in HIV-1 negative partners of HIV-1 infected individuals. They further went on to show lower levels of susceptibility of PBMC from the exposed-uninfected individuals, compared to control PBMC, when exposed in culture to laboratory strains of HIV-1 in an activation independent infection model[272]. In a cohort of exposed-uninfected female sex workers from Cote d'Ivoire, Jennes et al demonstrated lower levels of CD69+ CD8+ T cells and lower production of IFNγ and MIP1β after allogenic stimulation in a mixed lymphocyte reaction[270]. However, a previous study by this same group actually showed higher levels of activation, as measured by levels of CD38+ CD8+ T cells, in unstimulated PBMC of exposed-uninfected sex-workers, and no difference in the CD4+ compartment[342]. Finally, a study of exposed-uninfected IV drug users from Vietnam showed higher activation, in the form of higher levels of CD38+ HLA-DR+ cells in both the CD4+ and CD8+ compartments. However, this study used normal blood donors as a comparison group raising the concern that IV drug use may be a confounder[343]. As well, the differing

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routes of exposure make comparison between this study and our own difficult. However, what is clear from these studies is that evidence exists to suggest that differing levels of T cell activation may have an impact on host-level susceptibility to HIV-1 infection.

6.3.3 Study rationale and description

On the whole, the studies that have investigated immune activation suggest that lowered activation may contribute to lowered susceptibility to sexual transmission of HIV-1. However, all of these studies have focused on cell surface markers of immune activation and have not investigated the consequences of this lowered activation intracellularly. Thus, the cellular consequences of this lower activation and the identification of particular pathways that may be either responsible for or impacted by this lowered activation remains un-explored.

Overall, the above stated rationale lead us to hypothesize that HIV-R CSW have functionally distinct populations of CD4+ T cells compared to HIV-1 susceptible controls. We further hypothesize that identification of genes shown to be differentially expressed in the HIV-R cohort will show evidence of reduced expression of genes involved in immune activation.

To address these hypotheses, two separate studies were carried out. The first study compared the gene expression profiles of CD4+ T cells of HIV-R women to HIV-1 negative low-risk controls (HIV-LRN), both at baseline and in response to antigenic stimulation, using immune focused nylon microarrays. Subsequently, expression profiles of unstimulated CD4+ T cells from HIV-R women were compared to HIV-1 negative

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sex-workers not meeting the definition of HIV-1 resistance outlined in Section 4.2.1.2 (so called high-risk negatives, HIV-HRN) using the Affymetrix U133 Plus 2.0 GeneChip. For simplicity, these studies will be discussed individually first with general conclusions made at the end of this section.

6.3.4 Gene expression analysis demonstrates differential CD4+ T cell function in HIV-R women compared to low-risk controls

A small pilot study (N=5 in both groups) was carried out in order to determine broadly if differences in immune gene expression could be measured between HIV-R and HIV-1 negative (in this case HIV-LRN) individuals. Based on those findings, a larger study was conducted (HIV-R N=17, HIV-LRN N=10) to expand the dataset and add statistical rigor. To this end the following hypotheses were made:

- CD4+ T cells of HIV resistant commercial sex-workers will show an inducible pattern of gene expression that differs from HIV-LRN women.

- Unstimulated levels of gene expression in CD4+ T cells of HIV-R women will show evidence of higher relative expression of genes known to inhibit steps in the HIV-1 life cycle and lower expression of genes involved in T cell activation.

- Single gene and gene interaction analyses will allow for discrimination of individuals into phenotypic categories based on HIV susceptibility.

- HIV-R women will show lower levels of secretion of key immune mediators both at baseline and after stimulation.

To address these, in both the pilot study and the larger sample set we investigated overall patterns of gene expression and individual genes that were differentially expressed both after antigenic stimulation (PHA, *C. albicans*, flu peptide and HIV-1 p24) and at baseline. In the larger study we were also able to determine gene pair interactions that significantly discriminated between the HIV-R and the HIV-LRN populations and then, based on multiple gene pair interactions describe a network of gene interactions that could be used to predict the resistance phenotype in prospective studies.

6.3.4.1 Sources of variation and correlation analysis of HIV-R compared to HIV-LRN individuals

A rather general means by which to address the hypothesis that HIV-R and HIV-LRN individuals would show distinct patterns of gene expression in the CD4+ T cell compartment is to compare the entire, unfiltered datasets. This was performed first by examining overall variation between sample groups over all stimulation conditions (Section 5.4.7) and then by correlating gene expression in each stimulation condition both within and between sample groups (Section 5.4.8).

Sources of variation analysis showed that the two main variables contributing to the variation seen in the dataset were sample identity and HIV status (Figure 12). In the first case, this suggests that a given individual varies significantly compared to any other given individual. This is to be expected in a human population as there is obviously heterogeneity in terms of the immune response. The large contribution of HIV status (i.e. HIV-R or HIV negative) to the overall variation is interesting. This suggests that a gene

expression pattern exists that underlies all stimulation conditions that may characterize HIV-1 resistance.

Correlation of gene expression values within a given group demonstrated relatively similar impacts of each stimulation condition within a group (Figure 13). When comparisons were made between groups (Figure 14), it was seen that both HIV-R individuals and HIV-LRN individuals respond differently to stimulation, particularly in response to PHA.

Overall these data demonstrate that HIV-R and HIV-LRN individuals show differential gene expression both at baseline and in response to stimulation. Determining the individual genes and pathways that define this differential expression is of great interest.

6.3.4.2 Cluster analysis HIV-R vs HIV-LRN

In both the initial pilot study and in the larger study hierarchical cluster analyses of genes and arrays were performed on gene lists containing either all genes (Figures 6 and 10) and on gene lists filtered to include only those genes showing at least a two fold change in expression after stimulation in at least 1 individual (Figures 7 and 11). Similar principals have been previously applied to demonstrate gene expression patterns at different clinical stages of HIV-1 infection[344], and to distinguish between viremic and aviremic HIV-1 infected individuals[345, 305, 304]. However, since genomic studies in HIV-R individuals are limited, this technique has not been previously applied to these groups.

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Initial analysis of 5 samples in both the HIV-R and HIV-LRN groups demonstrated

clustering together of 4 of the 5 HIV-R individuals based on expression of all genes

(Figure 6) and on the expression of the genes that passed the 2-fold cutoff in at least 1

individual (Figure 7) after either *C. albicans* stimulation (Figures 6a, 7a) or stimulation

with flu peptide (Figures 6c, 7c). This initial finding was quite interesting as it suggested

an antigen-induced gene expression pattern that was non-HIV-1 specific, i.e. based on

CD4+ T cell recall response to two common non-HIV antigens. Thus it was concluded

that CD4+ T cells of HIV-R women behave distinctly from HIV-LRN individuals. It is

interesting to note that after both *C. albicans* and flu peptide stimulation the one HIV-R

sample that did not cluster with the others was the same, ML 767. However, this sample

often fell just outside of the main cluster of HIV-R individuals (Figures 6a, 6c, 7a, 7c).

That no considerable clustering of individuals into groups based on HIV status was seen

after PHA stimulation is not surprising, as the activation seen after mitogenic stimulation

would likely overwhelm any subtle pathways involved in HIV-1 resistance. Conversely,

the lack of clustering of HIV-R individuals after HIV-1 p24 stimulation was not

expected. As these individuals have previously been exposed to HIV-1, one would expect

a clear association of all individuals after p24 stimulation, indicative of a recall response.

However, it is important to point out that the HIV-1 specific CD4+ T cell responses

previously reported in HIV-R individuals in this cohort were only seen in a proportion of

individuals[268] and thus may either not be present in all individuals or may wane

depending on the length of time from exposure.

Upon expansion of this initial study to include larger numbers of individuals in both the HIV-R (N=17) and HIV-LRN (N=10) populations, the initial clustering results were not maintained (Figures 10, 11). Although clusters of subsets of HIV-R individuals could be identified in most conditions (boxes within Figures 10 and 11) there was no clear division between all individuals based on either total gene expression or expression of genes meeting the 2-fold cutoff. There are two possible explanations for this observation; first it is possible that the lack of discrimination between sample groups is due to the large numbers of genes being considered i.e. the resistance phenotype is determined by a smaller number of genes than is identified through either filtering method. Second, the act of stimulation may alter the gene expression profiles of the cells under study such that the expression of genes that contribute to resistance to infection in the unstimulated condition is altered. Either of these explanations is possible separately or in combination and was explored further by more focused analyses as discussed later on in this section.

Overall, the lack of a definable expression pattern as determined by cluster analysis is not evidence that such a pattern does not exist. For example Hyrcza et al, only saw definable clusters in their HIV-1 viremic vs aviremic patients once a select set of genes, the interferon stimulated genes, were considered[305]. As well, the findings by other studies that such expression patterns exist in HIV+ individuals with varying levels of viremia does not directly contradict our findings. It is likely in these cases that HIV-1 infection itself is what distinguishes these populations. As shown in Section 5.2 and discussed in Section 6.2, HIV-1 infection has a dramatic impact on the function of immune cells. It is likely that the HIV-1 resistance phenotype is much more subtle. Therefore, focusing on those genes shown to be significantly differentially regulated in the HIV-R population

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upon stimulation and at baseline is necessary to define specific genes and pathways involved in mediating resistance.

6.3.4.3 Antigen-stimulated gene expression differences between HIV-R and HIV-LRN individuals

The type of immune response generated by HIV-R women after antigenic stimulation may play an important role in determining susceptibility to HIV-1 infection. A previous study on this group of HIV-R CSW showed evidence for lower production of IL-4 in response to a variety of different antigenic stimuli, suggesting a bias towards type-1 immunity in response to recall antigens[346]. As well, CD4+ T cells of HIV-R women who responded to p24 peptide showed an increased ability to proliferate compared to HIV-1 infected women[268]. Thus, gene expression profiles of CD4+ T cells after stimulation with a general immune stimulus and both HIV-1 and non-HIV-1 antigens may provide insight into specific genes and pathways that mediate this differential function. This being the case, PBMC cultures of HIV-R and HIV-LRN women were exposed to mitogenic stimulation (PHA), two recall antigens to which exposure in these populations was assumed to be similar, *C. albicans* and flu peptide, and an HIV-1 specific antigen (HIV-1 p24). Gene expression differences were measured after antigenic stimulation in order to identify genes and pathways that may play a role in meditating HIV-1 resistance, both in the initial pilot study and in the expanded study.

In the initial study set of 5 HIV-R and 5 HIV-LRN individuals, HIV-R individuals showed a trend towards down-regulation of a variety of cytokine, chemokine and transcription factor genes, with many of these genes being down-regulated in more than

one stimulation condition (Table 15), with several of these genes having been previously shown to interact with HIV-1. Firstly, previous studies have shown that HIV-1 Tat increases expression of IL-6 and IL-8 likely in an NF-κB-dependant manner[347]. That these genes showed a down-regulation after *C. albicans* stimulation that was not mirrored in the HIV-LRN population may suggest a subtle differential function of NF-κB in the HIV-R population. This is of interest as NF-κB has been shown to be crucial for HIV-1 replication in the early stages of infection[80]. Next, the reduced expression of STAT5b and IRF-2 after PHA and p24 stimulation respectively would seem to agree with previous results as both STAT5b and IRF-2 have been shown to be expressed at lower levels in CD4+ T cell lines shown to be resistant to HIV-1 infection[348]. As well, STAT5b has been shown to interact with the HIV-1 LTR enhancing transcription and replication[324]. Finally, and in contrast to what might be expected, two genes shown previously to inhibit HIV-1 replication, MIP-1β and SP3, were down regulated after stimulation, with SP3 being down regulated in all conditions. It has been well documented that MIP-1β, a beta chemokine that interacts with CCR5, is able to inhibit HIV-1 infection by competitive inhibition of the co-receptor interaction[183]. SP3 is a member of the SP family of transcriptions factors, which are responsible for regulating transcription of a variety of human genes. As well both SP1 and SP3 have been shown to bind the HIV-1 LTR, but with opposite effects, whereas SP1 has been shown to enhance HIV-1 LTR transcription, SP3 inhibits this activation[325]. However, it is possible that the apparent down regulation of these genes after stimulation is related to a higher expression in HIV-R women prior to antigenic stimulation. To address this issue unstimulated levels of gene expression need to be considered.

Following these initial analyses, further sample data were acquired and the datasets were combined to gain statistical confidence in those genes differentially expressed between the HIV-R and HIV-LRN populations. Comparison of gene expression levels after antigenic stimulation between the HIV-R and HIV-LRN populations again showed cytokine, chemokine and transcription factor genes to be differentially regulated (Table 20). However, few of these genes confirmed the observations seen in the analysis of the pilot dataset (Table 15). In fact, SP3, highly down regulated after p24 stimulation in the initial analysis, showed a higher expression level in HIV-R individuals after p24 stimulation, once the larger number of samples were analysed. Although the fold difference is small, 1.09, the p value is quite significant, 0.0004, reflective of low variation between HIV-R samples. As these analyses include a larger number of samples, 17 compared to 5, this is likely more indicative of the actual expression levels of SP3 in the HIV-R population when compared to the HIV-LRN population. Several other interesting observations, particularly in the HIV-1 p24 stimulated samples were made. The higher expression of NFKBIE, a known inhibitor of NF-κB activity, is of interest as previous data presented here suggest several genes regulated by NF-κB to be expressed at a lower level in the HIV-R population. As well, the apparent lower expression level of the T cell receptor alpha and delta locus after p24 stimulation could be evidence of lower activation levels of CD4+ T cells, specifically in response to HIV-1, as T cell receptor signaling is the major mechanism of activation.

Overall, these data suggest some interesting gene regulation differences between HIV-R and HIV-LRN individuals particularly in response to HIV-1 p24 stimulation. However, in the *in vivo* condition, the CD4+ T cells of these HIV-R women likely come into contact

with HIV-1 in an antigen-unstimulated manner. Thus, the analysis of how these cells are behaving at baseline may provide interesting findings that will help to better define the HIV-1 resistant phenotype.

6.3.4.4 Unstimulated gene expression differences between HIV-R and HIV-LRN individuals

As mentioned above, baseline gene expression data of CD4+ T cells may be of interest as it likely represents the manner in which these cells would encounter HIV-1 *in vivo*. This being the case, unstimulated levels of gene expression were compared between the HIV-R and HIV-LRN populations in both the initial pilot study and in the larger sample set.

In the pilot study, the comparison of 5 HIV-R and 5 HIV-LRN individuals revealed differential baseline CD4+ T cell expression levels of 73 genes. Manual evaluation of these lists revealed several genes, previously implicated in blocking HIV-1 replication, to be expressed at a higher level in HIV-R women (Table 16). Specifically, MIP-1β and SP3, as discussed above, are of interest due to their proven abilities to block very early stages of HIV-1 replication, entry and LTR transactivation respectively[349, 325]. The finding that these genes are expressed at a higher level in HIV-R women prior to stimulation, and then are down regulated after stimulation is of interest. It is possible that the high baseline levels of these genes, those that would function to prevent the early stages of HIV-1 infection, are shut off by some regulatory mechanism, in order to allow for normal immunologic stimulation in the presence of antigen.

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The finding that STAT5b, a known enhancer of HIV-1 replication[324], is expressed at a higher level in HIV-R women at baseline is counter to what would be expected. However, it is known that the initial stages of induction of HIV-1 replication require several different host factors, and the various contributions of these and their ability to either complement or interfere with each other, particularly in the presence of known inhibitors of LTR interaction, such as SP3 is not known. Therefore, it is possible that the enhancing effects usually attributed to STAT5b are not sufficient to counteract the inhibitory effect of genes such as SP3, and that lower levels of other genes required by HIV-1 for replication contribute to limit HIV-1 susceptibility. Further studies addressing these issues are necessary in order to answer this important question.

Another interesting finding is the higher expression of defensin alpha 6 (DEFA6). The alpha defensins are known to be small molecules involved in innate antiviral defense. Previous studies have shown certain members of this family, specifically alpha defensins 1, 2, and 3 to be expressed at a higher level in both HIV-1 exposed seronegative women[350] and HIV-1 long-term non-progressors[188]. As well, a recent study has attributed the anti-HIV-1 properties of these molecules to their ability to bind both gp120 and CD4, thus inhibiting the initial steps necessary for infection[351]. However, to date little is known about the effects of DEFA6, although it is thought to possibly be involved in the immune response in the gut[352]. It is well known that mucosal immune responses early on in infection, particularly the extent of infection and immune depletion in the gut, play a large role in determining the outcome of HIV-1 infection[95, 345, 94]. Thus, if this higher expression of DEFA6 seen in the periphery were mirrored in the gut of HIV-R women, it is possible that it could substantially impact infection. Despite the lack of

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available data, the structural and functional relatedness of this molecule to those known to inhibit HIV-1 make its higher expression in the HIV-R population significant.

Analysis of those genes expressed at a lower level in HIV-R women also showed several interesting findings. The overall trend in these genes is towards lower expression of genes involved in cell cycle regulation, signal transduction and T cell signaling, suggesting an overall lower level of activation of CD4+ T cells in HIV-R women (Table 17). This theory is supported by the lower expression of CD69, a protein expressed almost exclusively on activated T cells[353].

As well, several cytokine genes were expressed at a lower level. The finding of lower levels of IL-2 is consistent with the theory of lower T cell activation, as this cytokine is well known to be involved in CD4+ T cell activation and has been shown to enhance replication of HIV-1. Also, IL-16, expressed at a lower level by HIV-R women, is involved in T cell activation further supporting this hypothesis. Interestingly, IL-16 has also been shown to inhibit HIV-1 promoter activity *in vitro*[354]; however, the *in vivo* significance of this is unknown. Similarly, the lower level of IL-1β transcripts detected in HIV-R women is indicative of lower activation levels in these women. IL-1β has been shown to activate T cells and to enhance the production of HIV-1 in latently infected cells[355]. Finally, the lower expression of IRF-1, an innate cytokine involved in the interferon response, is consistent with previous findings by this group, of lower production of this molecule in HIV-R women, which may function to limit early replication by HIV-1[297].

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Pathway analysis including all genes that were differentially expressed between HIV-R and HIV-LRN individuals further supports the lower activation hypothesis. These analyses identified three genes all expressed at a lower level in HIV-R women that are known to be involved in T cell activation and cytokine signaling (Figure 8), specifically; SHC, Raf1 and c-Jun. These genes are all poly-functional signal transduction molecules involved in a variety of biological processes (reviewed in Ravichandran, 2001[356]). Of particular interest here is the lower expression of c-Jun, a component of the transcription factor AP-1 (reviewed in Hess J 2004[357]). AP-1, required for the transcription of several human genes, has been shown to interact with NFAT and bind to the HIV-1 LTR thus enhancing LTR transcription levels[358]. Thus, the lower expression of this gene could have an impact not only on the level of T cell activation but on the ability of HIV-1 to replicate during the early stages of infection as well.

As in the antigen-stimulated gene expression section, after the pilot study analyses described above, additional samples were run to strengthen the confidence in gene expression changes found. Although not all specific genes previously shown to be expressed at different levels in HIV-R in comparison to HIV-LRN women were confirmed in these larger sample sets, the overall trend towards lowered levels of genes involved in cellular activation was consistent.

Firstly, analysis of lists of genes expressed at a higher level in HIV-R women (Table 21) showed three main groups of genes to be differentially regulated. Genes involved in inhibition of cell growth, cellular activation and transcription were expressed at a higher level in HIV-R individuals. Specifically, CDKN2A, a known growth inhibitor involved in

tumor suppression[359], PSTPIP1 (also known as CD2BP1) shown to be a negative regulator of T cell activation in murine cells[328] and NFKBIE, a member of the IκB family of NF-κB inhibitors. Although little has been published on NFKBIE, the closely related proteins NFKBIA and NFKBIB (also known as IkBA and IkBB) are well known to inhibit the formation of NF-κB complexes thus inhibiting NF-κB mediated transcription (reviewed in Li et al, 2002[360]). As mentioned previously, cellular activation in general and NF-κB activity in particular, have a large impact on the early stages of HIV-1 infection.The presence of these inhibitors may either directly play a role in limiting HIV-1 infection in its early stages or may be evidence of an overall trend towards lower levels of activation.

When analyzing the list of genes expressed at a lower level by HIV-R women compared to HIV-LRN women (Table 22), further evidence of lower levels of cellular activation were found. The lower expression levels of the cell proliferation gene PCNA, transcription factors NFIC and AP2S1, adenosine receptor ADORA2B, and signal transduction molecule MAPKAPK3, is consistent with this hypothesis.

Of these genes, perhaps the most interesting are the NFI family transcription factor member NFIC (also known as CTF) and the receptor ADORA2B (also known as A2B). The NFI transcription factors have been implicated in regulation of transcription in a variety of organisms and have been shown to enhance transcription in several virus models (reviewed in Gronostajski 2000[361]). Perhaps most interestingly, NFI proteins have been implicated in the TGFβ and TNF signaling pathways[362, 363] and in adenoviral replication [364]. As well, NFIC has been shown to interact synergistically

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with the HIV-1 Tat protein to enhance transcriptional activity and splicing in an *in vitro* setting[365]. The role this gene may play in regulating HIV-1 transcription in a more natural situation remains to be explored.

Furthermore, the adenosine receptor ADORA2B has been shown to be involved in cellular activation resulting in increased production of TNF and IL-6 which in turn further up regulate ADORA2B in some cell types[366, 367]. As both TNF and IL-6 expression are linked to NF-κB activity (discussed in detail later), the lower expression level of ADORA2B supports the lower activation theory and further defines a role for specific cytokine signaling pathways involving NF-κB.

Also of note, the largest negative fold difference between HIV-R and HIV-LRN individuals is seen in the gene caveolin 1 (CAV1). CAV1 is a scaffolding protein found in the membrane of several cell types and involved in protein accumulation into membrane rafts[368]. This protein has also been found to accumulate in endomembranous compartments and contribute to endocytosis and signal transduction[369]. In the context of HIV-1 infection CAV1 has been shown to specifically interact with gp41, and is likely involved in the initial stages of virus entry. Furthermore, antibodies directed against the region of CAV1 that binds gp41 were shown to prevent infection of primary CD4+ T cells[370, 371]. Conversely, an earlier study showed that co-expression of CAV1 and HIV-1 in transfected 293T cells actually inhibited HIV-1 proviral DNA production in a dose-dependant manner[372]. The *in vivo* significance of this finding is unknown. However, in the context of natural resistance to infection, it is more likely that inhibition of infection, i.e. reduction in viral entry plays a

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greater role than the possible enhancing effects of lower CAV1 expression post-integration.

Overall, results seen in the comparison of unstimulated CD4+ T cell function between HIV-R and HIV-LRN individuals demonstrates evidence of lower levels of cellular activation and modified expression of genes possibly involved in HIV-1 replication. Further exploration of the interaction of these genes with one another and examination of their downstream effects, in terms of cytokine production will add to the definition of the HIV-R phenotype.

6.3.4.5 Confirmation of gene expression differences in HIV-R compared to HIV-LRN by qRT-PCR

In order to confirm gene expression changes as seen by microarray, quantitative real-time reverse-transcriptase PCR is generally employed. Due to the limited sample source and the resulting limited quantity of RNA remaining after the array experiments carried out over the course of this thesis, qRT-PCR confirmation was a significant challenge. Of the genes we chose for confirmation studies, we were unable to amplify several from the remaining sample material. Furthermore, the genes that were shown to amplify were often only present at low-levels. It is well accepted that the quality of qRT-PCR data is related to the amount of transcript available for amplification. It is likely this fact that contributed to the limited success in confirming gene expression levels by qRT-PCR (Table 23). This point is underscored by the relative success of qRT-PCR confirmation in the later array study (discussed later). However, it should be noted that the two genes showing the same trend in both array and qRT analysis, JUN and NFIC (Table 23) both

had relatively high transcript levels, as assessed by mean crossing point, and are thus likely to reflect actual transcript levels. As well, both these genes support the overall theme of lower levels of activation in the HIV-R population put forth thus far in this thesis. Further confirmation of the other genes would require follow-up sample acquisition, including the complimentary protein samples, which should certainly be a future direction of this study.

6.3.4.6 Predictive interaction analysis for discrimination of HIV-R from HIV-LRN individuals

The ability to predict which individuals, upon exposure to HIV-1, might resist infection is a goal of obvious importance as it would allow for the identification of individuals for prospective studies. As well, the parameters used to define the resistance phenotype could serve to educate preventative treatments such as vaccines. As HIV-1 resistance is likely a multi-parametric trait, techniques that involve more than just single gene analysis, i.e. those discussed in the previous section, are useful in outlining the HIV-1 resistant phenotype. Recently, predictive interaction analysis (PIA) has been used to predict which patients may develop graft-versus-host disease following organ transplant[318]. PIA is a data-driven approach that attempts to describe a particular condition of interest based on competitive or synergistic gene-pair interactions predicted from microarray data. These interactions are often much better at predicting sample class than are the component single genes, as measured by standard statistical methods. Furthermore, these predicted interactions can be used to determine larger networks of genes involved in establishing a particular trait. In this thesis, PIA was used to determine significant two-gene interactions

and gene interaction networks that could be used to discriminate between HIV-R and HIV-LRN individuals, based on baseline expression of CD4+ T cells.

Based on our dataset, 38 significant interactions, either synergistic or competitive, were identified (Table 24). All gene interaction p values were substantially smaller than those of either component gene, and in many cases at least one member of the gene pair was not significantly differentially expressed in the single gene analysis. The power of this type of analysis to improve class prediction over that seen by single gene analysis is demonstrated in the competitive interaction measured between ADORA2B and PSTPIP1 (Figure 15). Based on expression of either gene individually, discrimination of HIV-R from HIV-LRN individuals shows a sensitivity (defined as percentage of HIV-R individuals correctly identified by linear discriminant analysis) of 70.5% and sensitivity values (defined as the percentage of individuals correctly identified as HIV-LRN) of 70% for ADORA2B and 60% for PSTPIP1. However, when the gene pair interaction is considered, the resulting sensitivity and specificity values are 88% and 100% respectively. This is further reflected in the p values associated with either ADORA2B (p=0.01) or PSTPIP1 (p=0.05) versus the gene pair interaction p value (p=0.00009). This particular gene pair is also of interest functionally as ADORA2B, expressed at a lower level by HIV-R individuals has been shown to be unregulated in instances of immune activation and is increased in response to TNF and IL-6[366, 327]. As well, PSTPIP1 has been shown to be a negative regulator of T cell activation[328]. This functional data lends support to the statistical interaction determined by PIA, as well as further strengthening the observation of lowered activation in HIV-R individuals.

A further benefit of PIA is the ability to expand gene pair interactions into networks of genes shown to interact through this type of analysis. Several networks can be built based on the expression data presented in Table 24 the largest of these is shown in Figure 16. Several genes comprising this network have been previously discussed, specifically NFIC, PSTPIP1 and ADORA2B, and support the hypothesis of lowered baseline activation levels.

Further investigation of the function of other members of this network reveals another interesting theme. The genes DDX1, CD59 and ITPR3 (all expressed at a lower level in HIV-R individuals) have all been shown to interact with various HIV-1 proteins and play important roles in viral replication. DDX1, an RNA binding protein involved in translation, has been shown to interact with Rev and aid in the export of long HIV-1 transcripts from the nucleus[373]. CD59, a complement regulatory protein, has been shown in *in vitro* studies to interact with HIV-1 p17 and gp41 likely aiding in viral assembly and membrane budding[374]. Finally, ITPR3 a gene involved in calcium signaling was shown to be involved in Tat mediated TNF production in macrophages[375]. Thus, this network, as defined by gene interaction analysis, contains both evidence of lowered cellular activation and lower levels of host proteins involved in HIV-1 replication. Investigating the various contributions of these genes to establishing reduced susceptibility at the cellular level would be of interest.

Overall, the ability to predict individuals that may be classified as HIV-R based on the expression of a small number of genes is an enticing prospect. Due to the large sample numbers required for cross-validation of the predicted two-gene interactions and network

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this was not possible in the given study. Future studies including a larger number of individuals would serve to refine the model presented here, possibly leading to a predictive test for the HIV-R phenotype that could be used for prospective studies and to evaluate immune responses generated by preventative methods such as vaccines and microbicides.

6.3.4.7 HIV-R individuals show lower baseline production of key immune mediators *ex vivo*

To this point, gene expression data comparing CD4+ T cell function of HIV-R and HIV-LRN individuals has revealed a theme of generalized lower expression of genes involved in immune activation accompanied by a higher level of expression of activation inhibitors, particularly in unstimulated cells. However the question remains, can this trend only be visualized at the level of gene expression or is it reflected in the general immune milieu as measured by soluble immune mediators? To address this issue, plasma samples (when possible) and cell culture supernatants from PBMC cultures were collected at the same time, and from the same samples, as RNA collection for the microarray experiments. Cytokine levels were measured at baseline and in response to antigen stimulation using BD's CBA technology. This technology allowed for the simultaneous measurement of 14 cytokines and chemokines and was carried out for both the initial pilot study and in the larger sample sets. It is important to reiterate that these measurements reflect levels of cytokines secreted by the mixed cell (PBMC) population, not just the CD4+ T cell compartment, thus directly implicating a particular cell type is not possible. However, important information into the possible *in vivo* causes or effects of the pre-defined lowered immune activation can be gained.

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In both the smaller pilot study (Figure 9) and in the larger study (Figure 17) the levels of the proinflammatory cytokines IL-1β, Il-6 and TNF were significantly lower in the HIV-R population compared to the HIV-LRN individuals. As well, plasma levels of IL-1β were also significantly lower in the HIV-R women (Figure 18 and Table 25) suggesting the results seen in culture reflect the *in vivo* condition. Unfortunately this technique was not sensitive enough to detect the very low plasma levels of either IL-6 or TNF.

The proinflammatory cytokines, typically secreted by activated monocytes and macrophages, play a critical role in innate and adaptive immunity and are key mediators of lymphocyte activation. Interestingly, both IL-1β and TNF are well known to stimulate different signaling cascades that activate transcription through NF-κB and AP-1, with IL-1β signaling though a pathway closely related to TLR signaling and TNF signaling through TNFR1. These signaling cascades result in the production of TNF, IL-1β and IL-6 (reviewed in Li Q, 2002[360]). This is particularly interesting, as data in this thesis have shown indirect evidence for altered NF-κB signaling, and direct evidence for the lower expression of JUN, a component of active AP-1. Taken together these data further support the hypothesis of lower levels of immune activity in HIV-R women, possibly related to reduced activity of NF-κB and AP-1

In addition to their obvious immunomodulatory effects, these proinflammatory cytokines have been implicated in enhancing HIV-1 infection at the cellular level. Studies of latently infected monocytic and T cell lines have shown that addition of TNF can activate replication of HIV-1, mediated through the LTR, which can then serve to stimulate

further TNF production in both an autocrine and paracrine fashion[376, 377]. As well,

both HIV-1 Tat and Nef proteins have been shown to specifically induce production of

TNF[378, 379]. These data strongly suggest a role for the induction of this cytokine in

HIV-1 replication in quiescent cells. As well, IL-6 and IL-1β have been implicated in

induction of HIV-1 replication in both monocytes and resting CD4+ T cells *in vitro*[380,

381] with IL-6 possibly acting in an NF-κB independent manner[381]. The *in vivo*

relevance of levels of these cytokines has been demonstrated as it has been shown that

levels of proinflammatory cytokines directly correlate with levels of viral shedding at

mucosal surfaces[382, 383]. Furthermore, the chemoattractive properties of these

cytokines have also been shown to be involved in recruiting susceptible cells to the sites

of HIV-1 infection[336]. Overall, lower levels of these cytokines at baseline may both

interfere with early rounds of HIV-1 replication and reduce the number of susceptible

cells *in vivo*.

The observation of lower levels of certain proinflammatory cytokines in unstimulated

PBMC of HIV-R women leads to the question of whether the production of these

cytokines is defective in these individuals. After antigenic stimulation, CBA analysis

showed no difference in production of any of these cytokines between HIV-R and HIV-

LRN individuals in either the pilot study (data not shown) or the larger sample set (Table

25 and Figure 18). This suggests that the lower baseline production of these cytokines

observed in HIV-R women does not compromise their ability to mount an antigen-

specific response and thus, when challenged by other pathogens, they are able to mount a

normal immune response. Whether this is a generalizable observation, or is antigen-

specific is an important question. Specific studies looking at the ability of HIV-1

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proteins, specifically Tat and Nef to induce these cytokines in HIV-R individuals would be of great interest.

A final observation made in this section is the apparent lower expression of IL-10 as seen in the larger sample study (Figure 17). IL-10 is considered to be an immunoregulatory cytokine and has been shown to be anti-inflammatory[163]. Thus, the lower level of this cytokine in HIV-R cell culture supernatants is paradoxical. Two possible explanations exist for this apparent discrepancy. First it is possible that the kinetics of IL-10 expression did not allow for detection of elevated levels of this molecule in supernatants. It has been shown that IL-10 is upregulated in response to higher levels of proinflammatory cytokines and functions to control production of these cytokines[384]. Second, it is possible that the regulation of IL-1β, IL-6 and TNF is, in this instance, independent of IL-10 and instead indicative either of a lower activity of a common member of their signaling pathways, such as AP-1 and NF-κB and/or through the activity of IL-10 independent regulatory T cells.

Overall, the lower baseline production of IL-1β, IL-6 and TNF in the HIV-R individuals constitutes a novel and interesting finding that deserves further study. Evidence presented in this section supports the theory of lower levels of immune activation at baseline in HIV-R women and outlines a possible role for both NF-κB and AP-1 in this phenotype.

6.3.5 Gene expression analysis demonstrates differential CD4+ T cell function in HIV-R

women compared to high-risk controls

Although the finding of lowered baseline activation in HIV-R compared to HIV-LRN

individuals is no doubt interesting, the criticism could be made that these results are

confounded by the variable of sex-work, as the HIV-LRN individuals were, by definition,

not involved in sex-work. Therefore, a second study was designed three years after the

initial study that compared CD4+ T cell gene expression in HIV-R individuals to HIV-1

negative sex-workers not meeting the definition of resistance. As these women are also

involved in sex-work, they are considered to be at high risk of infection and thus were

termed HIV-1 high-risk negatives (HIV-HRN). Based on the results presented in Sections

5.3 and 5.4 this study focused only on unstimulated gene expression in CD4+ T cells.

Based on previous findings the following hypotheses were generated:

- CD4+ T cells of HIV-1 resistant commercial sex-workers will express a distinct
  set of genes at baseline compared to HIV-1 high-risk negatives.
- HIV-1 resistant women will express lower levels of genes involved in cellular
  activation and host genes involved in HIV-1 replication and/or higher levels of
  inhibitors of these processes compared to high-risk HIV-1 negative women.
- Pathway analysis will demonstrate several genes involved in certain biological
  processes, such as immune activation; will be differentially expressed between
  groups.

As our initial comparison of HIV-R women to HIV-LRN controls showed lowered

baseline cellular activation, we wondered if this phenomenon extended beyond immune

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function and could be detected in cellular processes such as metabolism or cell division as well. This, coupled with the fact that significant advances have been made in recent years regarding signal amplification for array detection, allowed us to use the much larger total genome arrays commercially available from Affymetrix instead of the immune focused, nylon arrays. This allowed for the exploration of many more transcripts involved in many more biological processes. In order to determine genes and pathways involved in HIV-1 resistance, statistical analyses were performed comparing expression between the groups. Functional themes within the set of genes differentially expressed between HIV-R and HIV-HRN were determined using web-based pathway identification software.

6.3.5.1 Lower general gene expression characterizes HIV-R women compared to HIV-HRN individuals

Initial comparison of baseline gene expression of CD4+ T cells between HIV-R and HIV-HRN women showed a general trend of lower expression of a large number of genes in HIV-R women at every level of significance (Table 27). In fact, at the loosest definition of significance, $p<0.05$, 2,248 genes were differentially expressed between the groups with ~90% (2,024) expressed at a lower level by HIV-R individuals. This observation supports the hypothesis of lowered activation in HIV-R individuals.

In order to visualize this trend and to determine the ability of the differentially expressed genes to discriminate between sample classes, cluster analysis was performed. This was done using highly differentially expressed genes ($p<0.001$) and showed exact discrimination between samples in a given group (Figure 19). As the genes used to

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perform this analysis were preselected to be different between groups this is not all that surprising. However, inspection of the heatmap does show very good concordance in the level of expression of the selected genes within the HIV-R population (left half of Figure 19) compared to the HIV-HRN individuals (right half of Figure 19). As well, it strengthens the hypothesis of lowered activation in HIV-R individuals, as the genes that characterize HIV-R individuals are predominantly expressed at a lower level than high-risk controls. These data suggest that the trends noticed in the comparison between HIV-R and HIV-LRN will be upheld even when the variable of sex-work is controlled for. Investigation of the specific genes and pathways that are differentially expressed between these groups should further support this.

6.3.5.2 HIV-R women show lower expression of host genes involved in HIV-1 replication

As discussed in the previous section (6.3.4) it was seen that HIV-R individuals expressed higher levels of genes shown to suppress HIV-1 replication (specifically MIP1β and SP3) and lower levels of genes that aid in viral replication (specifically JUN and CAV1). As this may directly impact on the host cell susceptibility, it was of interest to see if similar genes could be found when comparing HIV-R and HIV-HRN individuals. Upon analyzing the list of significant genes for genes known to be involved in the HIV-1 life cycle, several genes were identified that were expressed at a lower levels in HIV-R individuals compared to high-risk controls (Table 28). In terms of their involvement in HIV-1 replication, these genes act at a variety of postentry steps.

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The petidyl-prolyl cis-trans isomerase cyclophilin a (CYPA) is a molecule known to aid in proper folding of several cellular proteins[385]. The importance of this protein in enhancing HIV-1 replication has also been demonstrated[386, 387]. In fact, it has been shown that CYPA is actually incorporated into mature virions[65]. The precise mechanism that mediates this enhancement is unknown. However, CYPA has been shown to directly bind to the HIV-1 capsid, and that its enhancing function occurs prior to reverse transcription[386]. Previously it was thought that the binding of CYPA to the HIV-1 capsid may protect it from the TRIM family of restriction factors, such as TRIM5α[388, 389], however this has proven not to be the case[330].

It has been shown that both PP1 and NF-κB (of which both the NFKB1 and RELA sub-units are expressed at a lower level in HIV-R, -1.7 and -1.4 fold respectively) are involved in HIV-1 early gene transcription. The protein phosphatase 1 (PP1) is a member of a family of serine/threonine phosphatases used to maintain cellular equilibrium of phosphorylated proteins. *In vitro* studies looking at PP1 showed that this protein can associate with the HIV-1 Tat protein and aid in dephosphorylation of the C-terminal domain of RNA polymerase II (RNAPII), thus enhancing early gene transcription[331]. Subsequent study has also put forth the possibility that PP1 facilitates the dephosphorylation of CDK9 allowing it to interact with Tat and aiding in the recruitment of RNAPII to the TAR element in the HIV-1 LTR thus aiding in Tat mediated gene transcription[390]. Regardless of its mechanism of action, this protein seems to be involved in enhancement of Tat-mediated HIV-1 early gene transcription.

In addition to its known immunologic importance, the transcription factor NF-κB has long been known to be important in early HIV-1 gene expression[391]. NF-κB is a transcriptional activator that is made up of heterodimers of Rel family proteins, most commonly the proteins NF-κB p50 and RelA (reviewed in Karin 2000[392]). In normal cells NF-κB is generally sequestered with inhibitory proteins known as IkB[393]. Activated cells then undergo a process of releasing NF-κB from IkB complexes through the action of IkB kinases (IKK). This form is then translocated to the nucleus and acts as a transcriptional activator. The HIV-1 LTR contains several binding sites for various host transcriptional activators (reviewed in Cullen 1991[394]) including NF-κB. Several studies have investigated the importance of NF-κB binding to the HIV-1 LTR in the viral life cycle. The general consensus from these studies is that NF-κB is absolutely required for regulating HIV-1 gene transcription in the early stages of infection both in primary T cells[395, 396, 332] and macrophages[397]. The fact that HIV-R individuals show lowered expression of both NFKB1 (-1.7 fold p=0.01) and RELA (-1.4 fold p=0.0009) is thus very significant. The lower levels of this molecule could negatively impact early evens in transcription required for establishment of systemic infection, allowing the infected cell to be eliminated prior to production of viable virus, limiting overall susceptibility. What is not apparent from the gene expression data is the amount of NF-κB sequestered in IkB containing vesicles. Were this also different between the two groups, with HIV-R individuals having more IkB/NF-κB complexes, the ability of HIV-1 to replicate would be further negatively impacted. Although this is beyond the scope of this study, the balance of evidence pointing towards some levels of differential function of NF-κB between HIV-R and both HIV-HRN and HIV-LRN controls makes this a particularly interesting future objective.

The double-stranded RNA binding protein staufen (STAU) has been shown to play roles in HIV-1 protein processing and viral assembly. During HIV-1 replication, *in vitro* experiments have shown that STAU associates independently with both genomic HIV-1 RNA and Gag proteins[398], and is incorporated into the HIV-1 virion[329]. It was further shown that both knock down of STAU expression, using RNAi, and transient over expression, impair HIV-1 replication and infectivity *in vitro* [329, 398]. This study also demonstrated that large increases or decreases in STAU expression have negative impacts on the multimerization of Gag proteins, and that this likely inhibits HIV-1 replication.

Finally, the signal transduction molecule Zap70, well known for its role in T cell signaling, was recently shown to be important for cell to cell transmission of HIV-1. This protein was shown to be present in the virological synapse, the accumulation of host and viral proteins at the site of direct cell to cell transmission. *In vitro* experiments using cells that do not express Zap70 or express a kinase dead mutant showed drastically reduced HIV-1 replication. Further analysis determined that, in the absence of functional Zap70, Gag localization was impaired. As well, PBMC isolated from infants with a rare form of severe combined immunodeficiency (SCID) where Zap70 is not expressed, were less able to support HIV-1 replication than healthy controls [334].

Recently, a full genome siRNA study was completed looking at the impact of over 21,000 host genes on the HIV life cycle[399]. This study examined both early and late stages of HIV replication and identified 273 genes considered to be crucial for viral

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replication. Interestingly only a small number, 36, had previously been implicated in HIV replication. Importantly to the studies presented here the requirement of NF- κB was confirmed in this study. Of further interest is that of the 273 genes identified by this method, 48 appear as expressed at a significantly lower level by HIV-R compared to HIV-HRN individuals. Of these, several genes of the Mediator complex, a cellular process involved in initiation of transcription that had not previously been implicated in HIV replication, are expressed at a lower level by HIV-R individuals. Although several host genes previously shown to be required for HIV replication did not appear on this screen it should be noted that the very *in vitro* nature of this study makes it of more value as an identification tool for new targets as opposed to a reconfirmation of previously well-defined HIV dependency factors.

Taken together the lower expression of several host genes used by HIV-1 at all stages of replication in HIV-R individuals likely contributes to reduced susceptibility, and thus our hypothesis that we will see altered expression of host genes involved in HIV-1 replication is supported. However, it is difficult to determine which of these may be *the most* important in determining HIV-1 susceptibility. Several of these genes also play critical roles in the immune response and thus whether their lower expression is more important in an immune context or in their impact on HIV-1 replication remains to be determined.

6.3.5.3 HIV-R women show lower expression of genes involved in immune activation

As discussed in Section 6.3.2, activation status of CD4+ T cells has been investigated in some HIV-1 exposed uninfected cohorts, with the common theme being that lower cellular activation associates with protection. Previous data presented here have

demonstrated a similar finding in our cohort, when comparing HIV-R to HIV-LRN individuals. This being the case, we hypothesized that the same trend would be seen when comparing HIV-R to HIV-HRN individuals. Manual inspection of the list of genes that were significantly differentially expressed between these two populations supported this hypothesis (Table 29). Several genes involved in cell signaling, gene transcription and the immune response were expressed at a lower level by HIV-R individuals. Of particular interest is the lower expression of HLA-DR, a cell surface marker upregulated during cellular activation that has been previously seen to be expressed at a lower level in other HIV-1 exposed uninfected cohorts[272, 271].

As well, several genes involved in T cell signaling including the T cell receptor itself were expressed at a lower level by HIV-R individuals. As engagement of the T cell receptor and signaling through the TCR pathway is the major method of activation of T cells, the lower expression of these key molecules is reflective of lower activation[163]. As outlined several times in this thesis, T cell activation not only plays a critical role in HIV-1 disease progression, but has shown to be important in the earliest stages of HIV-1 infection and is required for viral transmission and establishment of infection[273]. The overwhelming evidence presented here fully supports the hypothesis that CD4+ T cells from HIV-R women from the Pumwani cohort show lower levels of activation than do both low-risk and high-risk controls. This lowered activation phenotype may significantly impact the ability of HIV-1 to establish infection in these women.

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6.3.5.4 qRT-PCR confirms lower gene expression in HIV-R individuals

Previously (Section 6.3.4.4), only a small number of genes selected for confirmation actually showed the same trend by both microarray analysis and qRT-PCR. It was mentioned that this might have been due to the small amount of sample remaining after array analysis. Thus, for this portion of the study an amount of total RNA sufficient for qRT-PCR confirmation was withheld for this analysis. As shown in Table 30, we were able to confirm the lowered expression by qRT-PCR in 8 genes selected for this analysis. These data serve to confirm the expression data generated using the Affymetrix platform and further support the hypothesis of lowered activation in HIV-R individuals.

6.3.5.5 Pathway analysis supports lower levels of activation associates with HIV-1 resistance

Although single gene analysis is informative, it is necessarily biased as it relies on the knowledge of the data analyst to ascribe functional significance to individual genes. In order to enhance this, several tools are available that are able to scan all available information as to which genes have been shown to interact in given pathways, and then determine if a particular gene list is significantly enriched for genes in a pathway. To this end, we used the DAVID web tool (http://david.abcc.ncifcrf.gov/home.jsp) to scan the list of genes that were significantly differentially regulated and to determine which biological pathways may be significantly altered between our sample groups. This analysis returned several interesting findings both expected and unexpected (Table 31). Perhaps the most interesting of these, based on number of genes involved and biological and statistical significance are oxidative phosphorylation, the proteasome and the T cell receptor signaling pathway.

Oxidative phosphorylation is a biological process used in one form or other in almost all living organisms to produce ATP. In eukaryotes this is carried out in the mitochondria via the electron transport chain. In addition to the obvious importance of this process to cellular metabolism, modifying ATP production in various culture conditions has been shown to have drastic impact on viral production in both adenoviral and reoviral models[400, 401]. As reduction in oxidative phosphorylation necessarily results in lowered ATP production, the resulting impact on the cell would be lowered metabolism including lower protein production, both in the host cell and, in the case of infection, of viral proteins. The fact that HIV-R individuals express lower levels of several genes (31 in total) involved in this process (Figure 22 and Table 32) including ATPases, NADH oxidoreductases and succinate dehydrogenases is thus quite interesting. This is a novel finding as no other study has identified this process in a natural model of resistance to HIV-1 infection. It is likely that the lowered expression of these genes is a matter of the overall lower activity of CD4+ T cells in HIV-R individuals. Although the direct impact of this lower expression is difficult to determine, it is quite possible that it contributes to lowered cellular susceptibility to infection.

In addition to the lowered expression of several genes involved in oxidative phosphorylation, a number of different components of the proteasome (17 in total) are expressed at a significantly lower level by HIV-R compared to HIV-HRN women (Figure 23 and Table 33). The proteasome is an intracellular organelle, the normal function of which is to survey the cellular proteome, both regulating protein levels and processing proteins for antigenic presentation to the adaptive immune system. Previous studies have

shown that inhibition of proteasome function actually enhances HIV-1 infectivity *in vitro* likely by decreasing the proteasomally mediated degradation of ubiquitinated viral particles during viral entry and uncoating[402]. However, other studies have shown that the proteasome is actually required for proper proteolytic processing of Gag during viral assembly and that its inhibition at this stage of replication is deleterious to viral production[403, 335]. These seemingly contradictory effects make the significance of the observed lowered expression of proteasome genes questionable. It is possible that, in the context of HIV-1 resistance, any enhancement of infection seen initially after entry due to repression of the proteasome will be counteracted due to the various other blocks to infection discussed in the previous section. As well, the requirement for proteasome activity during viral assembly may act synergistically with other genes that act later in the viral life cycle, such as NF-κB or Staufen, in order to limit viral spread.

In addition to its roles in cellular protein processing and HIV assembly, the proteasome plays a large role in antigen presentation in the context of MHC class I[404]. A likely result of HIV-R individuals having lower abundance of proteasomal constructs would thus be lower levels of antigen processing. To date there is no evidence to suggest that HIV-R individuals respond differently to other infectious agents suggesting the ability to mount a normal immune response. It would be interesting to examine rates of protein processing and antigen presentation in the cells of HIV-R individuals as lower levels of this may result in lower immune activation contributing to the phenotype of lower cellular activation described here. Also, this may have an impact on the maintenance of cellular memory, partially explaining why not all HIV-R individuals maintain HIV-specific immune responses at all times.

Finally, and perhaps most interestingly, significantly lower expression of 10 genes involved in T cell receptor signaling were expressed at a lower level in HIV-R women compared to high-risk controls (Figure 24 and Table 34). As mentioned throughout this section, T cell receptor signaling is the primary mechanism of activation of T cells. This is of obvious importance to HIV-1 infection as HIV-1 has a requirement for activation of T cells particularly early on in infection. Of the genes involved in this process, the lower expression of the T cell receptor itself and NF-κB stand out, as both have been recurring themes throughout this thesis. The lower expression of these molecules, and the impact that would have on cellular activation state would no doubt limit the ability of HIV-1 to establish infection, and thus in all likelihood, this phenomenon would contribute to reduced susceptibility both at the cellular and host levels.

6.4 Summary

Overall, this thesis has used genomic technologies to attempt to answer three questions:

1. To what level do the gene expression profiles of particular cell subsets differ from the mixed cell population?

2. What level of immune dysfunction exists in CD4+ T cells of asymptomatic HIV-1 infected individuals?

3. How does CD4+ T cell function vary between HIV-1 resistant individuals compared to control individuals either at low-risk or high-risk of infection?

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In the first case, we showed significantly different subsets of genes are induced in either the CD4+ or CD8+ T cell subsets compared to the PBMC population. This has implications for future microarrays studies, as studies designed to measure gene expression in a mixed cell population may, in fact, overlook potentially interesting trends in specific cell subsets. This work resulted in the first publication directly comparing gene expression in cell subsets to the mixed cell population[322].

Secondly, we demonstrated large levels of differential CD4+ T cell function in relatively asymptomatic HIV+ individuals. These results support the body of knowledge accumulated that identify high levels of immune activation during HIV-1 infection, and suggests that this activation is present well before the onset of significant clinical pathology.

Finally, we investigated gene expression in CD4+ T cells of HIV-1 resistant women in comparison to both low-risk and high-risk HIV-1 negative individuals. The results of these comparisons support the handful of other studies showing lower levels of immune activation in exposed-uninfected cohorts. It should be mentioned that in comparing HIV-R to either HIV-LRN or HIV-HRN individuals it was not possible to control for age as a confounder. In fact the HIV-R individuals sampled were, on average, older than either the control populations (Tables 13, 18, 26). However, it has been shown by several studies that immune activation actually increases with age[405, 406, 407]. Thus, if age were to have an impact, we would expect to find higher activation in the HIV-R population, which is not the case.

In comparison to previous studies showing lower immune activation in HIV-1 exposed uninfected individuals, the study reported here has investigated this phenomenon in much greater detail. In fact, only one other population-based genomic study of reduced susceptibility to HIV-1 has been published[24]. This study focused on discordant couples and identified a small number of innate immune molecules that were associated with protection against infection. The differences in findings between that study and our own serve to underscore the importance of such studies in distinct populations with distinct transmission risks. Our findings that a general state of immune quiescence, extending beyond lowered expression of surface molecules, associates with reduced susceptibility is novel and has outlined several new genes and pathways that are associated with this phenomenon. Of particular interest is the lowered expression of genes involved in T cell signaling, specifically NF-κB and the T cell receptor. Understanding the complex interplay of all these factors will be crucial for design of novel therapies and vaccine candidates.

6.5 Model of HIV-1 Resistance

It would be impossible to carry out a study of this nature and not at least attempt to synthesize the available information into a coherent theory of HIV-1 resistance in this cohort. Briefly put, the major associations with HIV-1 resistance in this cohort, as outlined in the introduction, are an association of certain HLA types, repressed IRF-1 activity and effective adaptive responses in the form of both CD4+ and CD8+ HIV-specific T cells. This thesis has added the dimension of lower baseline levels of activation in the CD4+ T cell compartment, resulting in the lower expression of several host genes involved in HIV-1 replication. It has been shown previously that cells from HIV-R

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women can be infected, and produce virus *in vitro* after PHA and IL-2 activation[253]. Thus, the block in susceptibility likely occurs post entry. It is suggested here that the lower activation state of CD4+ T cells seen in HIV-R women results in a reduction of susceptible cells, i.e. cells that will produce sufficient amounts of viable virus to propagate infection. However, these cells may support some level of viral protein production that may aid in priming the adaptive immune response. Therefore, when an HIV-R woman encounters HIV, a much smaller number of cells become productively infected, i.e. not all cells are at this lower activation state, but the pool of susceptible cells is much smaller in an HIV-R woman. Cells that do support infection are then recognized and eliminated by the adaptive immune system, which has been primed both by productively infected cells and cells that only support marginal levels of viral protein production or defective viral particles (Figure 25). The root cause of this lower activation is still unknown. However, if it were caused by some degree of differential response (i.e. tolerance) in these individuals to constant antigenic exposure (either allo or otherwise), that may wane with reduced exposure, this would explain the phenomenon of late seroconversion after sex-break seen in a proportion of resistant women[408].

Were this theory to be correct, it would support a two-pronged approach to preventing HIV-1 infection in the general population. Topical agents such as microbicides that contained anti-HIV compounds as well as molecules that specifically targeted the negative impact of immune activation could be used in conjunction with an HIV-1 vaccine that produced HIV-specific cellular responses. This strategy, although admittedly difficult to employ on a population level, could have a dramatic effect on reducing HIV-1 transmission.

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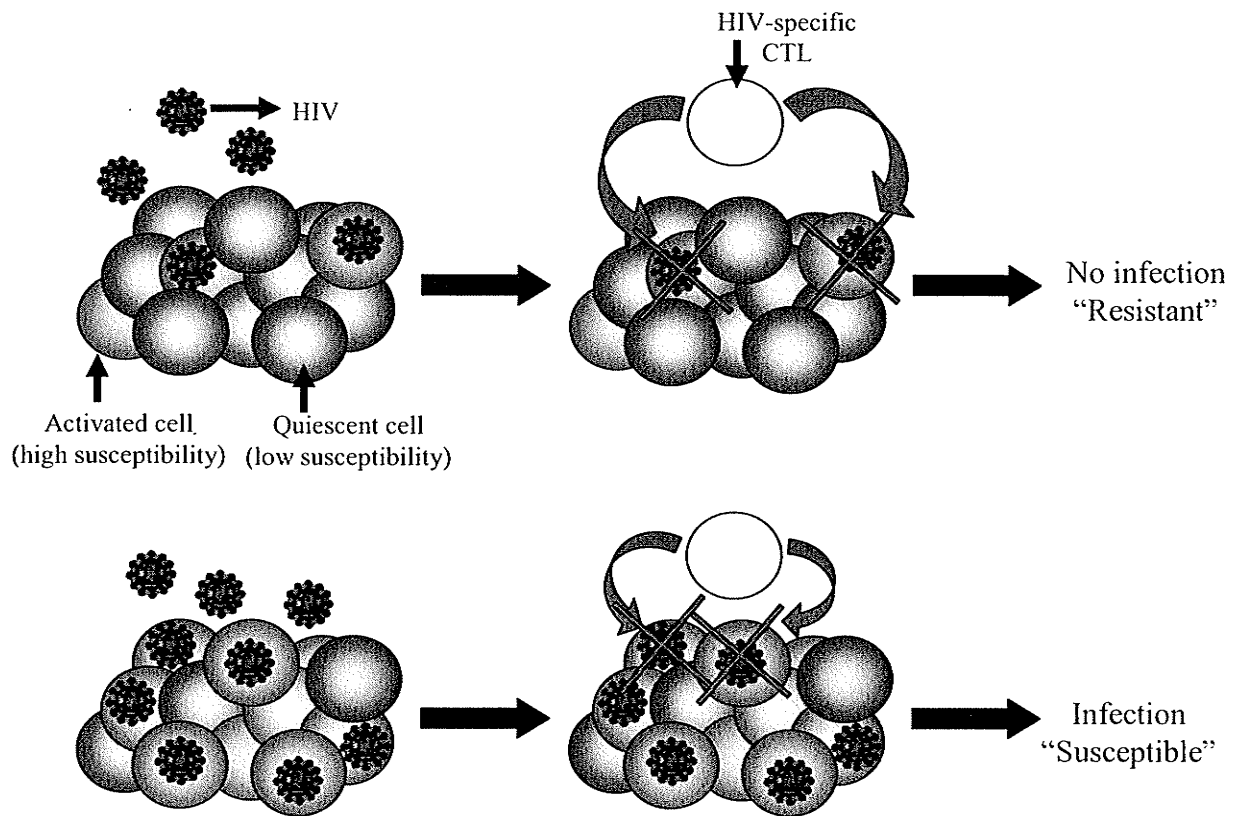
Figure 25: Model diagram of HIV-1 resistance. HIV-1 resistant individuals with the immune quiescent phenotype (top panel) have a larger proportion of quiescent CD4+ T cells (blue) that are not susceptible to HIV-1 infection. Those cells that are susceptible (red) are eliminated either by apoptosis or HIV-specific T cells (yellow) and infection is not established. In HIV-1 susceptible individuals (bottom panel) a sufficient pool of susceptible cells is available to establish HIV-infection.

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6.6 Future Work

As with many scientific investigations, in providing answers for the main hypotheses this study raises many new questions. Specific future directions associated with this project include analysis of the trends in gene expression at the protein level. Most interesting among these would be proteins such as staufen, NF-κB and the T cell receptor as the lower expression of these can be directly linked to lower HIV-1 replication and immune activation. Secondly, this study has focused on function of CD4+ T cells in the periphery. However, HIV-1 transmission in this cohort occurs at mucosal surfaces. Due to the difficulties in working with cells at these surfaces, studies of immune function at the cervix are only beginning in our group. Confirmation of the findings of this work at the mucosal level would greatly strengthen the model of resistance proposed.

Broadly, the two fundamental questions outlined in the introduction remain; what is the nature of this resistance and why does it develop? Although this work has shed significant light on the former, the latter remains unanswered. To answer this, a two-pronged strategy of investigating this phenomenon of lower activation in a controlled lab setting, by modifying the expression of individual genes of interest, and genetic studies in HIV resistant individuals and their family members, looking for polymorphisms associated with resistance in specific genes, would be of great interest. As the HIV-1 pandemic continues to grow, answering these fundamental questions of how resistance to infection occurs in nature, is an important step in developing a vaccine and stopping this already too deadly threat to global health.

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Section 8.0: Appendices

Appendix A: Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cell
APOBEC3G	Apolipoprotein B mRNA editing Enzyme Catalytic peptide-like 3G
CBA	Cytometric Bead Array
CPIA	Competitive Predictived interaction Analysis
CSW	Commercial Sex-Worker
CTL	Cytotoxic T Lymphocyte
DAVID	Database for Annotation, Visualization and Integrated Discovery
DC-SIGN	Dendritic-Cell-Specific ICAM3-Grabbing Non-integrin
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EMD	Empirical Mode Decomposition
ESN	Exposed Seronegative
EST	Expressed Sequence Tag
EU	Exposed Uninfected
FCS	Fetal Calf Serum
HIV	Human Immunodeficiency Virus
HIV+	HIV Positive
HIV-HRN	HIV Low-Risk Negative
HIV-LRN	HIV High-Risk Negative
HIV-R	HIV Resistant
HLA	Human Leukocyte Antigen
IFN	Interferon
IL	Interleukin
IMF	Intrinsic Mode Function
KEGG	Kyoto Encyclopedia of Genes and Genomes
KIR	Killer Immunoglobulin-like Receptor
LDA	Linear Discriminant Analysis
LTNP	Long-Term Non-Progressor
LTR	Long Terminal Repeat
MDM	Monocyte Derived Macrophage
MHC	Major Histocompatibilty Complex
MIP	Monocyte Inflammatory Protein
ml	Millilitre
ul	Microlitre
ug	Microgram
ng	Nanogram
NaOH	Sodium Hydroxide

NFAT	Nuclear Factor of Activated T cells
NFkB	Nuclear Factor of Kappa light polypeptide gene enhancer in B-cells
PBS	Phosphate Buffered Saline
PCA	Principal Components Analysis
PCR	Polymerase Chain Reaction
PHA	Phytohemagglutinin
PIA	Predictived interaction Analysis
PLIER	Probe Logarithmic Intensity Error
qRT-PCR	Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RMA	Robust Multi-array Analysis
RNA	Ribonucleic Acid
RP	Rapid Progressor
RPM	Rotations Per Minute
RPMI	Roswell Park Memorial Institute
RT	Reverse Transcriptase
SAPE	Streptavidin-Phycoerythrin
SDF	Stronla-Derived Factor 1
SDS	Sodium Dodoecyl Sulfate
SIV	Simian Immunodeficiency Virus
SPIA	Synergistic Predictived interaction Analysis
SSC	Saline Sodium Citrate
STI	Sexually Transmitted infection
TCR	T Cell Receptor
TNF	Tumor Necrosis Factor
TRIM5a	Tripartite Motif 5 alpha
UTR	Untranslated Region

Appendix B: Alphabetical Listing of Gene Symbols and Descriptions

Gene Symbol	Description
AATK	apoptosis-associated tyrosine kinase
ACVR2B	activin A receptor, type IIB
ADORA2B	adenosine A2b receptor
ALDH1	aldehyde dehydrogenase 1
AMY1	amylase alpha 1
ANNEXINV	annexin V
AOAH	acyloxyacyl hydrolase (neutrophil)
AP2	adapter protein 2
AP2S1	adaptor-related protein complex 2, sigma 1 subunit
APAF1	apoptotic protease activating factor
ARAF1	v-raf murine sarcoma viral oncogene homolog 1
ARHGAP1	Rho GTPase activating protein 1
ATF3	activating transcription factor 3
ATF5	activating transcription factor 5
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide
ATP5G2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 2
ATP5J	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F6
ATP5L	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit g
ATP5O	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit (oligomycin sensitivity conferring protein)
ATP6AP1	ATPase, H+ transporting, lysosomal accessory protein 1
ATP6V0D2	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d isoform 2
ATP6V0E	ATPase, H+ transporting, lysosomal 9kDa, V0 subunit e
ATP6V1B2	ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B, isoform 2
ATP6V1D	ATPase, H+ transporting, lysosomal 34kDa, V1 subunit D
ATP6V1E1	ATPase, H+ transporting, lysosomal 31kDa, V1 subunit E isoform 1
ATP6V1F	ATPase, H+ transporting, lysosomal 14kDa, V1 subunit F
ATP6V1G1	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G isoform 1
ATP6V1H	ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H
BCL2	B-cell CLL/lymphoma
BIRC1	baculoviral IAP repeat-containing 1
BIRC3	baculoviral IAP repeat-containing 3
BRCA1	breast cancer 1
BTF3	basic transcription factor 3
CASP1	caspace 1
CASP10	caspace 10
CASP5	caspase 5
CAV1	caveolin 1

CCL11	chemokine (C-C motif) ligand 11
CCL2	chemokine (C-C motif) ligand 2
CCL22	chemokine (C-C motif) ligand 22
CCR5	chemokine (C-C motif) receptor 5
CCR6	chemokine (C-C motif) receptor 6
CCR7	chemokine (C-C motif) receptor 7
CD3D	CD3D antigen, delta polypeptide (TiT3 complex)
CD59	CD59 molecule, complement regulatory protein
CD69	CD69 antigen (p60, early T-cell activation antigen)
CDC2	cell division cycle 2
CDC42	cell division cycle 42
CDK7	cyclin-dependent kinase 7
CDK8	cyclin-dependent kinase 8
CDK9	cyclin-dependent kinase 9
CDKN1A	cyclin-dependent kinase inhibitor 1A
CDKN2A	cyclin-dependent kinase inhibitor 2A
CDKN3	cyclin-dependent kinase inhibitor 3
CENPA	centromere protein-A
CHD1	chromodomain helicase DNA binding protein 1
CLK1	CDC-like kinase 1
COX4I1	cytochrome c oxidase subunit IV isoform 1 /// cytochrome c oxidase subunit IV isoform 1
COX6B1	cytochrome c oxidase subunit Vib polypeptide 1 (ubiquitous)
COX7C	cytochrome c oxidase subunit VIIc
COX8A	cytochrome c oxidase subunit 8A (ubiquitous)
CSF3	colony stimulating factor 3
CX3CR1	chemokine (C-X3-C) receptor 1
CXCL11	chemokine (C-X-C) ligand 11
CXCL2	chemokine (C-X-C) ligand 2
CXCR3	chemokine (C-X-C motif) receptor 3
CXCR4	chemokine (C-X-C motif) receptor 4
CYPA	peptidylprolyl isomerase A (cyclophilin A)
DDX1	DEAD (Asp-Glu-Ala-Asp) box polypeptide 1
DEFA6	defensin, alpha 6, Paneth cell-specific
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
E2F1	E2F transcription factor 1
E2F4	E2F transcription factor 4
E2F5	E2F transcription factor 5
EGF	epidermal growth factor (beta-urogastrone)
ELF2	E74-like factor 2
EPHA1	ephrin type-A receptor 1
	Bottom of Form

EPR1	effector cell protease receptor-1
EPR-1	effector cell protease receptor-1
ERCC2	excision repair cross-complementing rodent repair deficiency, complementation group 2
ERF	Ets2 repressor factor
FAF1	fas associated factor 1
FGFR3	fibroblast growth factor receptor 3
FLJ11904	hypothetical protein FLJ11904
FLJ13639	hypothetical protein FLJ13639
FLJ22004	hypothetical protein FLJ22004
FYB	FYN binding protein (FYB-120/130)
GAL	galanin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCA	grancalcin
GPSM3	G-protein signaling modulator 3
GRIK1	glutamate receptor, ionotropic, kainate 1
GRM3	glutamate receptor metabotropic 3
GTF2H2	general transcription factor IIH polypeptide II
HAS2	hyaluronan synthase
HLA-B	major histocompatibility complex, class I, B
HLA-C	major histocompatibility complex, class I, C
HLA-DPB1	major histocompatibility complex, class II, DP beta 1
HLA-DQB1	major histocompatibility complex, class II, DQ beta 1
HLA-DRA	major histocompatibility complex, class II, DR alpha
HLA-DRB1	major histocompatibility complex, class II, DR beta 1
HLA-DRB5	major histocompatibility complex, class II, DR beta 3
HLA-G	HLA-G histocompatibility antigen, class I, G
HRAS (RAS)	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
HRB	HIV-1 Rev binding protein
HSPA5	heat shock 70kD protein 5
HSPB3	heat shock protein beta 3
IFNGR1	interferon gamma receptor 1
IFNGR2	interferon gamma receptor 2
IGFBP2	insulin-like growth factor binding protein 2
IGFBP6	insulin-like growth factor binding protein 6
IL10	interleukin 10
IL10RA	interleukin 10 receptor, alpha
IL10RB	interleukin 10 receptor, beta
IL11	interleukin 11
IL12A	interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)

IL16	interleukin 16 (lymphocyte chemoattractant factor)
IL18	interleukin 18
IL1A	interleukin 1, alpha
IL1B	interleukin 1, beta
IL1R1	interleukin 1 receptor 1
IL1R2	interleukin 1 receptor 2
IL2	interleukin 2
IL22R	interleukin 22 receptor
IL23A	interleukin 23, alpha subunit p19
IL27RA	interleukin 27 receptor, alpha
IL2RB	interleukin 2 receptor, beta
IL4RA	interleukin 4 receptor, alpha
IL5	interleukin 5
IL5RA	interleukin 5 receptor, alpha
IL6	interleukin 6
IL8	interleukin 8
IL8RB	interleukin 8 receptor, beta
IL9R	interleukin 9 receptor
INPPL1	inositol polyphosphate phosphatase-like 1
IRF1	interferon regulatory factor 1
IRF2	interferon regulatory factor 2
IRF6	interferon regulatory factor 6
ISGF3G	interferon-stimulated transcription factor 3
ITGA9	integrin alpha 9
ITGAL	integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1
ITPR3	inositol 1,4,5-triphosphate receptor, type 3
JAK1	janus kinase 1 (a protein tyrosine kinase)
JUN	v-jun avian sarcoma virus 17 oncogene homolog
KCNMA1	calcium-activated potassium channel
KIF22	kinesin family member 22
LAT	linker for activation of T cells
LIFR	leukemia inhibitory factor receptor
MADH3	mothers against decapentaplegic homolog 3
MAP2K3	mitogen-activated protein kinase kinase 3
MAP2K4	mitogen-activated protein kinase kinase 4
MAP2K7	mitogen-activated protein kinase kinase 7
MAP3K1	mitogen-activated protein kinase kinase kinase 1
MAP3K14	mitogen-activated protein kinase kinase kinase 14
MAP3K5	mitogen-activated protein kinase kinase kinase 5
MAP3K7	mitogen-activated protein kinase kinase kinase 7

MAP3K7IP2	mitogen-activated protein kinase kinase kinase 7 interacting protein 2
MAP3K8	mitogen-activated protein kinase kinase kinase 1
MAP4K1	mitogen-activating protein kinase kinase kinase kinase 1
MAP4K2	mitogen-activating protein kinase kinase kinase kinase 2
MAPK1	mitogen-activating protein kinase 1
MAPK10	mitogen-activating protein kinase 10
MAPK3	mitogen-activating protein kinase 3
MAPK4	mitogen-activated protein kinase 4
MAPK7	mitogen-activated protein kinase 7
MAPK9	mitogen-activated protein kinase 9
MAPKAPK3	mitogen-activated protein kinase-activated protein kinase 3
MCP3	monocyte chemoattractant protein 3
MDM2	mouse double minute 2 homolog
MET	met proto-oncogene (hepatocyte growth factor receptor)
MIF	macrophage migration inhibitory factor
MIP1B	macrophage inflammatory protein-1beta
MKLP1	mitotic kinesin-like protein 1
MMP7	matrix metalloproteinase 7 (matrilysin, uterine)
MYBP-H	myosin binding protein H mRNA
MYH1	myosin Heavy Chain 1
N2RF1	nuclear receptor subfamily 2, group F member 1
NDRG	N-myc downstream regulated
NDUFA12	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12
NDUFA13	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13
NDUFA8	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8, 19kDa
NDUFB7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa
NDUFB9	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9, 22kDa
NDUFC1	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1, 6kDa
NDUFS3	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase)
NDUFS5	NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15kDa (NADH-coenzyme Q reductase)
NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductase)
NDUFV2	NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa
NFATC4	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4
NFIC	nuclear factor I/C (CCAAT-binding transcription factor)
NFRKB	nuclear factor related to kappaB binding protein
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p50)
NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon
NOTCH4	notch homolog 4

NR2F	nuclear receptor subfamily 2, group F, member 1
NRN1	neuritin 1
NTF3	neurotrophin 3
OSR1	oxidative-stress responsive 1
PAS8	peroxisomal Protein 8
PCNA	proliferating cell nuclear antigen
PDYN	pro-dynorphin
PEA3	polyomavirus enhancer activator 3
PGY3	P-glycoprotein-3/multiple drug resistance-3
PIN1	protein (peptidylprolyl cis/trans isomerase) NIMA-interacting 1
POLR2E	polymerase (RNA) II polypeptide E, 25kDa
PPP1CA	protein phosphatase 1, catalytic subunit, alpha isoform
PPP2R3	protein phosphatase 2 (formerly 2A), regulatory subunit B"
PRKCL2	protein kinase C-like 2
PSMA1	proteasome (prosome, macropain) subunit, alpha type, 1
PSMA2	proteasome (prosome, macropain) subunit, alpha type, 2
PSMA3	proteasome (prosome, macropain) subunit, alpha type, 3
PSMA4	proteasome (prosome, macropain) subunit, alpha type, 4
PSMA6	proteasome (prosome, macropain) subunit, alpha type, 6
PSMB1	proteasome (prosome, macropain) subunit, beta type, 1
PSMB4	proteasome (prosome, macropain) subunit, beta type, 4
PSMC2	proteasome (prosome, macropain) 26S subunit, ATPase, 2
PSMC3	proteasome (prosome, macropain) 26S subunit, ATPase, 3
PSMC4	proteasome (prosome, macropain) 26S subunit, ATPase, 4
PSMD1	proteasome (prosome, macropain) 26S subunit, non-ATPase, 1
PSMD11	proteasome (prosome, macropain) 26S subunit, non-ATPase, 11
PSMD2	proteasome (prosome, macropain) 26S subunit, non-ATPase, 2
PSMD3	proteasome (prosome, macropain) 26S subunit, non-ATPase, 3
PSMD4	proteasome (prosome, macropain) 26S subunit, non-ATPase, 4
PSMD6	proteasome (prosome, macropain) 26S subunit, non-ATPase, 6
PSMD8	proteasome (prosome, macropain) 26S subunit, non-ATPase, 8
PSTPIP1	proline-serine-threonine phosphatase interacting protein 1
PTGDR	prostaglandin D2 receptor
PTGER2	prostaglandin E receptor 2 (subtype EP2), 53kDa
PTPN6	protein tyrosine phosphatase, non-receptor type 6
PTPRC	protein tyrosine phosphatase, receptor type C
RAB11B	RAB11B, member RAS oncogene family
RAB2	RAB2, member RAS oncogene family
RAB36	RAB36, member RAS oncogene family
RAB3A	RAB3A, member RAS oncogene family
RAB6A	RAB6A, member RAS oncogene family

RAF1	v-raf-1 murine leukemia viral oncogene homolog 1
RAP1A	RAP1A, member of RAS oncogene family
RB1	retinoblastoma 1
RBBP1	retinoblastoma-binding protein 1
RBBP5	retinoblastoma-binding protein 5
RELA	v-rel nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65
RHOA	ras homolog gene family, member A
SCYB14	small inducible cytokine subfamily B 14
SDHA	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
SDHB	succinate dehydrogenase complex, subunit B, iron sulfur (Ip)
SELE	selectin E
SEMA3B	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B
SH3BP2	SH3-domain binding protein 2
SHC1	SHC (Src homology 2 domain containing) transforming protein 1
SNAP23	synaptosomal-associated protein, 23kDa
SP1	Sp1 transcription factor
SP2	Sp2 transcription factor
SP3	Sp3 transcription factor
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
STAT1	signal transducer and activator of transcription 1
STAT2	signal transducer and activator of transcription 2
STAT3	signal transducer and activator of transcription 3
STAT4	signal transducer and activator of transcription 4
STAT5B	signal transducer and activator of transcription 5b
STAU	staufen, RNA binding protein (Drosophila)
SYNGR1	synaptogyrin 1
TAF9	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 32kDa
TCEA2	transcription elongation factor A (SII), 2
TCEB2	transcription elongation factor B (SIII), polypeptide 2 (18kDa, elongin B)
TCEB3	transcription elongation factor B (SIII), polypeptide 3 (110kDa, elongin A)
TCF12	transcription factor 12
TCF3	transcription factor 3
TCF7	transcription factor 7
TCF8	transcription factor 8
TEAD1	TEA domain family member 1
TFAP4	transcription factor AP-4
TFAR15	Apoptosis-related protein TFAR15
TNF	tumor necrosis factor
TNFRSF10B	tumor necrosis factor receptor subfamily, member 10B
TNFRSF11	tumor necrosis factor receptor subfamily, member 11

TNFSF4	tumor necrosis factor (ligand) superfamily, member 4
TRA@	T cell receptor alpha locus
TRAF2	TNF receptor-associated factor 2
TRAF3	TNF receptor-associated factor 3
TRAF4	TNF receptor-associated factor 4
TRAP1	TNF receptor-associated protein 1
TRBC1	T cell receptor beta constant 1
TRD@	T cell receptor delta
TST	thiosulfate sulfurtransferase
TUBA1	tubulin, alpha 4a
TXK	TXK tyrosine kinase
UQCRFS1	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1
USP9X	ubiquitin specific protease 9 X
VAMP4	vesicle-associated membrane protein 4
VCAM1	vascular cell adhesion molecule 1
VDAC3	voltage-dependent anion channel 3
ZAP70	zeta-chain (TCR) associated protein kinase 70kDa
ZFP37	zinc finger protein 37