Sequence-Based Genotyping of Killer Cell Immunoglobulin-Like Receptors and Their Associations with HIV-1 Resistance and Disease Progression

By Rae-Anne Michelle Hardie

A thesis submitted to the Faculty of Graduate Studies of

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Abstract

An HIV-1 vaccine is desperately needed, and understanding the biological factors that contribute to protective responses against HIV-1 in a cohort of HIV-1 resistant sex workers from Nairobi, Kenya may provide new insight for vaccine design. HIV-1 resistance is thought to be multifaceted; therefore, a comprehensive understanding will be crucial. Killer cell immunoglobulin-like receptors (KIRs) on natural killer (NK) cells are ligands for human leukocyte antigen (HLA) antigens, which are major factors in the susceptibility to HIV-1. Based on their ability to mediate NK activity, and their associations with other viral and autoimmune diseases, we hypothesized that KIR2DL2/2DL3/HLA-C genotypes play a role in HIV-1 resistance in the Pumwani cohort.

A novel sequence-based method was successfully developed and used to type KIR2DL2/2DL3 genes and allele groups for 957 women in the Pumwani cohort. Eight new allele groups were also identified. We found that KIR2DL3/HLA-C1 homozygosity, which is thought to mediate weaker inhibition, was associated with HIV-1 resistance, possibly due to higher NK activity in the innate response to HIV-1. KIR2DL2 is strongly inhibitory, and can bind both HLA-C1 and HLA-C2. Women who were homozygous for KIR2DL2 had slower progression to AIDS, possibly due to lowering immune activation, which is known to be detrimental in the spread of HIV-1 during chronic infection. A novel variant of KIR2DL2 was associated with faster disease progression, possibly due to a change in the amino acid that that reduces its binding affinity for HLA-C2. The association of KIRs with HIV-1 emphasizes the importance of NK-mediated cytotoxicity in HIV-1 resistance, but also its potential detriment in disease progression.

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After climbing a great hill, one only finds that there are many more hills to climb.

-Nelson Mandela

Dedication

To my parents, and the rest of my family, for being so supportive and believing in me.

To Jos, thank you for your love and support, and never allowing me to give up, even from half a world away.

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Section 1: Introduction

1.1 Background

More than 25 years after the identification of Human Immunodeficiency Virus Type 1 (HIV-1) in 1981, a total of sixty million people have been infected by HIV-1 and almost half of those have died of AIDS. The HIV/AIDS pandemic continues to be the leading infectious disease challenge to public health. UNAIDS estimated that in 2007, 33 million (30.3-36.1 million) people were living with HIV/AIDS, with 2.7 million (2.2-3.2 new infections) new infections and 2.0 million (1.8-2.3 million) deaths [8]. Despite the enormous amounts of time and money spent on developing treatments, vaccines, and prevention strategies, the pandemic persists.

The region with the greatest proportion of HIV-1 infections is sub-Saharan Africa, where the average adult HIV-1 prevalence is 5%, but reaches staggering levels of over 20% in some countries, including Botswana, Lesotho, and Swaziland. While only 10% of the world's population live there, sub-Saharan Africa is home to more than two thirds of all people living with HIV/AIDS globally. Over three quarters of all AIDS-related deaths in 2008 [8] were in sub-Saharan Africa. In fact, AIDS is the primary cause of death in this region [8]. The disproportional impact on mortality in this region can be attributed to the generalized epidemic transmission and a failure of prevention. The financial burden of antiretroviral drugs and a lack of access to quality healthcare exacerbate this situation, and only about a quarter of individuals in this region and other developing or transitioning countries who need life-saving treatments are receiving them. For those who do have access to medical care and treatment, the highly effective

antiretroviral treatment (HAART) regimen reduces mortality and morbidity in HIV-1 positive individuals by slowing its progression, but they do not cure the HIV-1 infection or stop transmission.

Effective prevention and treatment programs have provided some hope, such as the World Health Organizations 3 by 5 initiative and the President's Emergency Plan for AIDS Relief (PEPFAR), but they have not been implemented at a large enough scale. Other prevention strategies shown to decrease transmission include treating concurrent sexually transmitted infections (STIs) [9], male circumcision, although not broadly implemented yet [10, 11], and condom programs [12]. However, these have not made a huge impact on infection rates in sub-Saharan Africa. Much effort has also been put into the development of a topical microbicide to prevent sexual transmission, but efficacy trials have been disappointing thus far [13]. Scaling up access to antiretroviral treatment would prolong lives and prevent new infections through reduction in viral load [14, 15], but this is currently not a financially viable option. Because of the high mortality rates associated with HIV/AIDS, limited access to expensive treatment, and continued spread of the virus, the best hope of ending this pandemic is an effective prophylactic vaccine. However, the road to the development of a vaccine continues to be a long and arduous one. The only candidate that has made it through to phase III trials, which was to induce antibodies against gp120, was a failure [16]. Recently, a recombinant, nonreplicating, human adenovirus serotype 5-vectored vaccine, known as MRKAd5, expressing three HIV-1 clade B-derived internal proteins underwent a Phase IIb proof-of-concept (STEP) trial. The vaccine was hoped to elicit cell-mediated immunity, but the trial was halted due to the observation that not only was the vaccine not effective, but it may actually

increase the risk of infection [17]. Developing new strategies will be essential if we hope to come up with a successful vaccine, and a unique opportunity for understanding protective mechanisms against HIV-1 lies in a group of individuals who remain uninfected even after many exposures to the virus.

Exposure to HIV-1 does not necessarily result in infection with the virus, and has been documented in several cohort studies, with the identification of highly exposed but uninfected individuals (HEPS) [18-22] as well as HIV infected long-term non-progressors (LTNPs) [23, 24]. A model of natural resistance to HIV-1 could act as an invaluable tool in designing a vaccine to induce protective immune responses against HIV-1. While this variation in susceptibility to infection can be attributed to both host and viral factors, the fact that certain individuals remain uninfected with HIV-1 after exposure to many different strains of the virus indicates that host immunity likely plays a major role in limiting infection. Polymorphisms in genes influencing the immune response to HIV-1 may account for the variation in susceptibility to HIV-1 infection between individuals with similar risk of exposure. A successful vaccine against HIV-1 will probably require the induction of innate immune responses, high titres of neutralizing antibodies, strong T-cell response, and mucosal immunity. A comprehensive understanding of the genetic factors of contributing to HIV-1 immunity will facilitate the design of a protective vaccine. This thesis aims to explore the role of immunogenetics of the innate Killer cell Immunoglobulin-Like Receptors (KIRs) on natural killer (NK) cells in Kenyan sex workers who are naturally resistant to HIV-1.

1.2 The History of HIV and AIDS

The first North American cases of AIDS were reported on June 1, 1981, after the US Centers for Disease Control (CDC) reported 5 cases of Pneumocystis pneumonia (PCP) caused by *Pneumocystis carinii* fungus, which has since been renamed *Pneumocystis jerovici*, in a group of previously healthy homosexual men in Los Angeles, California [25, 26]. Further cases of PCP, as well as other rare opportunistic infections and malignancies such as Kaposi's sarcoma, that are normally associated with immune deficiency were observed in more homosexual men [27].

The illnesses were initially thought to be restricted to homosexual transmission and were labeled in the general press as Gay-Related Immune Deficiency (GRID), but the underlying cause was unknown. One hypothesis was that an unidentified sexually transmitted infectious agent was causing the observed immunodeficiency; another was that the use of substances such as inhalants and street drugs, which were thought to be common in the so-called homosexual style of life, could lead to immunodeficiency [28].

However, cases of immunodeficiency-associated infections were soon observed in other populations: Haitian immigrants living in the United States[29], hemophiliacs who had received blood transfusions [30], and heterosexual injection drug users (IDU) [28]. It was soon clear that "GRID" was not an accurate descriptor of the demographic that the immunodeficiency was affecting, as it seemed to follow the infection pattern of blood-borne pathogens.

In 1982, the CDC introduced a more accurate name for the illnesses: AIDS or Acquired Immune Deficiency Syndrome. The agent causing AIDS was identified in 1983, by a group of scientists at the Pasteur Institute led by Dr. Luc Montagnier [31]. It

was a retrovirus, originally thought to be a member of the human T-cell leukemia virus family (HTLV) [32] and was named lymphadenopathy-associated virus (LAV) [33, 34]. Concurrently, Dr. Robert Gallo from the United States was also investigating the identity of the virus, which he called HTLV-III [35, 36]. Gallo's team also played a key role in linking the virus to the development of AIDS. Shortly afterwards, San Francisco researchers isolated a retrovirus that they called AIDS-associated retrovirus (ARV) from AIDS patients in different risk groups, but also from asymptomatic people from groups at risk for AIDS [37, 38]. These researchers had all identified the same virus, and in 1986, the International Committee of Viral Taxonomy renamed it the Human Immunodeficiency Virus (HIV-1) [39]. In 1987, after much controversy over the title of who made the first discovery of HIV, Montagnier and Gallo agreed to share the credit.

A second type, HIV-2 was discovered in 1986. HIV-2 is less virulent and less transmissible than HIV-1, and is found primarily in West Africa [40, 41]. In contrast, HIV-1 accounts for most HIV infections worldwide. Patients infected with HIV-2 also typically progress to AIDS more slowly, if at all, compared to those with HIV-1.

While these were the first recognized cases of HIV-1 in North America, the virus is hypothesized to have originated from a Simian Immunodeficiency Virus (SIV) in African non-human primates, then adapted and transferred as a zoonosis to humans over multiple transmission events, possibly through hunting practices. This likely occurred at some earlier time point in the early 20^{th} century. It is thought that HIV-1 originated in chimpanzees (*Pan troglodytes troglodytes*) in Southern Cameroon, after evolving from SIVcpz [42, 43]. Based on viral taxonomy, three main zoonotic transmissions are thought to have occurred, resulting in the three groups of HIV-1: M, N, and O [42]. HIV-

2 is believed to have evolved from an SIVsm from Sooty Mangabeys (*Cerocebus atys*) in Guinea-Bissau, Gabon and Cameroon [44].

1.3 HIV-1 Transmission

The most common routes for HIV transmission are sexual intercourse, exposure to infected body fluids or tissue (organ transplants or blood transfusions prior to screening tests, intravenous drug use or occupational exposure), from mother to fetus (during pregnancy or delivery) or mother to child through breastfeeding. High-risk body fluids include blood, male and female genital secretions, and breast milk. While HIV can be detected in other bodily fluids such as urine, saliva and tears of infected individuals, there are no documented cases of infection caused by these body fluids, thus the possibility of being infected by them is considered to be minor.

Worldwide, the most common mode of HIV-1 transmission is heterosexual insertive sexual intercourse [12]. Transmission may occur when infected secretions from one partner contact the mucous membranes in the rectum, genitals, or mouth of the other partner. As a consequence of the anatomy of intercourse, receptive sexual partners have a higher risk of becoming infected by an HIV-1 positive insertive partner than would an insertive partner with an HIV-infected receptive partner. Other factors that can increase the risk of HIV-1 acquisition include trauma to the genital or anal tract[45], infection with other sexually transmitted infections (STIs) [46], and lack of circumcision [10, 11, 47]. Reducing the risk of infection through efforts such as educational programs, condom distribution, and promoting circumcision is an important part of reducing the effects of the pandemic.

1.4 HIV-1 Structure and Genetics

HIV-1 is a member of the family of *Retroviridae* and genus *Lentivirus* [48] . Lentiviruses are characterized by long duration of illness and long incubation periods. They are single-stranded, positive-sense enveloped RNA viruses. As a retrovirus, the viral RNA genome of HIV gets converted into double-stranded DNA upon entry into the target cell. This reverse transcription is performed by virus-encoded enzyme reverse transcriptase (RT), which is transported in the virus particle [49]. The viral DNA is integrated into the host cellular DNA via a virus-encoded integrase enzyme, with the help of host cellular co-factors, enabling the genome to be transcribed. All retroviruses encode the structural proteins Gag, Pol (polymerase) and Env (envelope) [50].

HIV-1 is 120 nm in diameter and spherical. Its two copies of 9 kb single-stranded RNA (ssRNA) encode HIV's nine genes and are enclosed by a conical capsid, made up of the viral protein p24 (Gag) [51]. The p24 core also contains nucleocapsid proteins, p7, and enzymes required for virion development: reverse transcriptase (RT), proteases, integrase (IN), and ribonuclease. Surrounding the capsid, a matrix made of viral protein p17 (Gag) maintains the integrity of the virion. The viral envelope, made up of two layers of phospholipids acquired from the human host cell upon budding, surrounds the matrix. The envelope also contains proteins from the host, and envelope protein (Env), which forms a spike and protrudes through the surface of the viral particle. Env is a complex containing a cap made of three molecules of glycoprotein 120 (gp120) and stem made of three gp41 molecules anchored in the viral envelope [51]. These glycoproteins are both derived from the *env*-encoded gp160, and their complex facilitates attachment and fusion of the virus with target cells.

In addition to the *gag, pol,* and *env,* which encode the structural genes, HIV-1 also encodes the accessory proteins, *tat, rev, nef, vpr, vif* and *vpu* (or *vpx* in HIV-2) [51-53]. The proteins they encode affect the ability of HIV-1 to infect cells, replicate, or cause disease. Long terminal repeats (LTR) flank both the 5' and 3' ends of the HIV-1 RNA, acting as important regulators in transcription as well as insertion into the host genome.

1.5 HIV Groups and Clades

HIV-1 is divided genetically into 3 groups: M (main), N (non-M, non-O), and O (outlier) [54]. Group M is responsible for the majority of the HIV-1 pandemic, and is subclassified into 9 subtypes or clades: A (broken down further into A1 and A2), B, C, D, F (broken down further into F1 and F2), G, H, J and K [55, 56]. Clade C is the most prevalent and comprises around 60% of the world's infections [57]. Recombinant viruses have also emerged, probably as a result of recombination in individuals infected with two or more divergent strains of HIV-1 [58-60]. The worldwide distribution of the different subtypes is not evenly distributed, with B most prevalent in North America and Western Europe, and C most prevalent in sub-Saharan Africa. It is observed that the different subtypes can vary in their influence on pathogenicity and the rate of disease progression [61], but the exact reasons for this are not known.

1.6 HIV-1 Tropism and Replication

HIV-1 is capable of infecting several different types of immune cells, including $CD4^+$ T cells, macrophages, and dendritic cells [62, 63]. The virus gains entry into cells via interaction of their envelope gp120 with the CD4 molecule on the target cell, which acts as a receptor for HIV-1 [64]. In addition to the gp120-CD4 binding, binding of a chemokine co-receptor is also necessary for viral entry.

Two major co-receptors are utilized by HIV-1 for host cell entry. The β-chemokine receptor, CCR5 is used by Macrophage tropic (M-tropic) strains of HIV-1 [65]. Most primary HIV-1 isolates use CCR5 co-receptors, possibly because macrophages are among the first cells infected by HIV-1 [65, 66]. These strains are termed R5. The α-chemokine receptor CXCR4 is used by T cell tropic strains of HIV-1 [67, 68]. These strains are referred to as X4. Transitional dual-tropic strains of HIV-1, termed X4R5, also exist which can use both CCR5 and CXCR4 as co-receptors. In sexually-transmitted HIV-1, both X4 and R5 virus are present in an infected individual's secretions. However, R5 virus is predominantly transmitted, and then switches tropism later in infection to X4, likely due to an as yet unknown selection process.

As one of the first cells encountered by HIV-1 in sexual transmission, dendritic cells (DCs) are important in the early interactions between HIV-1 and $CD4^+$ T cells. DCs can be infected by the CD4-CCR5 route, but can also express and utilize mannose-specific C-type lectin receptors that bind gp120 for entry. The best-classified of these is called dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN) [69-71]. Immature DCs bind HIV-1 in the genital mucosa, and upon binding, transport HIV-1 to draining lymph nodes, where a primary immune

response is elicited [70, 72]. This allows for close interaction between HIV-1 and $CD4^+$ T cells, and thus facilitates the establishment of systemic infection [73]. DCs may also be directly infected with HIV-1, consequently acting as a reservoir for HIV-1 [74-76].

The start of HIV-1 replication is marked by the binding of the viral envelope proteins, gp41 and gp120, to the host CD4 receptor and a co-receptor (CCR5 or CXCR4). A conformational change occurs in gp41 and gp120 that allows the virus membrane to fuse with the host membrane, facilitating entry of the virus into the cell. The virus core is released into the cytoplasm, while the membrane and envelope remain on the outer surface of the cell. After entry, the core is uncoated via digestion by host enzymes, causing the release of viral RNA, reverse transcriptase (RT), integrase, and RNase H [49]. The error-prone reverse transcriptase, with a mutation rate of 3×10^{-5} per base per replication cycle, can result in at a mutation in each nucleotide of HIV in a day. The RT, primed by human tRNA(Lys,3), transcribes a single strand of (-) sense DNA from the viral (+) sense RNA, followed by degradation of the bound strand of RNA by RNase H. The RT then transcribes a second strand of DNA using the first strand as a template [77]. The double stranded DNA (dsDNA) enters the host cell nucleus, where it randomly integrates into the host genome by viral integrase. The integrated DNA may remain dormant, or it may actively produce virus. Replication occurs when the provirus is copied into mRNA which is spliced to produce the regulatory proteins Tat and Rev. Tat binds to the viral LTR and sets off further viral transcription, while Rev inhibits mRNA splicing as it accumulates, through interaction with alternate splicing factor/splicing factor 2 (ASF/SF2) and p32 [53, 78]. Host cell machinery is used to translate viral mRNA into long precursor protein strands, which are later cleaved into their mature

structural and enzymatic forms, by viral proteases, either during or following release from the host cell. Release of the new virions occurs by budding from the host cell and consequently acquires host membrane phospholipids and proteins [79]. This is an important mechanism in evading the host immune response because it allows the virus to disguise itself as a host cell. Finally, the mature virion is able to further establish systemic infection of the host.

The main reservoir for latent HIV-1 replication is in resting memory $CD4^+$ T cells [80-82]. This poses a huge challenge in HIV-1 eradication, as highly active antiretroviral treatment (HAART) is very effective at reducing active infection, it does not target these inactive cells [82]. These infected cells would also fail to be recognized by the host immune response. Latently infected cells do not typically recommence virus production until activation. Activation occurs upon recognition of previously encountered antigens, which allows HIV-1 to proliferate. Other reservoirs that allow HIV-1 to avoid the immune response and therapy during the host during clinical latency include macrophages [83] and follicular dendritic cells [84, 85].

1.7 Clinical Course of Infection and HIV-1 Pathogenesis

HIV-1 pathogenesis follows three general stages: primary infection following an initial incubation period, followed by latency, and finally increased viral replication leading to $CD4^+$ T cell decline and AIDS. The length of time for progression to each stage of disease is variable between individuals, and depends on a wide variety of factors, such as the immune system, socioeconomic status (which impacts diet and overall

health), viral strain, and presence or absence of consistent treatment. There are, however, some generalities in disease progression.

Following exposure, there is an asymptomatic incubation phase lasting about two to four weeks characterized by a huge amount of viral replication, during which primary HIV-1 infection of systemic $CD4^+$ T cells occurs. In particular, the effector-memory T cells in the gastrointestinal tract are massively depleted [86]. Infection is established in lymphoid tissues via transport from the mucosa by $CD4^+$ T cells and DCs. The primary infection involves a rapid viral amplification leading to high levels of virus in the peripheral blood with an accompanying decrease in the number of circulating $CD4^+$ T cells. Symptoms associated with primary infection are relatively non-specific, but during viremia, most infected individuals experience a flu-like illness characterized by fever, headache and lymphadenopathy. This illness is usually not diagnosed, as the symptoms are similar to many other viral infections. HIV-1 viremia is then reduced, possibly due to the production and cytotoxic activity of cytotoxic $CD8^+$ T cells (CTL). This is followed by seroconversion, or production of antibodies against HIV-1 [87]. Typically the symptoms disappear in conjunction with the emergence of the CTLs/antibodies and the subsequent drop in viremia. The viral setpoint following the resolution of the primary infection stage is indicative of the disease progression rate [88].

The initial infection stage is followed by the clinically latent stage. This is different from true viral latency, which would indicate a state of infection without ongoing viral replication, whereas with clinical latency, the virus does replicate. This stage is variable in length depending on the host and virus, and treatment, but can last for 8-10 years. The host appears healthy during this time, but over time the immune system

is unable to completely eradicate the HIV-1, and the patient eventually succumbs to the intense pressure of HIV-1.

The inability to control infection is due in part to HIV's high mutation rate (due to the error-prone RT), which generates about 1 error per 5 x 10^5 bases [77], resulting in mutant forms of the virus that are able to escape the initial immune responses. This results in uncontrolled viral replication and a steady loss of $CD4^+$ T cells, which are essential for a properly functioning immune system. This loss can occur through direct killing, due to the direct cytopathic effects of viral replication within the infected $CD4^+$ T cells. Also, recent evidence suggests that immune activation-induced apoptosis plays a substantial role in disease progression [89, 90], possibly due to the upregulation of apoptosis inducing molecules such as TRAIL, CD95, and DR4/5 on T cells [91]. Immune activation is also thought to be detrimental to the immune response via activation of naïve and memory T cells, which upregulates CCR5 and increases susceptibility of infection, disturbing homeostasis of naïve and memory T cell pools which may result in their depletion and thus reduced ability to mount adaptive immune responses, and also by increasing inflammation which can destroy secondary lymphoid tissues needed in T cell formation and homeostasis [91]. The AIDS definition is applied when an individual's $CD4^+$ count drops below a certain threshold, usually below 200 cells/μl, at which time they become susceptible to opportunistic infections or malignancies, which they eventually succumb to [92]. AIDS-defining illnesses include several infections that usually do not infect individuals with normal T cell immunity, thus they are termed opportunistic [93]. Examples include pneumonia caused by *Pneumocystis carinii*, an intracellular bacterium, atypical mycobacteria, or viruses such

as cytomegalovirus (CMV). Although $CD4^+$ T cells are the cells most depleted by HIV-1, the $CD8^+$ and antibody-mediated responses suffer as well due to a lack of helper T cells which shape their development [93]. Oncogenic viruses may also contribute to increased cancer susceptibility in AIDS, including Epstein-Barr virus-associated B cell lymphoma and herpesvirus-associated Kaposi's sarcoma [93].

Between HIV-1 positive individuals, there exists a great spectrum in terms of progression through the stages of infection. Some individuals progress to AIDS very soon after primary infection, within 1-3 years; these individuals are termed rapid progressors. Other infected individuals may continue to be clinically asymptomatic and maintain normal CD4 counts for greater than 10 years, and are termed long-term non-progressors [94]. Another distinct phenotype of HIV-1 contact is observed in about 1 in 300 HIV-infected individuals is the ability to control HIV-1 RNA replication to a level below 50 copies per ml in the absence of therapy for one year or longer [95, 96]. These individuals are termed elite controllers. Viremic controllers [96] are able to maintain HIV-1 RNA levels below 2000 copies per ml in the absence of therapy for at least a year. These elite and viremic controllers tend to have longer than average median durations of infection, generally more than 12 years. Understanding the immune mechanisms behind the variance in rate of progression to AIDS may provide insight into therapeutic vaccines.

In addition to differences in HIV-1 disease progression, heterogeneity also exists within populations in regards to the susceptibility to HIV-1 infection. A small proportion of individuals never become infected with the virus, even after frequent exposure over many years. This is an important phenomenon observed in our study population, which

will be discussed further. This highlights the importance of the immune response in controlling HIV-1.

1.8 The Immune Response to HIV-1

While both the adaptive and innate arms of the immune system work in concert to fight infection and maintain tolerance to self antigens, they differ in several key ways. Adaptive immunity is characterized by recognition of specific antigens and the ability to maintain memory cells, which can clonally expand upon subsequent exposures to a pathogen. While the innate immune system is less specific in its response and lacks memory, it is the fastest responder upon first contact with a pathogen, and plays an important role in directing the adaptive response.

1.8.1 Adaptive Immunity: Cellular and Humoral

The adaptive arm of immunity is comprised of two main types of lymphocytes: B lymphocytes, which secrete antibodies, and T lymphocytes, including $CD8^+$ T cells (CTLs, cytotoxic T cells) and $CD4^+$ T cells (T helper cells) which activate cellular and antibody responses. Adaptive immunity also results in memory cells to recognize and clonally expand upon encounter of previously encountered antigens. Recognition of viral and self-antigens is achieved through HLA antigen presentation. HLA class I molecules are found on all nucleated cells and present antigens to $CD8^+$ T cells, while HLA class II molecules are present only on professional antigen presenting cells (APCs), and present antigens to $CD4^+$ T cells.

Depending on the cellular environment, $CD4^+$ T cells can differentiate into effector cell subsets, which carry out different responses, and are defined by distinct sets of cytokines. These subsets include type 1 helper T cells (Th1), which correspond with a cell-mediated immune response, and type 2 helper T cells (Th2) cells, which correspond with a humoral or antibody-mediated response. Their differentiation depends on the stimuli of microbial antigens encountered by naïve $CD4^+$ T cells. Th1 development is stimulated by IFN-γ and IL-2 cytokines. This results in the recognition and destruction of infected cells presenting non-self antigens, via the CTL response. The Th1 cell-mediated response is thought to have the most significant contribution in controlling intracellular pathogens, including HIV-1, in infected individuals. In contrast, Th2 development arises due to the release of IL-4, which stimulates IgE, as well as IL-5, which activates eosinophils. Th2 responses are generally directed more towards extracellular pathogens and allergens [97]. A balance between the Th1 and Th2 responses determines the overall response, as several cytokines produced by Th2 cells, including IL-4, IL-10, and IL-13 actually act to inhibit the activation of macrophages and suppress Th1 immunity, and vice versa.

Additionally, a $CD4^+$ T cell subset called Th17, was recently identified as having a potential role in the defense against HIV-1. These cells secrete the proinflammatory cytokine Th17, and its levels increase during HIV-1 infection [98]. Another subset called CD4 regulatory T cells (Treg) known to suppress immune activation have been studied intensely recently. While their exact mechanisms have not been resolved yet, Tregs can be both protective, in terms of suppressing harmful immune activation, but also detrimental to the host if suppressing effective T cells [91].

1.8.2 Innate Immunity

The innate immune system constitutes the host's first response to HIV-1. The innate immune system consists of epithelial barriers, cells in circulation and tissues, as well as plasma proteins [99]. Extracellular components such as chemokines, cytokines, and lectin-binding proteins contribute to innate immunity. A previous misconception assumed that innate immunity was non-specific, weak, and ineffective at warding off infections. Recently, this has been refuted due to the discovery that innate immune cells are able to distinguish self from non-self cells (Lehner, 2008) and trigger signaling pathways to induce immune responses towards the pathogen. Many of these cells have pattern recognition receptors (PRRs) that target microbes and recognize structures or pathogen-associated patterns (PAMPS) that are shared by microbes, but that are not present on host cells. Other innate receptors, such as those on natural killer (NK) cells, may recognize the presence or absence of host molecules. The receptors for innate immunity tend to be germline-encoded with non-clonal distribution, and consequently they lack the diversity and potential for immunological memory of the adaptive immune system as well as the ability to recognize structural detail or antigens. In further contrast to adaptive immunity, subsequent encounters with a microbe do not result in more rapid responses by innate immunity. These limitations aside, this branch of immunity is a strong primary responder, with innate cells having effector functions initially in the control and even elimination of infections allowing time for adaptive immunity to form [99].

While innate immunity acts as the first defense against HIV-1, it is also important in shaping the adaptive immune response, acting as regulatory cells and instructing the

immune response through cross-talk between the APCs expressing CD4 and CD8 [93]. It is thought that an effective innate response to HIV-1 may prevent the early infection of the mucosal $CD4^{+}CCR5^{+}$ memory T cells [99, 100].

The first line of defense against HIV-1 during penetrative sexual transmission is the physical epithelial barrier lining the genital/rectal mucosa. The epithelial surface also contains cellular innate components, including γδ T-lymphocytes, dendritic cells (DCs), monocytes, and natural killer (NK) cells. Gamma-delta T-lymphocytes produce Th1/Th2 cytokines, and generate antiviral chemokines, including RANTES (CC-L5), MIP-1α (CC-L3) and MIP-1β [101, 102]. These γδ T-cells also express receptors that are typically associated with natural killer (NK) cells, and can have similar activating and inhibitory effects upon ligand binding [103, 104].

Dendritic cells (DCs) constitute another important group of cellular innate cells. These include Langerhans DCs in the vaginal lumen, oral mucosa, and foreskin [105], which act by capturing HIV-1 and presenting viral antigens on their surfaces. Other important DC subsets include the immature myeloid DCs (mDCs), as well as the circulating plasmacytoid DCs, which secrete interferons (IFN) α and β, which activate NK cells as well and may help mature other bystander DCs [106, 107]. These pDCs mature into antigen-presenting cells (APCs) that can regulate T cell development and other APCs [106]. This is an important link between the innate and adaptive immune responses. A decline in DCs is observed as HIV-1 infection progresses [108], and cannot be recovered after the initiation of HAART [109]. This impairs the host's innate immune system and thus its ability to fight other infections as HIV-1 progresses. In counterpoint to their protective role in inhibiting infection, DCs can also propagate infection, due to

their ability to be infected and transport HIV-1 from mucosal barriers to local lymph nodes, where the virus can infect more cells and propagate.

Macrophages are also important innate cells contributing to the immune response against HIV-1. These antigen-presenting cells express the HIV-1 CD4 and CCR5 receptors, which facilitate their infection by the virus. Macrophages act as an HIV-1 reservoir as the disease progresses [110, 111].

Innate cells such as macrophages, DCs, and NKs have the ability to recognize pathogens through the use of PRRs such as Toll-like receptors (TLRs), which then activate signaling cascades to activate the immune cell to coordinate systemic defenses via the release of cytokines, and control innate cell function [106, 112]. Single-stranded RNA from HIV-1 has been shown to bind and activate TLR7 and TLR8 on macrophages and DCs [113]. While this may be useful to combat viral replication during acute infection and may contribute to susceptibility to infection, it may actually result in an increase in viral replication during chronic infection through the activation of latent infected cells [114].

Further examples of innate germline-encoded receptors can be found on NK cells. Natural killer cells are innate cells that target virally infected and tumor cells for destruction [115], and contain specialized receptors that are able to control NK activity based on the presence or absence of their HLA class I ligands. Among these receptors, the most significant in terms of antiviral function are the Killer cell Immunoglobulin-like Receptors (KIRs), which are the focus of this study and will be discussed further.

Extracellular innate factors are also important in HIV-1 immunity. The complement system is included in this category [116, 117]. Also, type I interferons (IFN

α and β) are released due to stimulation of glycosylated envelope with mannose receptors on DCs and macrophages [118]. These interfere with viral growth, activate transcription factors to induce IFN-γ and promote Th1 development [119], and prevent apoptosis of activated T cells. There are 3 chemotactic cytokines (CC), which are produced by activation of macrophages, DCs, T, NK, and γδ T cells. These are RANTES, MIP-1α and MIP-1β. The CC chemokines can block the CCR5 co-receptors and thus prevent HIV-1 infection in vitro [120] or SIV in vivo [102], but they are also chemo attractants for T cells, which may in turn facilitate their infection. In addition, defensins are peptides excreted by epithelia cells, which may take part in mucosal protection against HIV-1 [121-124]. Two other host factors that act against HIV-1 infection include alpha1-antripsin alpha1-proteinase inhibitor (serpin A1), is a non-cytolytic antiviral factor found in the blood which inhibits neutrophil elastase, and CD91, its ligand, which internalizes serpin C-terminal fragments and α-defensins [125].

Recently, several intracellular innate antiviral factors have been identified, including apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G), tripartite motif 5-α (TRIM-5α), and alfa defensins. APOBEC3G is packaged into virions and deaminates viral cytidine to uridine [126-128], and it may also restrict HIV-1 infection in $CD4^+$ T cells [129]. However, its incorporation can be inhibited by HIV-1 protein Vif [130, 131], which downregulates APOBEC3G translation and targets the molecule for ubiquitination and degradation [132, 133]. Trim-5α is another host antiviral factor that is able to block HIV-1 capsid uncoating [134-136]. While this is a promising observation, human Trim-5α is not effective against HIV-1 [137]. More recently, protein-based tethers have been identified called CD317 or

"tetherin", which are able to retain fully-formed virions on the surfaces of infected cells and prevent their release but is counteracted by HIV-1 protein Vpu [138]. It is possible that these host antiviral factors evolved and were selected for along with human exposure to endogenous retroviruses.

1.8.3 Shortcomings: The Immune Response Does Not Effectively Clear HIV-1

Despite responses from the innate and adaptive arms of the immune system, there have been no effective immune mechanisms identified so far that are capable of the clearance of HIV-1. This is intensified by escape mutations in the virus as well as the presence of a latent reservoir of cells. While antibodies and CTLs are generated by infected individuals and may slow HIV-1 initially, they are not effective at preventing the chronic progression of the virus permanently [139]. The CTLs are the primary determinant of setpoint viremia. Antibodies mainly target the gp120 envelope glycoproteins and other viral proteins [140] to prevent viral entry, but the virus' surface proteins are capable of rapidly mutating due to the error-prone reverse transcriptase, thus allowing the virus to escape antibody recognition. HIV-1 is also able to downregulate the expression of HLA class I molecules on infected cells, resulting in unsuccessful CTL responses. Ironically, the host immune responses against HIV-1 actually assist the virus in its infection strategy. For example, viral particles coated with antibodies may bind Fc receptors on macrophages and follicular DCs in lymph organs, allowing the virus to enter these cells and create further reservoirs.

Additionally, the infection of immune cells directly and negatively impacts the functioning of the immune system, allowing HIV-1 to prevent its own clearance. Cytokines released in response to HIV-1 infection also enhance infection. Also, lysis of

infected cells by CTLs results in release of viral particles, subsequently further spreading the infection to other cells [93]. In the absence of therapy, patients eventually progress to AIDS.

It is clear that new vaccine strategies will need to be explored, and the discovery of rare individuals who have natural immunity to HIV-1 provide a unique model for this.

Section 2: Immunological and Host Genetic Contributions to Disease Susceptibility

2.1 The Pumwani Sex Worker Cohort

The Pumwani sex worker open cohort was established in 1985 in Nairobi, Kenya [141, 142] to prospectively study sexually transmitted infections, and continues to enroll sex workers with biannual follow-up at the Majengo clinic. The overall HIV seroprevelance in the Pumwani sex workers is approximately 70%, and most HIV negative women enrolled in the cohort seroconvert within three years. However, a subgroup of women remains seronegative and PCR negative despite frequent exposure to HIV-1, through high-risk sex work comprising an average of 5-6 sexual encounters per day. About 5-10 percent of highly-exposed sex workers in the Pumwani cohort are resistant to HIV-1, and are classified as such if they remain HIV-1 seronegative and PCR negative after at least three years follow-up while continuing active sex work, and remain as such at the time of study [19]. As seen in Figure 1, the risk of seroconverting for a sex worker who has remained HIV-negative for three years plateaus after further follow-up, indicating a decreased risk of infection corresponding with increased time in the cohort, and consequently increased exposure to HIV-1.

There are several features that make the women in this cohort an outstanding model for studying natural immunity to HIV-1, including their high exposure to many different HIV-1 subtypes, rate of exposure to high-risk infected men, extensive follow-up, and our comprehensive collection of information in regards to their clinical, behavioral, epidemiological, and biological data. Understanding the biological and genetic factors that contribute to this distinct resistance phenotype offers hope that effective vaccines or treatments could be developed based on natural immunity, or even tailored to an individual's genotype.

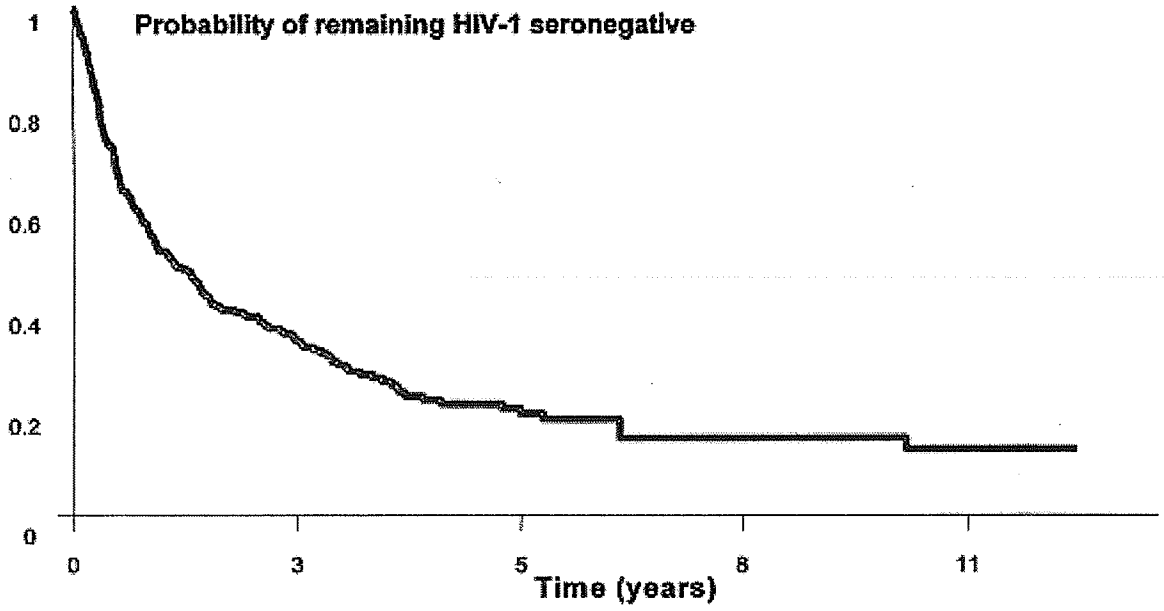

Figure 1. Kaplan Meier plot showing the probability of remaining HIV-1 seronegative with years of follow-up in the Pumwani sex worker cohort.

(Taken from [1]. Permission obtained August 22, 2008.)

2.2 Known Markers of Natural Resistance to HIV-1

All populations experience variation in susceptibility to infection and disease caused by infectious pathogens. Some individuals who are exposed to a pathogen will resist infection, and individuals who do become infected may experience different levels of morbidity and mortality. It is thought that the great diversity within the immune-related genes such as HLA and KIR has arisen throughout our evolution, to ensure survival of at least some members of a population due to selective pressure exerted by exposure to infectious agents. As observed with other infections, exposure to HIV-1 does not necessarily result in infection with the virus. Heterogeneity in HIV-1 susceptibility has been observed in several cohorts, including commercial sex workers (CSW) [18, 143-146], injection drug users [147], HIV-1 discordant couples [148], and HIV-1 negative babies of infected mothers [149], as well as individuals exposed but uninfected after occupational exposure [150].

The women of the Pumwani sex worker cohort provide an excellent opportunity to study natural immunity to HIV-1, due to their high levels of exposure to multiple viral variants. What is allowing these women to resist HIV-1 infection? Characterizing the host genetic and immunological mechanisms of resistance and susceptibility to HIV-1 infection may provide the answer, and will hopefully generate new ideas for vaccine design.

2.2.1 The Role of Genetics in Susceptibility to HIV-1 Infection

Human genetic make-up has been implicated in HIV-1 resistance. A well known example is a 32 base pair deletion in the CCR5 chemokine receptor, which has been found in some exposed, uninfected (EU) individuals [151-153]. This polymorphism is known is CCR5-delta32, and results in a truncated CCR5 which is not expressed on the surface of $CD4^+$ cells. In homozygotes, this prevents infection by R5 tropic viruses which require the receptor for entry [154]. Slower disease progression is seen in infected heterozygotes for CCR5-delta32, as well as people with mutations in the minor CCR2 coreceptor (CCR2-64I) [155, 156]. While the CCR5-delta32 mutation can be found at a heterozygote frequency of up to 20% in Eastern European populations and about 1% of Eastern Europeans are homozygous for the mutation, it is not detected in African or Asian populations to an appreciable extent [157, 158]. Mutations in the ligands for the major chemokine receptors, CCR5 and CXCR4, have also been identified. These include a promoter mutation in RANTES (ligand for CCR5) which is associated with delayed disease progression [159] and HIV-1 EU individuals [160], as well as a polymorphism in the 3' untranslated region of a ligand for CXCR4, stromal derived factor 1 (SDF-1). The SDF-1 3'A mutation results in increased SDF-1 translation, which may result in reduced T-tropic HIV binding due to competition from the more-prevalent SDF-1, and thus a lesser chance of infection [161]. Another suppressive chemokine called CCL3L1 is a ligand for the CCR5 coreceptor, and its copy number is known to differ between populations. Lower than average CCL3L1 copy numbers have been associated with an increased susceptibility to HIV-1 and faster disease progression in HIV-1 positive individuals [162]. In addition, polymorphisms in the IRF-1 gene, located in the IL-4

gene cluster, have been found to associate with lower levels of IRF-1 protein expression and resistance to infection by HIV-1 in the Pumwani cohort [163]. As well, a genetic variant in the DC-SIGN repeat region, which may cause decreased binding of HIV-1 to DCs, was associated with a decreased risk of infection [164].

While the genetic factors identified thus far shed light on protection to HIV-1, they do not explain all cases of altered susceptibility to infection. Thus, it is clear that other factors also contribute to altered susceptibility to HIV-1.

2.2.2 The Role of Immunology in Susceptibility to HIV-1 Infection

Several innate factors have been found to associate with susceptibility to HIV-1, including the beta chemokines RANTES [165], MIP-1α, MIP1β and SDF-1, as a consequence of their interactions with HIV-1 co-receptors including CCR5 (RANTES, MIP-1α and MIP-1β) [166] and CXCR4 (SDF-1) [167]. The CCR5-blocking beta chemokines were initially found in the supernatants of activated $CD8^+$ T cell cultures from HIV-1 positive patients, which could inhibit HIV-1 infection *in vitro* [168]. It was shown that this anti-HIV effect was due to their binding of the coreceptor, and thus inhibited binding and entry of HIV-1 [169, 170], an observation that is supported by the fact that several cohorts studies have shown them to be expressed at high levels in HIV-1 EU individuals [171].

HIV-1-specific adaptive immune responses have been observed in exposed uninfected subjects, including $CD4^+$, $CD8^+$, and antibody responses, suggesting that these individuals have encountered the virus, but may have successfully cleared it before the establishment of chronic infection. HIV-1 specific $CD4^+$ T cell responses have been found in HIV-1 EU individuals [148, 172-174] including the resistant women in the

Pumwani cohort, demonstrated by the release of IL-2 when exposed to HIV-1 Env or HIV-1 peptides, or shown to proliferate upon exposure to p24 [172]. These sex workers tend of have depressed levels of immune activation markers such as HLA-DR and CD38. This may help to prevent chronic infection, since activated T cells are necessary for HIV-1 replication. These $CD4^+$ T cells may also contribute to protection by enhancing the CTL response. HIV-1 resistance in the Pumwani cohort has been associated with broadly cross-reactive CTL to HIV-1, which are weaker and less frequent than the responses seen in infected subjects, and which may recognize different epitopes [173, 175-178]. HIV-1 specific CTL response is measured by IFN-γ secretion or cytotoxic ability. Interestingly, HIV-1 specific CTL responses in the genital tract of these sex workers have been shown to be more frequent than those in the systemic compartment, and more frequent than responses in HIV-1 positive sex workers [177]. However, whether the CTL contributes to protective immune responses against HIV-1 remains to be elucidated.

Studies have also shown that HIV-specific antibodies are associated with altered susceptibility to HIV-1. Neutralizing IgA antibodies have been detected in HIV-1 EU individuals [148, 179-184], including in the vaginal and oral mocosa in HIV-1 resistant women of the Pumwani cohort [180, 181, 184]; however, they are not found in all EU groups [185]. While this HIV-1-specific IgA has been shown to inhibit HIV-1 infection in culture, its physiological role is less clear. Whether the presence of these antibodies is protective or simply a marker of exposure is still unknown. In addition to IgA, macaque models have shown that passive transfer of neutralizing IgG were protective against a simian HIV (SHIV) challenge [186]. HIV-specific IgG antibodies have not been shown to play a role in altered susceptibility to HIV-1 [182] in humans, although they have been

discovered in the genital tract of HIV-infected people [187]. Interestingly, non-neutralizing IgG have been shown to play an important role in HIV-1 replication *in vitro*, inhibiting replication in macrophages and immature DCs, via either Fab domain-mediated neutralization of HIV-1, or binding to the Fcγ I and II receptors on these immune cells [188]. Another SHIV study in macaques showed that *in vivo*, a mutated version of neutralizing IgG1 b12 that was not able to bind Fc receptors had impaired protection against the vaginal route of infection [189], supporting the involvement of b12 in protection, perhaps through a role in antibody-dependent cellular cytotoxicity (ADCC). These findings suggest that both neutralizing and non-neutralizing antibodies are important in mediating protection from HIV-1.

A recent study showed that HIV-1 resistant women in the Pumwani cohort overexpressed several innate factors in genital secretions [190], including serpin B proteins, cystatin A, which is known to have anti-HIV-1 properties. Polymorphisms in the regulatory factor IRF-1, which is thought to be able to activate HIV-1 replication, have been found to be overrepresented in HIV-1 resistant cohort members as well [163, 191]. HIV-1 resistant women in the cohort also had elevated T cell recruitment and have elevated levels of RANTES in the genital mucosa, which were not observed in the systemic compartment [192].

2.2.3 The Role of Host Immunogenetics in Susceptibility to HIV-1

One of the most -studied aspects in susceptibility to HIV-1 has been the HLA system, and both class I and class II genotypes have been implicated in protective immunity [193, 194]. Mismatches in HLA type between discordant couples, as well as class I B53 and class II DR5, DR1, and DQ4 have all been associated with protection

from infection [195-197], as well as class I B18 in Thai CSW [18]. In addition, delayed disease progression has been observed in infected individuals with the HLA-B*35 or B*57 genes [198]. In the Pumwani cohort, the genotypes that have been shown to associate with HIV-1 resistance include the HLA class I A2/6802 supertype [193], as well as class II DQB1*050301, *0603, *0609, and DQA1*010201-DQB1*0603 [199], DPA1*010301, DPB1*3001, DPA1*010301-DPB1*3001, DPA1*0301-DPB1*5501 [200], DRB1*01, *1102, *030201, *070101, *1503, and DRB5*010101 [201].

Recently, another marker has been implicated in HIV-1 immunity- the KIR family of receptors, which are expressed on NK cells. While several studies have demonstrated that certain KIRs are associated with delayed disease progression in HIV-1 positive individuals, they have also been shown to associate with lowered susceptibility to HIV-1 infection. The role of KIRs in HIV-1 resistance appears to be very important, and will be discussed further.

2.2.4 Markers of HIV-1 Resistance in the Pumwani Cohort

A subset of women enrolled in the Pumwani sex worker cohort remains seronegative and PCR negative for HIV-1, despite repeated high-risk exposure through active sex work [143]. This resistance phenotype is likely due to a combination of a multitude of traits, and indeed, we have identified several genetic, immune, and immunogenetic factors associated with resistance in the Pumwani cohort, as summarized in 2.2, while ruling out others that were found to be protective in other populations.

Resistance in the Pumwani cohort cannot be attributed to safer sexual behavior or other sexually transmitted infections. We also know that resistance in the cohort is not due to chemokine polymorphisms or expression levels that have been associated with

protection in other populations, including CCR2-64I [202], CCR3, CXCR4 and CCR5 (delta 32) [203].

Despite the many genetic and immune components that are associated with natural protection from HIV-1 infection, the exact mechanism is unclear. Since the resistance phenotype cannot likely be narrowed to a single factor, comprehensive insights into each factor that contributes to resistance, taken both independently and as a whole, will be critical in understanding this phenomenon and ultimately informing vaccine design.

2.3 Natural Killer Cells

Natural killer (NK) cells are bone marrow-derived lymphocytes that are important in the early innate immune response against viral infection and tumour cells [204-206]. They are found in the liver, peritoneum and placenta, and they comprise about 15% of peripheral-blood lymphocytes [204]. They are also present in lymph nodes and have the ability to migrate to inflamed tissues and organs. Natural killer cells were discovered based on their ability to kill tumour cells, but were soon found to also play an important role in killing virus infected cells in infected hosts [207]. Once thought to be non-specific in their killing, evidence to the contrary has shown that NK cells actually contain receptors which impart specificity to their cytolytic activity. [208]

The inactivated mature NK cell is prepared for activation; constitutively expressing cytokine transcripts, including IFN-γ [208], granzymes and perforin. Granzymes are proteolytic enzymes that activate caspases in target cells, causing apoptosis, and perforin is a protein subunit that assembles to create a pore-forming

structure that damages the target cell. Granzymes and perforin are stored in intracellular granules [209, 210] and are also important components of cytotoxic T cells. NK cell activation can be achieved through exposure to type I interferons (IFN-α or IFN-β) or pro-inflammatory cytokines (IL-15, IL-12, or IL-18) [211]. These are often produced by DCs, and reciprocally, the NK-derived IFN-γ helps in the maturation and function of DCs [211]. Cytokines produced by NK cells can also affect other leukocytes, such as macrophages, granulocytes, and other lymphocytes. NK cells may also act as bystanders due to activation by the surrounding cytokines and IFNs. Additionally, NK cells may attack virus-infected cells coated in IgG due to the presence of an activating Fc receptor for IgG (FcγR CD16) on the surface of the NK cell [211].

Overall, NK cells are important in the host's innate defence against viruses, including HIV-1, due to their rapid cytokine and chemokine secretion, their ability to directly kill infected cells, and their stimulatory and regulatory effects on other important adaptive immune cells. Some viruses have even developed evasion mechanisms from NK cells. This is exemplified by the detrimental effects that HIV-1 viremia can have on NK function, including reduced expression of activating receptors and increased expression of certain inhibitory NK receptors, which can result in impaired ability to lyse infected cells, as well as inhibition of the release of both CC-cytokines and pro-inflammatory cytokines [212]. For example, a decline in IFN-γ producing NK cells has been observed as HIV-1 progresses [213]

2.3.1 NK Receptors: Killer cell Immunoglobulin-Like Receptors

Recently, considerable attention has been paid to the observation that NK cells may directly recognize infected cells through binding of NK receptors to infected cells,

thus mediating their activity. This has been one of the most important discoveries in terms of understanding how and why NKs attack their targets while sparing self-cells. The first prediction of potential NK-cell receptors with inhibitory effects came after the observation that while T cell lymphomas grew in syngeneic mice, that a variant deficient in HLA class I was rejected by the host [214]. Eventually, it was shown that NK-cell receptors could block the cytolytic functions of NK cells, due to their recognition of HLA class I molecules [103, 215-218]. The NK receptors include C-type lectin receptors, including CD94/NKG2 on chromosome 12 [206], and the Immunoglobulin Superfamily located in the leukocyte receptor complex on chromosome 19, which includes the leukocyte immunoglobulin-like receptors (LILRs), leukocyte-associated inhibitory receptor (LAIR), FcαR, and the activating NK receptor NKp46 and the Killer cell Immunoglobulin-like Receptors (KIRs) [206]. KIRs are the most abundant surface receptors expressed on human natural killer cells, and they have specific HLA class I molecules as their ligands. This allows self versus non-self recognition, or the missing-self hypothesis, or the targeting and elimination of cells that do not express self-HLA molecules [206]. KIRs have also been shown to be expressed on a minor subset of $CD8^+$ T lymphocytes.

The ability of KIRs to activate or inhibit NK activation and function, depending on the receptor as well as presence or absence of its HLA ligand, is important in the balance between fighting infection and avoiding autoimmunity.

2.3.2 How KIRs Work: Signaling and Effector Functions

How do KIRs function to allow to NK cells to target infected cells for destruction, while leaving normal self cells untouched? Upon binding a normal healthy cell, the NK

cell is inhibited. Despite binding of an activating receptor, which may be a short-tailed KIR or belong to another NK activating receptor family, an inhibitory KIR on the same NK cell will also bind to an HLA Class I molecule which is normally expressed on the surface of healthy host cells (Figure 2A). This overrides the activating signal and inhibits the NK cytolysis. However, activation of the NK cell occurs when an activating receptor on the NK cell binds to a stress ligand on the infected host cell (Figure 2B). In this case, the inhibitory KIR is not engaged due the absence of the HLA Class I ligand on the surface of the host cell, which is typically downregulated by the virus, or in the case of tumor cells, by cellular dysfunction. This triggers the release of cytolytic perforin and granzymes from the NK cell and subsequent destruction of the infected cell. In addition to being downregulated, another activation mechanism proposes that HLA class I can present specific peptides, such as those from pathogens, which can disrupt the KIR inhibitory ligand from binding [219].

KIR signaling determines whether the NK cell will be activated to kill an infected or tumour cell, or inhibited against self-cells. Most long-tailed, or inhibitory KIRs, contain two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) [220] (except 2DL4 which has only one ITIM) which operate in tandem to control inhibition. Upon binding its HLA class I ligand, the tyrosine residues in the ITIM are phosphorylated, resulting in the recruitment of Src-homology domain-bearing tyrosine phosphatase 1 (SHP-1), a tyrosine phosphatase that inhibits the proteins involved in the intracellular activation cascade [221]. An example of this is shown in Figure 3, for the inhibitory KIR2D receptors. In contrast, the short-tailed KIRs are generally activating, with their signaling mediated by the transmembrane adaptor protein DAP12, which contains two or

more immunoreceptor tyrosine-based activating motifs (ITAMs) [222, 223]. When the activating KIR binds its ligand, DAP12 is phosphorylated and ZAP-70/Syk kinase gets recruited, causing the downstream intracellular signaling cascade to be activated [224].

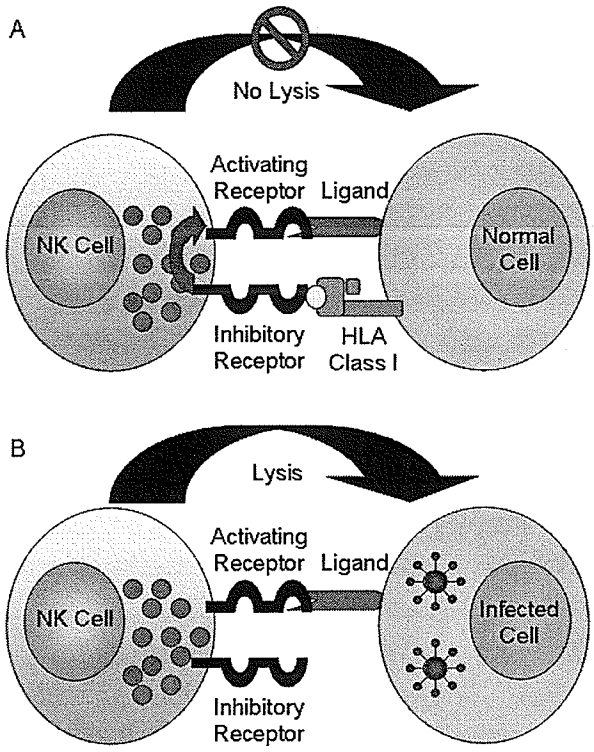

Figure 2. KIR effects on NK cytolysis in the control of HIV-1 infection.

(A) Upon binding a normal cell, the NK cell is inhibited: despite binding of the activating receptor to the host ligand, the HLA class I receptor binds the inhibitory KIR, which overrides the activating signal and inhibits the NK cytolysis.

(B) Upon contact with an infected cell, the NK cell is activated, causing the release of perforin and granzymes: the activating receptor is bound to the ligand on the infected host cell, but the inhibitory KIR is not engaged due to a lack of HLA class I ligand on the surface of the host cell.

A review of NK recognition of target cells can be found in Fauci et al [212].

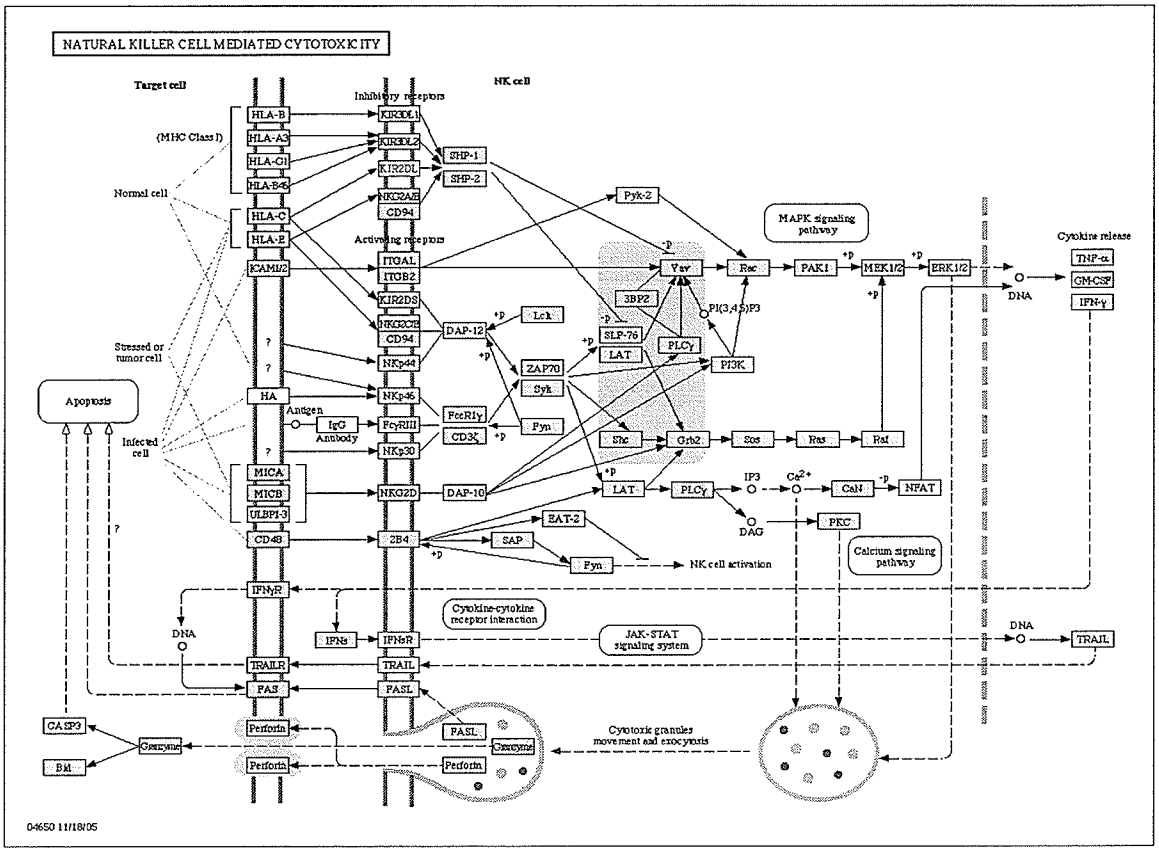

Figure 3. KIR2DL in the signaling pathway for natural killer cell cytotoxicity.

Note: Arrows represent molecular interactions between gene products, which are depicted in rectangles.

(Pathway diagram reproduced from Kyoto Encyclopedia of Genes and Genomes (KEGG) [2-5]. Permission obtained August 28, 2008.)

2.3.3 KIR Genetics and Structure

Much like the genes of the HLA complex, the KIR genes are highly polymorphic and have evolved and diversified, providing a basis for natural selection. The KIR gene family is located in the Leukocyte Receptor Complex (LRC) on chromosome 19q13.4. It is polygenic, consisting of 15 KIR genes and 2 pseudogenes. These are shown in Table 1 with each KIR's known ligands. The KIR genes are arranged in tandem over about 150 kb (Figure 4) [225-227]. They share a high level of sequence similarity (85-99%), and evolve via non-allelic homologous recombination, which may explain their polygenic nature. The exon and domain structure for each KIR gene is shown in Figure 5. Each gene is also polyallelic, or contains several alleles, adding a further layer of complexity. Consequently, the number of KIR combinations identified thus far is staggering, and the likelihood that two individuals have identical KIR haplotypes is extremely low. The KIR cytoplasmic tails have differing lengths which correspond with their functions. Although the exon sequence lengths are similar between short- and long-tailed KIRs, the presence of stop codons due to single-nucleotide polymorphisms or short insertions/deletions (indels) result in shorter tails. Other structural polymorphisms may be important in the specificity of ligand binding. Long-tailed inhibitory KIRs, have immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Figure 5). In contrast, short-tailed activating KIRs (Figure 5) are associated with the DAP12 signaling molecule through interactions with a positively charged lysine residue in their transmembrane domain [223, 228]. DAP12 contains immunoreceptor tyrosine-based activating motifs (ITAMs), which mediate KIR signaling.

Table 1. The KIRs and their known ligands.

Gene and Protein Symbol	Extracellular Ig-Like domains	Description	Aliases	# Alleles	Ligand
KIR2DL1 [229, 230]	D1-D2	2 domains, long cytoplasmic tail, 1	47.11, CD158a, cI-42, nkat1, p58.1	10	HLA-C group 2 ($HLA\text{-}C^{Lys80}$)
KIR2DL2 [230, 231]	D1-D2	2 domains, long cytoplasmic tail, 2	CD158b1, cI-43, nkat6	5	HLA-C group 1 ($HLA\text{-}Cw^{Ser77}$ and $^{Asn\ 80}$)
KIR2DL3 [229, 230]	D1-D2	2 domains, long cytoplasmic tail, 3	CD158b2, cI-6, nkat2, nkat2b, p58	10	HLA-C group 1 ($HLA\text{-}Cw^{Ser77}$ and ^{Asn80})
KIR2DL4 [232, 233]	D0-D2	2 domains, long cytoplasmic tail, 4	15.212, 103AS, CD158d	18	HLA-G
KIR2DL5A [234, 235]	D0-D2	2 domains, long cytoplasmic tail, 5A	CD158f, KIR2DL5.1		Undefined
KIR2DL5B [234, 235]	D0-D5	2 domains, long cytoplasmic tail, 5B	KIR2DL5.2, KIR2DL5.3, KIR2DL5.4		Undefined
KIR2DS1 [233]	D1-D2	2 domains, short cytoplasmic tail, 1	CD158h, EB6ActI, EB6ActII	4	HLA-C group 2 ($HLA\text{-}C^{Lys80}$)
KIR2DS2 [230, 231]	D1-D2	2 domains, short cytoplasmic tail, 2	183ActI, CD158j, cI-49, nkat5	8	HLA-C group 1 ($HLA\text{-}C^{Asn80}$)
KIR2DS3 [231]	D1-D2	2 domains, short cytoplasmic tail, 3	nkat7	3	Undefined
KIR2DS4 [231, 236]	D1-D2	2 domains, short cytoplasmic tail, 4	CD158i, cI-39, KKA3, nkat8	9	C*04
KIR2DS5 [231]	D1-D2	2 domains, short cytoplasmic tail, 5	CD158g, nkat9	3	Undefined

KIR2DP1 [235]		2 domains, pseudogene 1	KIR2DL6, KIR15, KIRY, KIRZ		
KIR3DL1 [229]	D0-D1-D2	3 domains, long cytoplasmic tail, 1	AMB11, CD158e1, cI-2, cI-11, KIR, nkat3, NKB1, NKB1B	22	HLA-Bw4-80I (strong), HLA-Bw4-80T (weaker)
KIR3DL2 [229]	D0-D1-D2	3 domains, long cytoplasmic tail, 2	CD158k, cI-5, nkat4, nkat4a, nkat4b	20	HLA-A3, A11
KIR3DL3 [237]	D0-D1-D2	3 domains, long cytoplasmic tail, 3	CD158z, KIR3DL7, KIR44, KIRC1	7	Undefined
KIR3DS1 [231]	D0-D1-D2	3 domains, short cytoplasmic tail, 1	CD158e2, nkat10	6	HLA-Bw4-80I (weak)
KIR3DP1 [235]		3 domains, pseudogene 1	CD158c, KIR2DS6, KIR3DS2P, KIR48, KIRX	4	

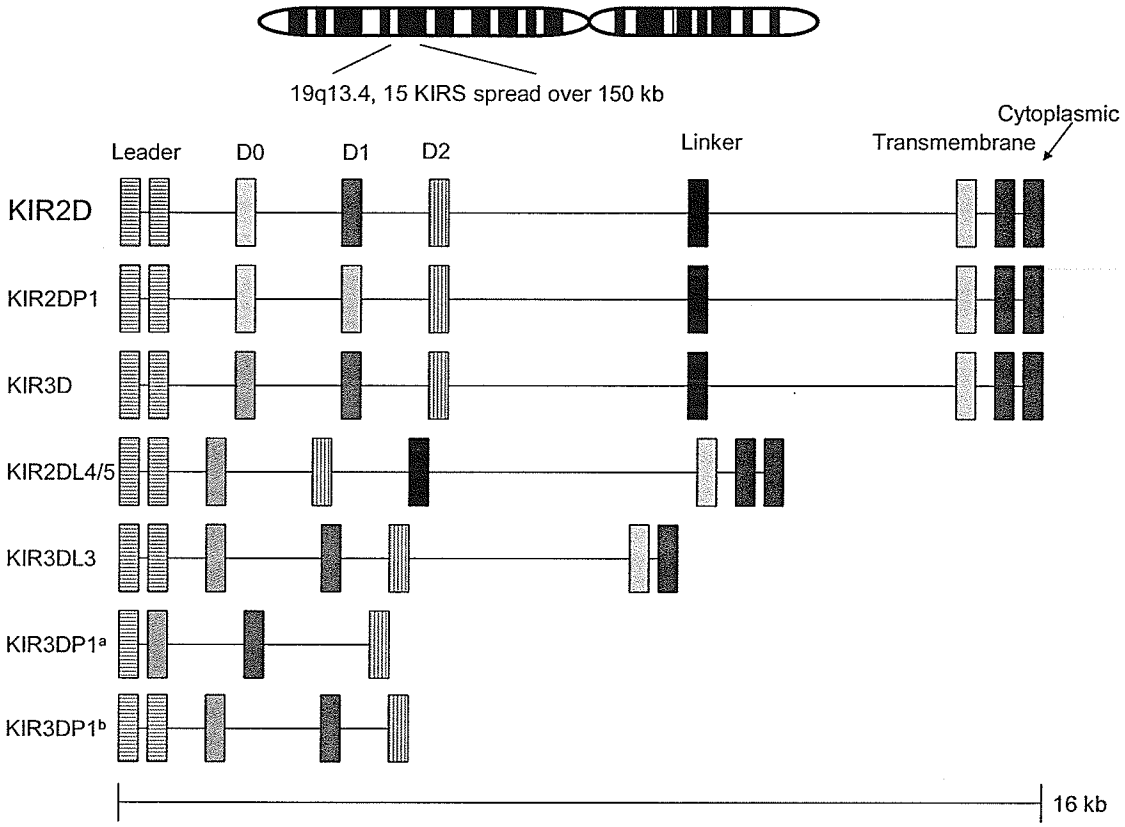

Figure 4. KIR exon-domain structure. KIRs are both polygenic and polyallelic.

For 2D (including 2DL2 and 2DL3):

Exons 1 & 2: Leader sequence

Exon 3: Domain 0 (D0), which is a pseudoexon

Exon 4: Domain 1 (D1)

Exon 5: Domain 2 (D2)

Exon 6: Linker

Exon 7: Transmembrane Anchor

Exons 8 & 9: Cytoplasmic Domain

A full description can be found in the National Library of Medicine (NLM) web book by Carrington et al [238, 239]..

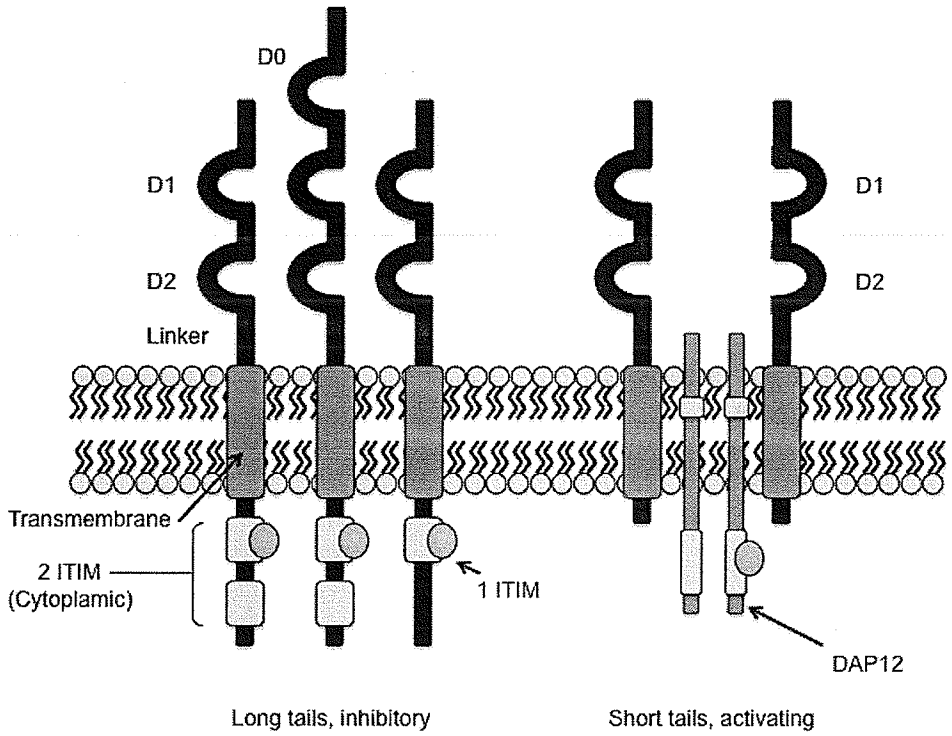

Figure 5. The structure of inhibitory (2DL and 3DL) and activating (2DS) KIR receptors spanning the cytoplasmic membrane.

ITIM: Immunoreceptor tyrosine-based inhibitory motif, ITAM: Immunoreceptor tyrosine-based activating motif

A full explanation of KIR receptor structure can be found in [222, 238, 240].

2.3.4 KIR Gene and Allele Nomenclature

The acronym KIR officially stands for Killer-cell Immunoglobulin-like Receptor", but originally it stood for "Killer-cell Inhibitory Receptor", when it was assumed that all KIRs were inhibitory. We now know that there are activating KIRs, hence the name change [241]. KIR nomenclature is summarized in Figure 6. KIR genes are named by the HUGO Genome Nomenclature Committee (HGNC) [6, 242, 242-244]. The KIR gene family consists of 15 genes and two pseudogenes, which are classified based on the structures of the molecules they encode. After the KIR acronym, follows '2D' or '3D', depending on the number of extracellular Ig-like domains [6, 245-247]. Next, 'L' or 'S' indicate a long or short cytoplasmic tail, respectively, while pseudogenes are indicated with a 'P'. Finally, the last digit indicates the number of the gene encoding a protein with this structure. KIR alleles are named analogously to the HLA nomenclature. Following the gene name, an asterisk acts as a separator before the numerical allele designation. The first three digits of the numerical designation represent alleles that differ in the sequences of the proteins they encode. The next two digits represent alleles that are different by synonymous (non-coding) differences within the coding sequence. The last two digits distinguish the alleles which only differ by substitutions within an intron, promoter, or other non-coding region.

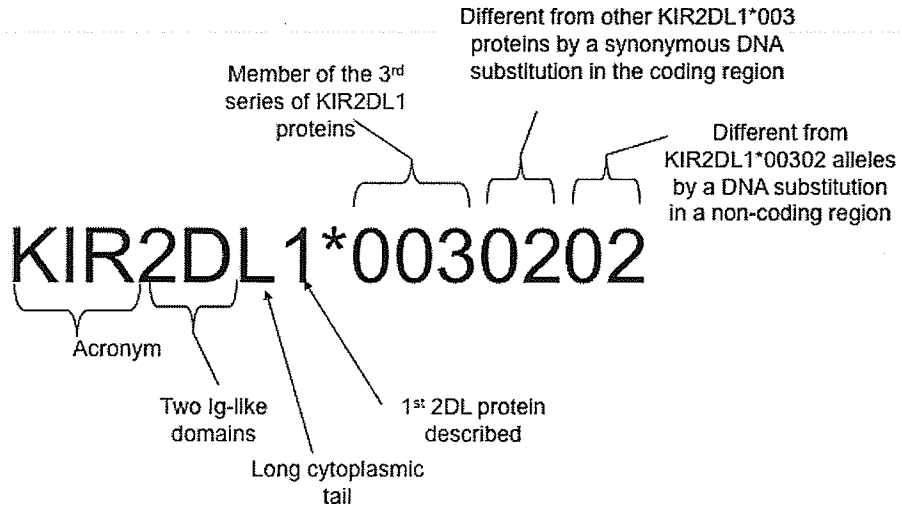

Figure 6. KIR Allele Nomenclature.

(This figure is reproduced in modified format by kind permission of Dr. Steven Marsh, Anthony Nolan Research Institute, London from the IPD-KIR Database http://www.ebi.ac.uk/ipd/kir/alleles.html [6, 7]. Permission obtained September, 2008.)

2.3.5 KIR Haplotypes

Over 100 KIR haplotypes have been identified, when gene and allele differences are considered. While the number and type of genes present varies, almost every haplotype contains the framework loci 3DL3, 3DP1, 2DL4, and 3DL2 [237]. There are two main haplotypes, A and B, present in approximately equal frequencies in Caucasian populations [238]. Haplotype A is mostly inhibitory, with fixed gene content, but can differ at the allele level. It contains the framework genes, as well as 3DL3, 2DL3, 2DP1, 2DL1, 3DL1, and 2DS4. The only activating gene in haplotype A is 2DS4, however there is a null version of this gene which is present at a population frequency of 84%, therefore some homozygotes for haplotype A actually have no functional activating KIRs [238]. Haplotype B is predominantly activating, and has much greater subtype diversity due to its variable gene content. It contains different combinations of 2DS2, 2DL2, 2DP1, 2DL1, 3DS1, 2DL5, 2DS3, 2DS5 and 2DS1 as well as the framework genes and potentially 2DP1, 2DL1, and 2DL5. These categories are broad, and vary with the number of possible KIR genes possessed on a haplotype ranging from 6-16 [7].

2.3.6 KIR Expression on NK Cells

Each individual's polyclonal NK cell population contains the full complement of transcribed KIR receptors from every KIR gene in their genome [225] (with the exception of 2DL5 [235]), however, individual NK cell clones express only a subset of these. This introduces challenges in studying individual NK receptors, and functional studies usually require single-cell cloning to distinguish them. Even between individuals, the proportion of cells expressing a given KIR can vary greatly [248], as can the quantity

of a given KIR on an NK cell. It has been shown that certain alleles of a given gene may be expressed at higher levels than others [249, 250], and that having two copies of a gene results in higher expression as well, in a dose-dependant manner [249]. The only KIR shown to be ubiquitously expressed on NK cells is the framework gene 2DL4.

Expression is thought to be stochastic, as shown by their combinatorial frequencies [250, 251]. As well, expression is regulated at the transcriptional level [251] and stably maintained in an individual over time, after established during development [251]. While the inhibitory NK receptor CD94/NKG2A is upregulated by IL-15 [252], KIR expression is not thought to be influenced by cytokines. Tolerance, or self vs. non-self recognition, is thought to be key in expression, with HLA class I thought to have a role in educating NK cells in the bone marrow. While it was once assumed that every NK cell in an individual had at least one inhibitory receptor for a self HLA molecule [206], recent studies have shown exceptions to this in the form of NK cell subsets lacking inhibitory receptors for self-HLA [253, 254] which are hyporesponsive rather than self-reactive. Further studies are required sort out the mechanism of NK tolerance, which may reveal important insight about KIR expression.

While mouse studies showed that expression of specific major histocompatibility complex (MHC) genes decreased the frequency and expression of their KIR ligand equivalents (Ly49), human studies showed that HLA only influenced the frequency of cells expressing KIRs, but had no effect on KIR expression levels [255]. Yawata et al [256] showed that possession of a cognate HLA/inhibitory KIR pair resulted in more NK cells expressing that particular KIR. Despite these observations, the mechanism of HLA's influence on KIR frequency is not clear. Epigenetic mechanisms also play a role

in expression, and it has been shown that methylation of CpG islands that surround the start of each KIR gene result in restricted access for transcription factors, and consequently a lack of expression [257]. Conversely, the genes were expressed when methylation was removed. Sequence variations in the promoter regions [235] are thought to contribute to differences in expression as well. The main proximal promoter is bidirectional, and a second distal promoter has been identified as well [258]. Competition between the forward (transcription) and reverse (no transcription) promoter action is thought to correspond with expression. Another possible mechanism modulating expression includes the altered binding of transcription factors to polymorphic binding sites in the promoter region, including regulatory factor TCF-1 to the TCF-1 binding site, which has been found in the promoter of some KIR genes [234][235], or RUNX3 in KIR2DL5 [259].

2.3.7 KIR Associations with Diseases

What is the functional relevance of KIR polymorphism? As with HLA, the variance within the KIR genomic region is thought to be advantageous in ensuring that at the population level, a diverse range of immune responses can be elicited towards infectious disease threats, while still maintaining tolerance to self. However, since the KIR binding is thought to depend on HLA recognition, this suggests that complex HLA-mediated regulation may be more important than foreign antigen recognition. In general, activating KIRs tend to confer risk towards autoimmune and inflammatory disorders, while genotypes that lead to lower inhibition and higher activation appear to be protective in viral infections.

KIRs have been shown to increase the susceptibility to several autoimmune diseases such as psoriatic arthritis (KIR2DS1/2DS2 with HLA-Cw homozygosity) [260], insulin-dependant diabetes mellitus (IDDM) (KIR2DS2/HLA-C1) [261], and scleroderma (KIR2DS2+/2DL2-) [262]. They have also been shown to be very important in reproductive failure, due to the role of uterine NK (uNK) cells in the remodeling of spiral arterioles during pregnancy, as required for placental perfusion [263]. This includes preeclampsia in mothers with activating KIR genotypes and fetus with HLA-C2 [264]. They are also associated with miscarriages and spontaneous abortions in mothers having increased activating KIR2DS2 and decreased HLA-C2 with increased frequency of activating KIR [265], and mothers lacking KIR2DS1 but having increased HLA-C2 in both parents [266].

Downregulation of HLA class I molecules occurs on tumor cells. Consequently, it makes sense that KIR-induced inhibition has been associated with cancer. Examples include malignant melanoma (the inhibitory KIR2DL2/2DL3 with HLA-C1) [267] and leukemia (inhibitory KIR2DL1, KIR2DL2, KIR2DL3) [268]. The role of activating KIRs on carcinogenesis may depend on whether inflammation plays a role in the cancer's development. For example, the development of cervical cancer is thought to have a strong inflammatory component, due to human papilloma virus infection. Indeed, inhibitory KIR-HLA combinations have been associated with protection, perhaps due to less NK activation and thus less inflammation, as opposed to the activating combination of KIR3DS1 in the absense of HLA-C2 or HLA-Bw4, which increases susceptibility [269].

The role of KIRs in infectious diseases has not been well-characterized, however, several key observations have been made. In most cases, the associations are not attributed to KIR polymorphism alone, as the individual's HLA type seems to play a key role as well. Individuals having KIR2DL3 and its HLA-C group 1 ligand were more likely to be able to clear Hepatitis C virus (HCV) [270]. The functional basis for this observation has not been resolved, but one suggestion is that in the case of inhibitory KIRs with lower affinity for their ligands, which has been observed for KIR2DL3 and HLA-C; these individuals may be able to mount a stronger response to infection. This might be due to a lower threshold for activating signals to override the weaker inhibitory ones. Another important infection which has been studied in the context of KIR is the bacteria *Mycobacterium tuberculosis*, which was shown to associate with KIR2DL3 [271]. KIR3DL2 has also been shown to associate with higher levels of IFN-γ against red blood cells infected with malaria (*Plasmodium falciparum*) [272], possibly via indirect cross-talk with the myeloid component of the immune system.

These associations suggest a significant role for KIR variability in immunity and infection, and while KIRs are also expressed on a minor subset of T lymphocytes; functional data have suggested that NK cells are the main cell type that contributes to the genetic associations. Substantial associations have been generated from studying the KIRs and their synergy with HLA in HIV-1 susceptibility and disease progression [239, 273, 273-275], rendering these receptors as serious contenders in the putative protective immune response to HIV-1.

2.3.8 KIR Associations with HIV-1

Studies have shown that specific KIR and HLA class I combinations are associated with delayed disease progression in HIV-1 infection [276]. These are summarized in Table 2. The most-studied example of this is the KIR3DL1/3DS1 and HLA-B combination. While it was previously shown that HIV-infected individuals with the HLA-Bw4 epitope had slower progression to AIDS [277], Martin et al [273] showed that this association was strengthened if an individual's KIR3DL1/3DS1 type was also considered. Delayed progression to AIDS was observed if an individual had either a KIR3DL1 inhibitory receptor or its activating allele variant KIR3DS1, in combination with HLA-Bw4 with isoleucine at position 80 (HLA-Bw4-I80) [278]. While the 3DS1/HLA-Bw4-I80 combination was shown to protect against opportunistic infections in HIV-infected subjects, it had no affect on AIDS-associated malignancies [274]. Functional studies have shown that KIR3DL1 receptors recognize HLA-Bw4 [279], but attempts to show direct binding of KIR3DS1 with HLA-Bw4 have been unsuccessful [280, 281]. Despite the lack evidence that KIR3DS1 actually binds HLA-Bw4, *in vitro* experiments showed that KIR3DS1$^+$ NKs, could suppress HIV-1 replication in HLA-Bw4 cells, while KIR3DS1$^-$ NK cells could not. It is somewhat surprising that KIR3DL1 is associated with protection, especially considering that 3DL1*004, its most protective allele [282], is not even expressed on the surface of NK cells, and may be degraded intracellularly [283]. This suggests a putative role for KIR involvement in endosomal signalling, or it may just be a linked marker for activating or inhibitory haplotypes.

In addition to associations with disease progression in HIV-1 positive individuals, KIRs have also been implicated in resistance to HIV-1 infection. Certain KIR3DL1 high

expressing alleles (KIR3DL1*h) in combination with HLA-B*57 were found to be protective in a group of exposed uninfected individuals [284], as were higher proportions of the activating KIR3DS1 homozygotes, perhaps leading to protection via higher levels of NK activity [285]. In another study by Jennes et al, KIR2DL2/2DL3 heterozygosity in the absence of its HLA-C1 ligand was associated with HIV-1 resistance in sex workers from Côte d'Ivoire [286], as was KIR3DL1 homozygosity in the absence of its HLA-Bw4 ligand. In the same population, increased susceptibility to infection was also found in individuals with KIR2DL3 homozygosity in the presence of its HLA-C1 ligand. It is thought that the absence of ligands for inhibitory KIRs could potentially lower the NK activation threshold. In line with this is another study which proposed that genetic variants of non-classical HLA class I molecules HLA-E and –G, may have lower binding affinity for their ligands, CD94/NKG2A and KIR2DL4 respectively, and found that these variants indeed associated with a reduced risk of heterosexual HIV-1 infection [287]. These studies all support an involvement for KIRs in the host's anti-viral defense. Further functional characterization is needed to confirm the mechanisms of protection.

While these association studies are important in understanding the effects of KIRs on diseases, the current experimental methods available to detect these receptors have several limitations in terms of lack of specificity which will be further elaborated on, including labour-intensiveness and resources, and ability to discover novel genes/alleles.

Table 2. KIR/HLA associations with HIV-1.

KIR	HLA	Effect on HIV-1	Proposed KIR contribution
KIR3DL1*h	HLA-Bw4-I80	Slower disease progression	NK licensing- strong binding of inhibitory ligand=strong activation when signal absent
KIR3DL1*h	HLA-B*57	Decreased susceptibility to infection	
KIR3DS1	HLA-Bw4-I80	Slower disease progression	Increased activation
KIR2DL2/2DL3 heterozygous	Absence of HLA-C1	Decreased susceptibility to infection	Decreased inhibition
KIR2DL2/2DL3 homozygous	Presence of HLA-C1	Increased susceptibility to infection	Increased inhibition
KIR2DL4	HLA-G	Decreased susceptibility to infection	Endosomal

2.3.9 KIR2DL2/2DL3

Most of the human KIRs have 2 extracellular Ig-like domains, including type 1 KIR2D with domains D1 and D2 and type 2 KIR2D having domains D0 and D2 homologous to the corresponding domains of KIR3DL (Table 1). KIR2DL2 and KIR2DL3 are both members of the type 1 KIR2DL subfamily of inhibitory receptors. It is thought that KIR2DL2 evolved from a KIR2DL3-like ancestor which recombined with a KIR2DL1-like ancestor.

KIR2DL2 and KIR2DL3 each have 9 exons, however, exon 3, which shares approximately 80% homology with D0 of KIR3D [226], is considered a pseudoexon because it is missing one codon and gets spliced out of the functional mRNA. Another layer of complexity is added knowing that each individual may carry one or two alleles of each of these KIR genes, since one copy is obtained from each parent. The unique alleles for KIR2DL2 include KIR2DL2*001, *002, *003, *004, and *005. KIR2DL2 is usually associated with haplotype B, which contains mostly activating receptors. KIR2DL3 is usually associated with haplotype A, which mainly consists of other inhibitory receptors. Its unique alleles include KIR2DL3*001, *002, *003, *004, *005, *006, and *007. KIR2DL2 and KIR2DL3 share the same locus on KIR haplotypes, and both have the HLA-C group 1 (HLA-C1 allotype) epitope as their ligand (Table 1). The HLA-C1 epitope has serine at position 77 and asparagine at position 80, and is present in HLA-Cw1, -Cw3, -Cw7, -Cw8, -Cw12, -Cw13, and -Cw14. HLA-C group 2 (HLA-C2 allotype) has asparagine at position 77 and lysine at position 80, and includes HLA-Cw2, Cw4, Cw5, Cw6, Cw15, and Cw17. A recent study by Moesta et al [288] showed that KIR2DL2 and -2DL3 bound to all C group 1 alleles, but that they could also cross-react

and bind to some HLA-C group 2 (HLA-C2) (Cw5 and Cw2.2) and even some HLA-B allotypes (B*4601 and B*7301). Interestingly, they also found that KIR2DL2 was a stronger receptor than KIR2DL3.

2.3.10 KIR Evolution

HLA class I and KIRs are located on different chromosomes (chromosomes 6 and 19, respectively) and segregate independently, but even so they are thought to be co-evolving. For example, activating KIRs appear to have a strong negative correlation with their HLA ligands [289], while a positive correlation has been observed between inhibitory KIRs and their HLA ligands. Studies suggest that inhibitory KIRs appeared first and that activating receptors arose later from the inhibitory KIRs by recombination [290] and duplication [291]. It has been suggested that a recently discovered divergent KIR gene called KIR3DX1, might be the ancestral KIR gene from which all other KIR arose from, since it is present in all primates as a single copy [292].

2.4 Hypotheses

Like HLA, KIR genes have evolved and diversified. They are polymorphic and vary in the quantity of genes that they encode, which provides a basis for natural selection for resistance to pathogens. Synergistic and epistatic effects occur between HLA and KIR genes in HIV-1 progression, but little data is available on the effects of KIR-HLA on altered susceptibility to HIV-1 infection, especially for KIR2DL2/2DL3. KIRs are associated with autoimmunity, rheumatoid arthritis, organ/tissue transplantation, reproductive failure, and HIV disease progression, and are thus important

immune modulators. These observations suggest a role for KIR variability in immunity and infection, and warrant further study of KIRs in their involvement in HIV-1 infection.

Based on this rationale, the following hypotheses were developed:

- KIR2DL2 and KIR2DL3 genes and alleles can be detected and typed using a sequence-based genotyping method, to a higher level of resolution than other available methods.
- Certain specific KIR-HLA combinations are associated with resistance or susceptibility to HIV-1 infection and disease progression, through their role in mediating NK activity or NK-mediated immune activation against HIV-1 infection.

2.5 Objectives

To examine the hypotheses, several objectives were developed:

1. To develop a sequence-based genotyping method for the co-amplification of KIR2DL2 and KIR2DL3, which are genes present at the same locus in different haplotypes.
2. To use this method to identify KIR genes and alleles in subjects of the Pumwani sex worker cohort.
3. To ensure correct amplification and confirm novel alleles by cloning.
4. To investigate associations of KIR2DL2 and KIR2DL3 genes and alleles with HIV-1 infection and disease progression.
5. To consider the synergistic effect of HLA class I ligands by including HLA-C in the KIR analysis.

Section 3: Materials and Methods

3.1 Materials

3.1.1 Study Group- ML Cohort

The study was conducted among a subset of women in the Pumwani cohort, who were classified into 3 different study groups: 1) HIV-1 resistant if they remained HIV seronegative and PCR negative for at least 7 years while continuing active sex work, and were negative at the time of study, 2) HIV-1 susceptible if they were HIV-1 positive, and 3) HIV-1 negative if they were HIV-1 negative but had shorter than 3 years follow-up. All resistant women in this study were enrolled before 2001 with an average follow-up time of 9.6 ± 4.3 years. Thus, women considered resistant had been HIV-1 negative for at least 7 years.

Criteria for enrollment, sample collection and sexually transmitted infection testing have been described [19]. Ethics committees at the University of Manitoba and the University of Nairobi have approved this study. Informed consent was obtained from all women enrolled in the study.

3.1.2 DNA Preparation

DNA sequence typing was performed using DNA extracted from whole blood, buffy coat, B-cells, peripheral blood mononuclear cells and peripheral blood lymphocytes using either the QIAmp DNA Mini Kit (QIAgen Inc., Mississauga, ON) or the BioRobot EZ1 (QIAgen Inc., Mississauga, ON) following the manufacturer's instructions. DNA

was quantified using standard spectrophotometric analysis. Samples were maintained at -40°C.

3.1.3 Commercial Reagents

1) Custom Primers (Invitrogen, Burlington, ON, Canada)
2) dNTPs (Invitrogen, Burlington, ON, Canada)
3) Taq DNA polymerase recombinant (Invitrogen, Burlington, ON, Canada)
4) UltraPure™ agarose (Invitrogen, Burlington, ON, Canada)
5) Ethidium bromide (10 mg/ml) (Sigma-Aldrich, Oakville, ON, Canada)
6) DNA ladder, 1 kb (Invitrogen, Burlington, ON, Canada)
7) Agencourt® AMPure® Kits (Beckman Coulter, Mississauga, ON, Canada)
8) BigDye® 1.1 terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA)
9) Hi-Di™ formamide (Applied Biosystems, Foster City, CA, USA)
10) SYBR® Green I nucleic acid gel stain 10,000X concentrate in DMSO (Invitrogen, Eugene, Oregon, USA)
11) QIAquick Gel Extraction Kit (QIAgen, Mississauga, ON, Canada)
    Buffer QG
    Buffer PE
12) Expand High Fidelity PCR Buffer 2 (10x) with $MgCl_2$ (Roche Diagnostics, Indianapolis, IN, USA)
13) Expand High Fidelity DNA polymerase enzyme mix (Roche Diagnostics, Indianapolis, IN, USA)
14) S.O.C. Medium (Invitrogen, Burlington, ON, Canada)

15) TOPO TA Cloning® Kit for Sequencing (Invitrogen, Burlington, ON, Canada)

pCR®4-TOPO®

Salt solution

One Shot® TOP10 chemically competent *E. coli*

Sequencing primers, 3.2 pmol/μl:

i) M13F (5' GTA AAA CGA CGG CCA G -3', annealing temperature 50°C), and M13R (5'- CAG GAA ACA GCT ATG AC -3', annealing temperature 50°C)

ii) T3 (5'- ATT AAC CCT CAC TAA AGG GGA -3', annealing temperature 51°C) and T7 (5'- TAA TAC GAC TCA CTA TAG GG -3', annealing temperature 50°C)

16) Ampicillin

100 mg/ml

17) UltraPure™ X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (Invitrogen, Burlington, ON, Canada)

Diluted to 40 mg/ml using dimethyl formamide

18) QIAprep 96 Turbo Plasmid Miniprep Kit (QIAgen, Mississauga, ON, Canada)

P1 Re-suspension buffer

P2 Lysis buffer

N3 Neutralization buffer

TurboFilter 96 well plate

QIAprep 96 well plate

PE Wash buffer

EB Elution buffer

19) EcoR1 restriction enzyme (Invitrogen, Burlington, ON, Canada)

20) 10x REact®3 buffer for EcoR1 (Invitrogen, Burlington, ON, Canada)

21) BigDye® 3.1 terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA)

22) Expand Long Template PCR System enzyme mix (Roche Diagnostics, Indianapolis, IN, USA)

3.1.4 Laboratory Prepared Solutions

1) TE buffer (pH 8.0)

   216 g Triza Base

   110 g 0.5 M EDTA (pH 8.0)

   800 ml ddH_2O

2) Polymerase chain reaction (PCR) 2x master mix

   120 mM Tris-HCl (pH 9.0)

   3 mM $MgCl_2$

   30 mM $(NH_4)_2SO_4$

   50 µM each dNTP

   0.2 % gelatin

3) ddH_2O ddH_2O

   double distilled water

4) 6x PCR gel loading buffer

   0.25 bromophenol blue

   0.25 xylene cyanol

5) 30% Glycerol in H_2O10x Tris-borate EDTA (TBE) buffer

108 g Tris Base

55 g Boric Acid

7.44 g EDTA

ddH_2O to bring up to 1 litre volume

6) Long PCR 2x+ Master Mix

120 mM Tris-HCl pH 9.0

3 mM $MgCl_2$

30 mM $(NH_4)2SO_4$

100 µM each dNTP

0.2% gelatin

ddH_2O up to 45.5 µl

7) Sodium Acetate

408.3 g sodium acetate

800 ml ddH_2O

glacial acetic acid to adjust pH to 5.2

ddH_2O to final volume of 1L

8) Luria-Bertani (LB) agar plates

1 l LB medium

15 g agar

200 µg/ml ampicillin

9) LB medium

1.0 % tryptone

0.5% yeast extract

1.0% NaCl

pH 7.0 with NaOH

Add 100 μg/ml ampicillin before using

3.2 Methods

3.2.1 KIR Contig Assembly and Selection of Exons for Sequence-based Typing

To determine regions for amplification and sequencing, contigs were created to show the overlapping DNA sequences for KIR2DL2 and KIR2DL3 genomic and allelic sequences for all 9 exons, as seen in Figure 7 [7, 293, 294]. Databases were also created, using only the exon data, for sequence-based typing. Typing regions were selected on the basis of polymorphisms within each exon using the sequence-based typing software program CodonExpress (University of Manitoba, Winnipeg, MB, Canada).

Based on its functional role in coding for domain 1, in combination with its high number of typing codons, or codon sites where polymorphisms could differentiate the difference between two or more genes/alleles, exon 4 was selected as the main typing exon for KIR2DL2/2DL3. Exon 4 contained enough typing codons to differentiate 2DL2 and 2DL3 from each other at the gene level, and also KIR2DL2 into two allele groups: 2DL2*001/002/003/005 and 2DL2*004 and to distinguish 2DL3 into 3 allele groups: 2DL3*001/002/003/007, 2DL3*004/005, and 2DL3*006. 2DL2*001/002/003/005 could be further typed into 2DL2*001/002/005 and 2DL2*003 if codon 221 located in exon 5 (domain 2) was known, and 2DL3*001/002/003/007 could be further typed into

2DL3*001/003 and 2DL3*002/007 if codon 229 located in exon 6 (linker) was known, and 2DL3*004/005 could be typed into 2DL3*004 and 2DL3*005 if codons 194 (exon 5) and 242 (exon 6) were known. For these reasons, exons 5 and 6 were also targeted for sequence-based typing. While many of the typing codons that could be used to type KIR2DL2/2DL3 to the allele level were located within exons 8 and 9 (the cytoplasmic domains), they did not necessarily contain sites that could differentiate the two genes from each other, and we were most interested in polymorphisms in the domains that bind to their HLA ligands (exons 4 and 5), while keeping the number of reactions to a minimum. Clearly, difficulties were anticipated to link exons 4, 5 and 6 from one chromosomal strand, but given the vast size of each KIR gene (~14.8 kb), full amplification was not feasible.

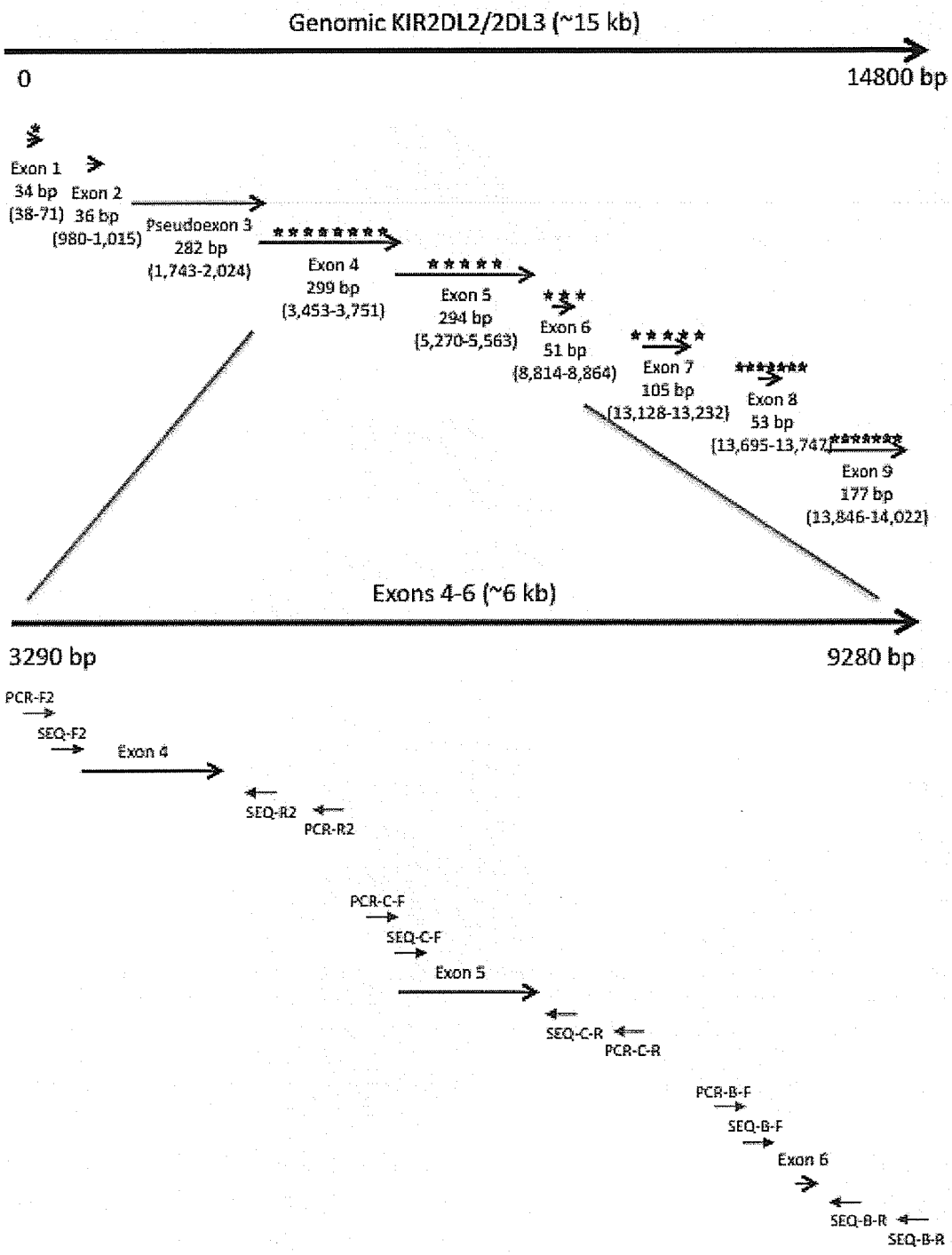

Figure 7. Contig of human KIR genes used to design 2DL2/2DL3 exon-specific primers. Note: Red stars indicate codon differences within that particular exon, either between 2DL2 and 2DL3 genes, or alleles of each gene. For exon 4 differences see Figure 8.

3.2.2 PCR Amplification Primers: Separate Amplification of Individual Exons

PCR amplification of KIR2DL2/2DL3 was accomplished using three sets of designed primers (Table 3) located in intron regions surrounding the polymorphic typing exons, or in the case of exon 5 forward primer, within the the exon's 5' end. Exons 4, 5 and 6 were amplified for KIR2DL2/2DL3. A second pair of primers was also designed for exon 6 (indicated –new in Table 3), when the first pair was found to have low specificity. The 50 µl PCR reaction mixture consisted of 22.75 µl 2x master mix, 1 µl each of forward and reverse primer at a concentration of 25 pmol/µl diluted in TE buffer, 2.5 Unit of *Taq* DNA polymerase (Invitrogen, Burlington, ON, Canada), 50 to 100 ng DNA, and ddH_2O. Annealing temperatures were calculated using the program PuTTy. The cycle parameters used in the PTC-100 programmable Thermal Controller (MJ Research, Inc., Watertown, MA, USA) were 96°C denaturation for 5 min, followed by 40 cycles of 1 min denaturing at 96°C, 1 min at the annealing temperature (Table 1), 2 min extension at 72°C, and then a final 10 min incubation at 72°C. Five µl of PCR reaction mixed with 6x loading dye was visualized on a 1.5% agarose gel using 1x TBE buffer and stained with ethidium bromide alongside a 1 kb ladder (Invitrogen, Burlington, ON, Canada) to check for the amplification of the correctly sized PCR products. The remaining PCR products were then purified using the Agencourt® AMPure® PCR magnetic bead purification system (Beckman Coulter, Mississauga, ON, Canada) according to the manufacturer's recommendations.

Table 3. KIR2DL2/2DL3 PCR primers.

Primer Name	Primer Sequence	Location	Domain	Annealing Temperature, °C
PCR-F2[1]	5'- GAA GGA CCT GCA CCA GGA GTT AA -3'	exon 4	D1	56.4
PCR-R2[2]	5'- TGT CTC TGT TGG TAC AAA CCT CAG -3'	exon 4	D1	56.4
PCR-C-F[3]	5'- AGG CCC ATG AAY GTA GGT TCT -3'	exon 5	D2	62
PCR-C-R[4]	5'- TGC ATC TGT CCA TGC TTC TC -3'	exon 5	D2	62
PCR-B-F[5]	5'- AGG ACT CCC AGG GCC CAA TA -3'	exon 6	linker	60
PCR-B-R[6]	5'- GTT GCT TCA TGA CCA ACA GTA AT -3'	exon 6	linker	60
PCR-B-F-new	5'- GAC ART GGG CGT CAC ATA C -3'	exon 6	linker	58
PCR-B-R-new	5'- TTC TCT GTT ACG GCA AGG CTG -3'	exon 6	linker	58

[1] Unique to 2DL2/2DL3

[2] Unique to 2DL2/2DL3

[3] Also homologous to 2DS2, 2DS3, and 2DP1

[4] Also homologous to 2DS2

[5] Also homologous to 2DL1, 2DS2, 2DS3, 2DS1, 2DS5, and 2DP1

[6] Also homologous to 2DP1, 3DS1, 3DP1

3.2.3 DNA Sequencing and Sequence Analysis

Nested sequencing was performed using sequencing primers located within the fragments amplified by PCR in 3.2.2. 2DL2-SEQ-F2 and 2DL2-SEQ-R2 were used to sequence exon 4 of 2DL2/2DL3 into gene and allele groups, while further allele typing was achieved by sequencing exon 5 using 2DL2-C-SEQ-F and 2DL2-C-SEQ-R and exon 6 using 2DL2-B-SEQ-F and 2DL2-B-SEQ-R (Table 4). Sequence specificity was achieved both by PCR and sequencing primers. The BigDye® terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) was used, with each reaction consisting of 2 µl BigDye® 1.1 (Applied Biosystems, Foster City, CA, USA) and 1.5 µl sequencing primer, diluted to a concentration of 3.2 pmol/µl with ddH_2O, as well as 2 µl template purified PCR product. The PTC-100 programmable Thermal Controller (MJ Research, Inc., Watertown, MA, USA) was used to incorporate fluorescent sequence markers. Parameters included a 96°C denaturation for 3 min, followed by 80 cycles of 30 sec denaturing at 96°C, 1 min at the annealing temperature (Table 4), and 4 min at 60°C.

After BigDye® incorporation, samples were prepared for sequencing. Samples were precipitated by adding 1 µl sodium acetate and 21 µl 95% Ethanol, then incubating in the dark for 3 hours at room temperature. Samples were spun in a centrifuge at 4000 rpm at 20°C for 1 hour, and the supernatant was removed. This was followed by two purification washes in 150 µl 70% ethanol, spun for 10 min at 4000 rpm. 20 µl Hi-Di™ formamide was added to each well and vortexed. Samples were heated for 1.5 minutes at 90°C, followed by 3 min on ice. Samples were transferred to MicroAmp Optical 96-well reaction plates (Applied Biosystems, Foster City, CA, USA), and sequenced using the ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Table 4. KIR2DL2/2DL3 sequencing primers.

Primer Name	Primer Sequence	Location	Domain	Annealing Temperature, °C
SEQ-F2[1]	5'- CAA GGG GAA GCC TCA CTC ATT CT -3'	exon 4	D1	60
SEQ-R2[2]	5'- TCC TTA CAA CTA CCT GGG GGT TC -3'	exon 4	D1	60
SEQ-C-F[3]	5'- CCA AGG TCA ACG GAA CAT TC -3'	exon 5	D2	58
SEQ-C-R[4]	5'- TGC ATC TGT CCA TGC TTM TC -3'	exon 5	D2	58
SEQ-B-F[5]	5'- TCC ATT GAG TAG AGG ACA GAC -3'	exon 6	linker	58
SEQ-B-R[6]	5'- ATC CTC CAG TTA GGA ATG CAG -3'	exon 6	linker	58
SEQ-B-R-new	5'- GTT GCT TCA TGA CCA ACA G -3'	exon 6	linker	58

[1] Also homologous to 2DS2 and 2DP1

[2] Also homologous to 2DS2 and 2DP1

[3] Also homologous to 2DS1, 2DS4, and 2DP1

[4] Also homologous to 2DL1, 2DL5, 2DS1, 2DS2, 2DS3, 2DS5, and 3DL2

[5] Unique to 2DL2/2DL3

[6] Also homologous to 2DS1, 2DS2, 2DS4, 2DP1, 2DS5, 3DL2, and 3DS1

3.2.4 PCR Co-amplification and Purification of Large KIR Fragments

For a sample of two of the cases in which the assignment of a particular codon did not fit the correct typing result across exons 4, 5 and 6 after separate typing reactions were completed (3.2.2,3.2.3), a co-amplification reaction was used. The purpose of this was to segregate the typing sequences into specific alleles, and was achieved by amplifying a long 6 kb fragment of genomic DNA containing all three exons. The 50 µl long PCR reaction mixture consisted of 22.75 µl 2x+ master mix, 1 µl each of forward and reverse primers at a concentration of 25 pmol/µl diluted in TE buffer, 0.25 µl Expand long template enzyme mix (Roche Diagnostics, Indianapolis, IN, USA), 2 µl (50 to 100 ng) template DNA, and 23 µl ddH_2O. The cycle parameters used in the PTC-100 programmable Thermal Controller (MJ Research, Inc., Watertown, MA, USA) included a 94°C denaturation for 2 min, followed by 9 cycles of 10 sec at 94°C, 30 sec at the annealing temperature (Table 5) and 8 min extension at 68°C. This was followed by 29 cycles of 94°C for 15 sec, 30 sec at the annealing temperature, and 8 min 20 sec at 68°C + 20 sec per cycle. The last step was a final 7 min extension at 68°C. Products from the PCR reaction were run on a on a 1% agarose gel and then stained in 25 µl SYBR® Green I nucleic acid gel stain (Invitrogen, Eugene, Oregon, USA) in 250 ml TBE buffer for 1.5 hrs to check for the amplification of the correctly sized PCR products. Gel purification was performed to isolate the desired 6 kb band from smaller bands, in preparation for insertion into a TOPO TA cloning® vector. The gel purification was performed using the QIAquick Gel Extraction Kit using a microcentrifuge, according to manufacturer's recommendations (QIAgen, Mississauga, ON, Canada).

3.2.5 PCR Co-amplification of Exons 4 and 5

A 4 kb genomic fragment containing exons 4 and 5 was amplified using a modified exon 4 forward primer (Table 5) and exon 5 reverse primer lengthened at the 5'end in the same two patients as in 3.2.4, in an attempt to resolve cloning problems with the 6 kb fragment containing exons 4, 5, and 6. This was amplified and purified using the methods from 3.2.2, with an annealing temperature of 62°C.

3.2.6 Gene and Allele Analysis

The sequence-based typing program CodonExpress was used to genotype KIR2DL2 and KIR2DL3 based on the sequence in exons 4, 5 and 6. Chromatograms were each manually analyzed to check for codon-assignment accuracy, as well as to identify any new sequences that did not match those in the database. Each given file contained two sequences, one from each KIR2DL2/2DL3 gene/allele present. Homozygotes would appear to have only one specific sequence, while heterozygotes had two peaks at certain codons. The program distinguishes alleles based on comparisons to the gene/allele database and the presence of the polymorphic typing codons, indicated in red in Figure 8. Quality controls on were performed on each sequence chromatogram using Sequencher 4.8 Software (Gene Codes Corporation, Ann Arbor, MI, USA).

Table 5. KIR2DL2/2DL3 PCR primers for co-amplification of a 6 kb fragment from exons 4, 5, and 6.

Primer Name	Primer Sequence	Location	Domain	Annealing Temperature, °C
exon4-F-long	5'- ATG **GAA GGA CCT GCA CCA GGA GTT AA** -3'	exons 4,5,6	D1, D2, Linker	57
exon6-R-long	5'- AGG **GTT GCT TCA TGA CCA ACA GTA AT** -3'	exons 4,5,6	D1, D2, Linker	57

Note: Bold text indicates the homology to single-exon-specific primers from Table 1.

Figure 8. Codon assignment for the exon 4 sequence-based genotyping of KIR2DL2 and KIR2DL3.

	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
2DL2*001	TCC	CTC	CTG	GCC	CAC	CCA	GGT	CGC	CTG	GTG	AAA	TCA	GAA	GAG	ACA	GTC	ATC
2DL2*002	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL2*003	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL2*004	...	...	...	...	...	...	...	CCC	...	...	...	...	...	...	...	...	...
2DL2*005	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*001	...	...	...	...	...	...	...	CCC	...	...	...	...	...	...	...	...	...
2DL3*002	...	...	...	...	...	...	...	CCC	...	...	...	...	...	...	...	...	...
2DL3*003	...	...	...	...	...	...	...	CCC	...	...	...	...	...	...	...	...	...
2DL3*004	...	...	CGG	...	...	...	...	CCC	...	...	...	...	...	...	...	...	...
2DL3*005	...	...	CGG	...	...	...	...	CCC	...	...	...	...	...	...	...	...	...
2DL3*006	...	...	...	...	...	...	...	CCC	...	...	...	...	...	...	...	...	...
2DL3*007	...	...	...	...	...	...	...	CCC	...	...	...	...	...	...	...	...	...

	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63
2DL2*001	CTG	CAA	TGT	TGG	TCA	GAT	GTC	AGG	TTT	GAG	CAC	TTC	CTT	CTG	CAC	AGA	GAA
2DL2*002	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL2*003	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL2*004	...	...	...	...	...	...	...	...	...	CAG	...	...	...	...	...	ACA	...
2DL2*005	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*001	...	...	...	...	...	...	...	...	...	CAG	...	...	...	...	...	...	...
2DL3*002	...	...	...	...	...	...	...	...	...	CAG	...	...	...	...	...	...	...
2DL3*003	...	...	...	...	...	...	...	...	...	CAG	...	...	...	...	...	...	...
2DL3*004	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*005	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*006	...	...	...	...	...	...	...	...	...	CAG	...	...	...	...	...	...	...
2DL3*007	...	...	...	...	...	...	...	...	...	CAG	...	...	...	...	...	...	...

	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
2DL2*001	GGG	AAG	TTT	AAG	GAC	ACT	TTG	CAC	CTC	ATT	GGA	GAG	CAC	CAT	GAT	GGG	GTC
2DL2*002	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL2*003	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL2*004	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL2*005	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*001	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*002	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*003	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*004	...	...	...	...	...	...	...	CGC	...	...	...	...	...	...	...	...	...
2DL3*005	...	...	...	...	...	...	...	CGC	...	...	...	...	...	...	...	...	...
2DL3*006	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	GTT
2DL3*007	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97
2DL2*001	TCC	**AAA**	GCC	AAC	TTC	TCC	**ATC**	GGT	CCC	ATG	ATG	CAA	GAC	CTT	GCA	GGG	ACC
2DL2*002	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL2*003	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL2*004	...	**AAG**	...	...	...	...	**ATT**	...	...	...	...	...	...	...	...	...	...
2DL2*005	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*001	...	**AAG**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*002	...	**AAG**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*003	...	**AAG**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*004	...	**AAG**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*005	...	**AAG**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*006	...	**AAG**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*007	...	**AAG**	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114
2DL2*001	TAC	AGA	TGC	TAC	GGT	TCT	GTT	ACT	CAC	TCC	CCC	TAT	CAG	TTG	TCA	GCT	CCC
2DL2*002	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL2*003	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL2*004	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL2*005	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*001	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*002	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*003	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*004	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*005	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*006	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2DL3*007	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

	115	116	117	118	119	120	121	122	123	124
2DL2*001	AGT	GAC	CCT	CTG	GAC	ATC	GTC	ATC	ACA	GGT
2DL2*002	...	...	...	...	...	...	...	...	...	...
2DL2*003	...	...	...	...	...	...	...	...	...	...
2DL2*004	...	...	...	...	...	...	...	...	...	...
2DL2*005	...	...	...	...	...	...	...	...	...	...
2DL3*001	...	...	...	...	...	...	...	...	...	...
2DL3*002	...	...	...	...	...	...	...	...	...	...
2DL3*003	...	...	...	...	...	...	...	...	...	...
2DL3*004	...	...	...	...	...	...	...	...	...	...
2DL3*005	...	...	...	...	...	...	...	...	...	...
2DL3*006	...	...	...	...	...	...	...	...	...	...
2DL3*007	...	...	...	...	...	...	...	...	...	...

Note: Dots indicate redundancy with KIR2DL2*001.

Bold red text indicates typing codons used to distinguish genes and alleles.

3.2.7 TOPO TA Cloning®

TOPO TA Cloning® was used to clone and sequence the regions of interest and to determine the correct PCR primer binding and amplification of the region of interest. In addition, this strategy was utilized to identify putative new alleles. This method allowed each individual allele in a patient to be analyzed separately for its gene and allele content.

3.2.8 TOPO TA Cloning® of a Large Genomic Fragment Containing Exons 4, 5 and 6 (Domain 1, Domain 2, and Linker regions)

A long 6 kb fragment containing exons 4, 5, and 6 was examined to confirm the typing results for each allele in each exon. A three-day cloning protocol was performed using the TOPO TA Cloning® Kit for Sequencing (Invitrogen, Burlington, ON, Canada). Upon consultation with the manufacturer, it was revealed that the only appreciable differences between the pCR®-XL-TOPO® vector, which is suggested for inserts between 4-10 kb, and the pCR®4-TOPO® vector, which is suggested for inserts up to 4 kb, were in the manufacturer's instructions. Thus, it was decided to use the pCR®4-TOPO® vector according to the manufacturer's instructions, but with modifications to accommodate a large insert.

For long inserts, gel purification was used to ensure that small fragments present in the PCR reaction, which would clone more efficiently than the fragment of interest, were minimized. This was achieved using SYBR® green stain (see 3.2.4 for full methods) to visualize the band rather than ethidium bromide (EtBr), to prevent the nicking of DNA. Nicked DNA is a poor cloning substrate and can be quickly degraded by bacterial nucleases. For a trial sample of two patients, KIR2DL2 and 2DL3 PCR products containing a 6 kb genomic fragment of exons 4, 5, and 6 (including introns)

were extended to add 3' adenine (A) overhangs to PCR products that were purified or amplified more than 24 hours prior to cloning. To extend, the following were incubated at 72°C for 15 min, iced, then quick spun: 1 µl Expand High Fidelity PCR Buffer 2 (10x) with $MgCl_2$ (Roche Diagnostics, Indianapolis, IN, USA), 2 µl dNTPs (1.25 mM), 1.5 µl ddH_2O, 5 µl PCR product from gel purification, and 0.5 µl Expand High Fidelity DNA polymerase enzyme mix (Roche Diagnostics, Indianapolis, IN, USA). To decrease competition of the insert for the vector during ligation, the insert:vector molar ratio was kept low, approximately 1:1. This was to increase the chances of both ends of the insert attaching to the open vector. Diluting the reaction, while increasing the salt solution to compensate for the increase in volume, reduces the competition for vector ends. To ligate PCR products with the vector, 3.7 µl salt solution, 1 µl pCR®4-TOPO® vector (Invitrogen, Burlington, ON, Canada), and 11.3 µl ddH_2O were added to 4 µl extended or fresh PCR products. To further increase the efficiency of the reaction, this the incubation time at room temperature was increased to 1 hr. Next, 3 µl of the ligation mixture was added to a vial of One Shot® TOP10 chemically competent *E. coli* cells (Invitrogen, Burlington, ON, Canada). This was incubated on ice for 30 min, heat shocked in a 42°C water bath for 30 sec, then transferred back to ice for 2 min. Next, 250 µl S.O.C. media (Invitrogen, Burlington, ON, Canada) was added and the tubes were shaken horizontally at 200 rpm at 37°C for an increased incubation time of 3 hours. Finally, bacteria from each tube was spread onto 3 pre-warmed LB Miller plates (80 µl transformed cells per plate) containing Ampicillin, which had been spread with 40 µl of 40 mg/ml X-gal at least 30 min prior. The plates were incubated upside down, overnight at 37°C in the dark to prevent degradation of X-gal.

After 24 hrs, 48 white colonies were picked using autoclaved toothpicks and each placed into a separate plastic culture tube containing 2.5 ml media and 2.5 µl Ampicillin (100 µg/µl). Cultures were grown in the tubes for 24 hours at 37°C, on a 300 rpm shaker.

The bacterial cells were centrifuged at 3600 rpm for 6 min to pellet the cells, then the supernatant was poured off. Plasmids were purified using the 96 Turbo Plasmid Miniprep Kit (QIAgen, Mississauga, ON, Canada) according to manufacturer's instructions, using a microcentrifuge. Purified plasmids were re-suspended in 130 µl EB Elution buffer.

The purified plasmids were checked for successful fragment insertion by performing a restriction digest using EcoR1, which cuts specifically on both sides of the insert. Each reaction contained 0.5 µl EcoR1 enzyme (Invitrogen, Burlington, ON, Canada), 1 µl 10x REact®3 (Invitrogen, Burlington, ON, Canada), 4.5 µl ddH_2O, and 4 µl purified plasmid. This was incubated at 37°C for 1 hr, then run on a 1% agarose gel containing ethidium bromide to check for the correctly-sized inserts. Sequencing was performed to determine the sequence of the gene fragment, using T3 and T7 primers, according to the manufacturer's specifications (Invitrogen, Burlington, ON, Canada). Sequencher 4.8 Software (Gene Codes Corporation, Ann Arbor, MI, USA) was used to trim and align sequences into contigs with the genomic template.

3.2.9 TOPO TA Cloning® of Exons 4 and 5 (Domains 1 and 2)

The 4 kb PCR-amplified fragment containing exons 4 and 5 was cloned using the same protocol as in 3.2.8. for a sample of 48 patients.

3.2.10 TOPO TA Cloning® of Exon 4 (Domain 1)

The 560 bp PCR amplified fragment for exon 4 was cloned using a three-day cloning protocol was performed using TOPO TA Cloning® Kit for Sequencing (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions with some modifications. This was used to determine the correct PCR primer binding and amplification in 4 patients (48 clones each) initially, then used to identify novel alleles in a subset of 21 patients who had exon 4 codon variants (48 clones each). KIR2DL2 and KIR2DL3 exon 4 PCR products were extended to add TA overhangs to PCR products that were purified or amplified more than 24 hours prior to cloning. To extend, the following were incubated at 72°C for 15 min, iced, then quick spun: 1 µl Expand High Fidelity PCR Buffer 2 (10x) with $MgCl_2$ (Roche Diagnostics, Indianapolis, IN, USA), 2 µl dNTPs (1.25 mM), 1.5 µl ddH_2O, 5 µl PCR product, and 0.5 µl Taq DNA polymerase recombinant (Invitrogen, Burlington, ON, Canada). To ligate PCR products with the vector, 1 µl salt solution and 1 µl pCR®4-TOPO® vector (Invitrogen, Burlington, ON, Canada) were added to 4 µl extended or fresh PCR products. This was incubated at room temperature for 10 minutes, and then 3 µl of the ligation mixture was added to a vial of One Shot® TOP10 chemically competent *E. coli* cells (Invitrogen, Burlington, ON, Canada). This was incubated on ice for 20 min, heat shocked in a 42°C water bath for 30 sec, then transferred back to ice for 2 min. Next, 250 µl S.O.C. media (Invitrogen, Burlington, ON, Canada) was added and the tubes were shaken horizontally at 200 rpm at 37°C for 2 hours. Finally, bacteria from each tube was spread onto 3 pre-warmed LB Miller plates (80 µl transformed cells per plate) containing Ampicillin, which had been

spread with 40 µl of 40 mg/ml X-gal at least 30 min prior. The plates were incubated upside down, overnight at 37°C in the dark to prevent degradation of X-gal.

After 24 hrs, 48 white colonies were picked using autoclaved toothpicks and each placed in a separate plastic culture tube containing 2.5 ml media and 2.5 µl Ampicillin (100 µg/µl). Cultures were grown in the tubes for 24 hours at 37°C, on a 300 rpm shaker.

The bacterial cells were centrifuged at 3600 rpm for 6 min to pellet the cells, then the supernatant was poured off. Plasmids were purified using the 96 Turbo Plasmid Miniprep Kit (QIAgen, Mississauga, ON, Canada) according to manufacturer's instructions, using a vacuum apparatus. Purified plasmids were re-suspended in 130 µl EB Elution buffer.

The purified plasmids were checked for successful fragment insertion by performing a restriction digest using EcoR1 restriction enzyme, which cuts specifically on both sides of the insert. Each reaction contained 0.5 µl EcoR1 (Invitrogen, Burlington, ON, Canada), 1 µl 10x REact®3 buffer (Invitrogen, Burlington, ON, Canada), 4.5 µl ddH_2O, and 4 µl purified plasmid. This was incubated at 37°C for 1 hr, then run on a 1% agarose gel containing ethidium bromide to check for the correctly-sized inserts. Sequencing was performed to determine the sequence of the gene fragment, using T3 and T7 primers, according to the manufacturer's specifications (Invitrogen, Burlington, ON, Canada). Sequencher 4.8 Software (Gene Codes Corporation, Ann Arbor, MI, USA) was used to trim and align sequences into contigs with the genomic template.

3.2.11 HLA Class I Typing

Previous studies from our group identified HLA class I alleles in the Pumwani cohort [295, 296]. Since HLA class I antigens are the ligands for KIR2DL2 and KIR2DL3, the data from these previous studies were incorporated into analysis of the associations of KIRs with HIV-1. In particular, certain HLA-C alleles act as ligands for KIR2DL2/2DL3. These alleles were categorized into two functional groups, or "allotypes": The C group 1, (HLA-C1) which includes alleles with serine at position 77 and asparagine at position 80 is known to be the ligand for KIR2DL2/2DL3. C group 2 (HLA-C2) is characterized by alleles with asparagine at position 77 and lysine at position 80, and is the known ligand for KIR2DL1. While we abbreviate C group 2 as HLA-C2 and C group 1 as HLA-C1, this is not referring to the serological groups [7].

3.2.12 Statistical Analysis

KIR2DL2/2DL3 allele/genotype frequencies, and Hardy-Weinberg calculations were estimated using Python for populations-32-0.6.0 (PyPop) [297]. Genotyping results were analysed with biological data using SPSS 16.0. Cross-sectional analysis was performed to identify associations of KIR genes, alleles and genotypes with HIV-1 susceptibility, using χ^2, Fisher's exact test and crosstabs analysis (odds ratio (O.R.), 95% confidence interval (95% C.I.)), to determine the relationship between binary outcomes and explanatory variables. The same testing was performed after recoding new variables to combine the KIR2DL2/2DL3 and HLA-C typing results in each individual. To add further stringency to the definition of HIV-1 resistance, crosstabs analysis was limited to include only women who enrolled in the cohort before 2001, thereby increasing the definition to seven years. Multivariate binary logistic regression analysis was performed

to determine potential linkage with the HLA alleles that we previously reported to associate with HIV-1 resistance or susceptibility. Kaplan-Meier plots with log rank testing were used to determine associations with time to seroconversion, as well as disease progression in positive women as measured by number of days with $CD4^+$ counts 400 or more as well as decline to AIDS as defined by number of days with $CD4^+$ counts of 200 or more.

Section 4: Results

4.1 KIR Genotyping

The first molecular characterizations of KIRs were performed serologically [230, 298]. However due to the homology between the KIRs, most of the antibodies are not exclusive to one specific KIR. For example, there are two antibodies (GL183 and DX27) that are able to bind to KIR2DL2 and KIR2DL3 [248], but they cannot distinguish differences between these two KIRs and thus bind to both, as well as to KIR2DS2. This lack of specificity makes serology a poor choice for studying these receptors in isolation. Thus, genetic typing is a more accurate method for characterizing KIRs, and can be used as a control in functional studies alongside antibodies, or on its own for detection of an individual's KIR genotype.

Genetic studies thus far have included sequence specific primers (SSP) [225, 299], or sequence specific oligonucleotide probes (SSOP) [300, 301]. SSP methods rely on detecting the presence or absence of a gene or allele by gel electrophoresis of PCR products amplified by primers that span typing codons, with the polymorphic site to be detected corresponding with the 3' annealing end of the primer. Only genes with an exact match at this site should be detected a given reaction, and thus SSP typically requires very high quality DNA to be accurate. In contrast, SSOP methods use probes that bind precisely to complimentary strands of PCR-amplified gene targets, and is more time-consuming than SSP, and including all probes to identify every allele at a given locus is very expensive. However, it requires that the sequence is known, so that probes can be designed to match them exactly. While these methods are specific for typing

KIRs at the gene level, they have several limitations, including the requirement of large amounts of template if multi-gene or multi-allele typing is desired, the inability to detect novel alleles, time-consuming protocols, the requirement of many reaction mixtures to detect, and difficulty in optimizing reaction conditions to ensure accurate detection of only the desired product.

Here, we describe the development of a sequence-based genotyping method, as a superior alternative tool for KIR genotyping, especially for cohort studies, where limited supply of DNA template for each patient is often an issue. The advantages of sequence-based typing methods over SSP and SSOP methods include its higher resolution, ability to identify new genes and alleles, more rapid screening, and the ability to co-amplify certain genes and alleles (thus eliminating the need to design new primers and probes for each new allele discovered) which reduces the number of reactions needed, especially as more new alleles are identified. Zhu et al [302] developed a sequence-based typing method for KIR2DL4 genes, one of the framework KIR genes, but currently, no sequence-based typing method exists for the other genes in the KIR family, including KIR2DL2/2DL3.

4.1.1 Rationale of Primer Design for KIR2DL2/2DL3

Each individual has two alleles total belonging to the KIR2DL2/2DL3 locus, since one copy is obtained from each parent. Thus, it is possible to have two KIR2DL2, two KIR2DL3, or one allele of each gene. Nested sequence-specific PCR and sequencing primers located in surrounding intron regions were used, allowing these genes to be co-amplified in individuals to qualitatively detect their presence or absence. The primers selected for the amplification of exons 4, 5, and 6 were designed to be specific

for both KIR2DL2 and KIR2DL3 but not for other KIRs, at as many base sites within the primer as possible, especially at the 3' annealing end.

As seen in Figure 9, the exon 4 forward PCR primer was specific for only 2DL2 and 2DL3, while its reverse primer was specific for both 2DL2 and 2DL3, but also for 2DS2. Based on this combination, the only sequence expected to amplify would be 2DL2/2DL3. For exon 5, the forward PCR primer matched 2DL2/2DL3 but was also homologous to 2DS2, 2DS3 and 2DP1. Its reverse PCR primer was homologous to 2DL2/2DL3, but also 2DS2, thus it was possible for KIR2DS2 to be amplified with this set of primers. The exon 6 forward primer shared specificity with 2DL2/2L3, but also 2DL1, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, and 2DP1. However, the exon 6 reverse PCR primer was more specific, matching 2DL2/2DL3, 2DP1 and 3DS1. Based on the combination of the two primers, amplification of the 2DL2/2DL3 target could be expected along with 2DP1 amplification.

Sequencing primers were designed to be nested within the intron region of the fragment containing exon 4 in order to further increase the specificity of the sequencing (Figure 10). Both the exon 4 forward and reverse sequencing primers were specific for 2DL2/2DL3, but also shared homology to 2DS2 and 2DP1. However, it was expected that only 2DL2 and 2DL3 would be amplified due to the specificity of the PCR primers, as the amplicon being sequenced should have only contained 2DL2/2DL3. The exon 5 forward sequencing primer was homologous to 2DL2 and 2DL3, but also shared homology with 2DS1, 2DS4, and 2DP1. The exon 5 reverse sequencing primer was homologous to 2DL2/2DL3, but also to 2DL1, 2DL5, 2DS1, 2DS2, 2DS3, 2DS5, and 3DL2. Based on PCR primer specificity, the only sequence other than 2DL2/2DL3 that

may have been expected in the PCR products was 2DS2, but the exon 5 forward sequencing primer should not have sequenced 2DS2 as it was different by 1 base pair. Thus, the overall sequencing reaction was expected to be specific for 2DL2/2DL3, at least in the forward direction. The exon 6 forward sequencing primer was designed to be specific for only 2DL2/2DL3, however, the exon 6 reverse sequencing primer shared homology with not only 2DL2/2DL3, but also 2DS1, 2DS2, 2DS4, 2DS5, 2DP1, 3DL2, and 3DS1. Based on the combined exon 6 PCR primer specificity, it was expected that there may be some 2DP1 amplified in addition to the target 2DL2/2DL3, and the exon 6 reverse sequencing primer was homologous to this gene as well. However, the forward sequencing primer should have been specific enough to only sequence 2DL2/2DL3. However, no sequence differences were observed when the forward and reverse sequencing primers were compared for each sample. This was true when forward and reverse sequences were compared for all 3 exons.

Due to the vast homology within the KIRs, the design of these primers faced many challenges. First, in some instances no "ideal" (i.e. sites completely unique to KIR2DL2/2DL3) existed for one or more primers in the set of four nested primers. In general, PCR primers were made to be as specific as possible, with the nested sequencing primers (Figure 10) or combination of forward and reverse primers used as another layer of selection for the desired product. Second, at the beginning of the study, there were fewer published sequences available, and some of the genomic KIR sequences were incorrect or missing, a problem which is described further in 4.1.2. In theory, however, the combinations of PCR primers and sequencing primers designed should have yielded the selection needed to detect the correct 2DL2/2DL3 target sequences.

Exon 4 PCR-F 5'-3' (3,292-3,314)

Primer F	GAA	GGA	CCT	GCA	CCA	GGA	GTT	AA
2DL2	---	---	---	---	---	---	---	--
2DL3	---	---	---	---	---	---	---	--
2DL1	---	---	---	---	---	---	---	-T
2DS1	---	---	---	---	---	---	---	-T
2DS2	---	---	---	---	---	---	---	-T
2DS3	---	---	---	---	---	---	---	-T
2DS5	---	---	---	---	---	---	---	-T
2DP1	---	---	---	---	---	---	---	-T
3DL2	--G	---	---	---	---	--G	-A-	-T
3DL3	--G	---	---	---	A--	--G	---	-T
3DS1	---	---	---	---	---	---	-A-	-T
3DP1	---	---	---	-T-	---	---	---	AT

Exon 4 PCR-R 5'-3' (3,874-3,897)

Primer R	TGT	CTC	TGT	TGG	TAC	AAA	CCT	CAG
2DL2	---	---	---	---	---	---	---	---
2DL3	---	---	---	---	---	---	---	---
2DL1	---	---	---	---	C--	-G-	---	--C
2DS1	---	---	---	---	C--	-G-	---	--C
2DS2	---	---	---	---	---	---	---	---
2DS3	---	---	---	---	---	-G-	---	--C
2DS5	---	---	---	---	---	-G-	---	--C
2DP1	---	---	---	---	---	---	---	--C
3DL2	---	---	---	---	---	-G-	---	--C
3DL3	--G	--T	---	---	---	-G-	---	--C
3DS1	---	---	---	---	---	-G-	---	--C
3DP1	---	---	---	---	---	-G-	---	--C

Exon 5 PCR-F 5'-3' (5,393-5,413)

Primer F	AGG	CCC	ATG	AAY	GTA	GGT	TCT
2DL2	---	---	---	--T	---	---	---
2DL3	---	---	---	--C	---	---	---
2DL1	---	---	---	---	---	--C	---
2DL4	-A-	---	---	---	T--	--C	--C
2DL5	G--	---	---	---	C--	--C	--C
2DS1	---	---	---	---	---	--C	--C
2DS2	---	---	---	---	---	---	---
2DS3	---	---	---	---	---	---	---
2DS5	---	---	---	---	---	--C	--C
2DP1	---	---	---	---	---	---	---
3DL2	---	---	---	---	---	--C	--C
3DL3	---	--G	G--	---	T--	--C	--A
3DS1	GA-	---	---	---	---	--C	--C
3DP1	---	-T-	---	---	T--	---	--C

Exon 5 PCR-R 5'-3' (5,737-5,754)

Primer R	GGG	CCT	TCA	TGC	AGG	CCG
2DL2	---	---	---	---	---	---
2DL3	---	---	---	---	---	---
2DL1	---	---	C--	---	---	--A
2DL4	AA-	-T-	C--	---	---	--A
2DL5	---	---	C--	---	---	---
2DS1	---	---	C--	---	---	--A
2DS2	---	---	---	---	---	---
2DS3	---	---	C--	---	---	--A
2DS5	---	---	C--	---	---	--A
2DP1	A--	---	---	---	---	--A
3DL2	---	---	C--	---	---	--A
3DL3	---	---	C-T	---	---	--A
3DS1	A--	---	C--	---	---	--A

Exon 6 PCR-F 5'-3' (8,693-8,712)

Primer F	AGG	ACT	CCC	AGG	GCC	CAA	TA
2DL2	---	---	---	---	---	---	--
2DL3	---	---	---	---	---	---	--
2DL1	---	---	---	---	---	---	--
2DS1	---	---	---	---	---	---	--
2DS2	---	---	---	---	---	---	--
2DS3	---	---	---	---	---	---	--
2DS4	---	---	---	---	---	---	--
2DS5	---	---	---	---	---	---	--
2DP1	---	---	---	---	---	---	--
3DL2	-A-	---	---	---	-T-	---	--
3DL3	GAT	---	-A-	--C	C-T	TGG	--
3DS1	-A-	---	---	---	-T-	---	--

Exon 6 PCR-R 5'-3' (9,253-9,275)

Primer R	GTT	GCT	TCA	TGA	CCA	ACA	GTA	AT
2DL2	---	---	---	---	---	---	---	--
2DL3	---	---	---	---	---	---	---	--
2DL1	---	---	---	---	--T	---	-G-	GC
2DL4	---	-GA	---	---	-AG	---	-G-	GC
2DL5	--C	--A	---	---	---	---	-GG	GC
2DS1	---	---	---	---	--T	---	-G-	GC
2DS2	---	---	---	---	---	-T-	-G-	GC
2DS3	---	---	---	---	---	-T-	-G-	GC
2DS4	---	---	---	---	--T	---	-G-	GC
2DS5	---	---	---	---	--T	---	-G-	GC
2DP1	---	---	---	---	---	---	---	--
3DL2	---	--C	-G-	---	---	---	---	--
3DS1	---	---	---	---	---	---	---	--

Figure 9. Regions of primer homology for KIR2DL2/2DL3 PCR primers, exons 4, 5, 6. Note: Dashed lines indicate base pairs identical to primer. Bold font indicates complete homology with primer. KIRs not listed have no known homology.

Exon 4 SEQ-F 5'-3' (3,369-3,391)

Primer F	CAA	GGG	GAA	GCC	TCA	CTC	ATT	CT
2DL2	---	---	---	---	---	---	---	--
2DL3	---	---	---	---	---	---	---	--
2DS1	G--	---	---	---	-G-	---	-A-	-C
2DS2	---	---	---	---	---	---	---	--
2DS4	GG-	---	---	---	---	---	-A-	-C
2DS5	G--	---	---	---	---	---	-A-	-C
2DP1	---	---	---	---	---	---	---	--
3DL2	GG-	---	---	C--	---	---	---	-C
3DL3	GG-	---	---	---	---	--T	---	TC
3DS1	GG-	---	---	C--	---	---	---	-C
3DP1	-G-	---	---	---	---	---	---	--

Exon 4 SEQ-R 5'-3' (3,813-3,835)

Primer R	TCC	TTA	CAA	CTA	CCT	GGG	GGT	TC
2DL2	---	---	---	---	---	---	---	--
2DL3	---	---	---	---	---	---	---	--
2DS1	---	---	--G	-C-	---	x--	-CC	--
2DS2	---	---	---	---	---	---	---	--
2DS4	---	---	---	-C-	---	---	CCx	--
2DS5	---	---	---	-C-	---	x--	-CC	--
2DP1	---	---	---	---	---	---	---	--
3DL2	---	-C-	TG-	-C-	---	---	---	--
3DL3	---	-C-	TG-	-C-	---	---	---	--
3DS1	---	-C-	TG-	-C-	---	---	---	--
3DP1	---	---	T--	-C-	---	x--	-CC	--

Exon 5 SEQ-F 5'-3' (5,423-5,442)

Primer F	CCA	AGG	TCA	ACG	GAA	CAT	TC
2DL2	---	---	---	---	---	---	--
2DL3	---	---	---	---	---	---	--
2DL4	---	GCA	---	-T-	---	---	--
2DL5	---	GC-	--G	-T-	---	---	--
2DS1	---	---	---	---	---	---	--
2DS2	---	---	---	--A	---	---	--
2DS4	---	---	---	---	---	---	--
2DS5	---	---	---	--A	---	---	--
2DP1	---	---	---	---	---	---	--
3DL2	---	---	---	--A	---	---	--
3DL3	TG-	G--	---	-T-	---	---	--
3DS1	G--	---	---	--A	---	---	--
3DP1	---	---	---	-T-	---	-C-	--

Exon 5 SEQ-R 5'-3' (5,639-5,658)

Primer R	TGC	ATC	TGT	CCA	TGC	TTM	TC
2DL2	---	---	---	---	---	--A	--
2DL3	---	---	---	---	---	--C	--
2DL1	---	---	---	---	---	---	--
2DL4	CA-	---	---	---	---	---	--
2DL5	---	---	---	---	---	---	--
2DS1	---	---	---	---	---	---	--
2DS2	---	---	---	---	---	---	--
2DS3	---	---	---	---	---	---	--
2DS5	---	---	---	---	---	---	--
2DP1	---	---	---	---	-C-	---	--
3DL2	---	---	---	---	---	---	--
3DL3	G--	---	---	---	---	---	--
3DS1	---	---	---	---	---	--T	--

Exon 6 SEQ-F 5'-3' (8,754-8,774)

Primer F	TCC	ATT	GAG	TAG	AGG	ACA	GAC
2DL2	---	---	---	---	---	---	---
2DL3	---	---	---	---	---	---	---
2DS1	---	-C-	---	-G-	---	---	--A
2DS2	---	-C-	---	---	---	---	---
2DS4	---	-C-	---	---	---	---	---
2DS5	---	-C-	---	---	---	---	---
2DP1	---	-C-	---	---	---	---	---
3DL2	---	---	---	---	---	---	AG-
3DL3	AA-	-G-	--C	CGA	CAT	GA-	A-T
3DS1	---	---	---	---	---	---	AG-

Exon 6 SEQ-R 5'-3' (9,144-9,164)

Primer R	ATC	CTC	CAG	TTA	GGA	ATG	CAG
2DL2	---	---	---	---	---	---	---
2DL3	---	---	---	---	---	---	---
2DL4	G--	---C	---	---	---	---	---
2DL5	---	---	--A	--T	---	---	---
2DS1	---	---	---	---	---	---	---
2DS2	---	---	---	---	---	---	---
2DS4	---	---	---	---	---	---	---
2DS5	---	---	---	---	---	---	---
2DP1	---	---	---	---	---	---	---
3DL2	---	---	---	---	---	---	---
3DS1	---	---	---	---	---	---	---

Figure 10. Regions of primer homology for KIR2DL2/2DL3 nested sequencing primers. Note: Dashed lines indicate base pairs identical to primer. Bold font indicates complete homology with primer. KIRs not listed have no known homology. x=deletion.

4.1.2 Individual Amplification and Sequencing of exons 4, 5, and 6 did not Resolve the Typing Results Correctly

PCR-based amplification and sequencing of 1009 women for exons 4, 5, and 6 using separate amplification and sequencing reactions for each exon were performed. However, there were numerous problems related to the primers used to amplify the sequences from all three exons. It was believed that selection by these primers would be sufficient, but there were numerous sequence anomalies. These showed that there were always several variant codons present in somewhat predictable patterns, which did not match the typing sequence database. Taken as individual exons, the sequences from exons 5 and 6 did match 2DL2/2DL3 alleles, but did not correspond with the results from the KIR2DL2/2DL3 sequences from exon 4. They also matched a number of other KIRs such as KIR2DS, which were identical to certain KIR2DL2/2DL3 alleles at these locations. Exons 5 and 6 were short and did not contain as many typing codons as exon 4, thus they were not diagnostic and were only being used to supplement and increase the allele typing resolution of the sequences found in the more polymorphic exon 4. The mismatches from the KIR consensus, or "codon variations" are shown in Table 6, with the number of patients indicated for each of the variations. Patient group A had codon variations from the expected sequences at codons 206 and 207 in individuals with KIR2DL2*001/002/005 and KIR2DL3*001/003, 004, 005, or 006. Group B had variations at codons 169, 206, 207, 236, and 237 that could not be resolved in individuals with KIR2DL2*003 and 2DL2*001/002/005 or *004. Group C contained patients with 2DL2*003 in combination with 2DL3*001/003, *002/007, *005, or *006, but like group A, they had variations at codons 206 and 207. Group D contained patients with the same

variance at codons 169, 206, 207, 236, and 237 as those in group B, but in KIR2DL2*003 homozygotes. Groups E and F had the same variations at codons 207 and 207 as groups A and C, but groups E and F had an additional variation at codon 37 in individuals with KIR2DL2*003 in combination with 2DL2*001/002/003/005, KIR2DL3*001/003 or 005. Individuals in group G had KIR2DL2*004 and KIR2DL3*001/003 with variation at codons 206, 207, and 237. Group H contained all individuals who had two KIR2DL3 alleles. A subset of this group had the same variation at 206 and 207 as groups A-G, but the majority of individuals in this group had a single variation at codon 237, identical to that in group G.

Later in the study, when new sequence data was published for alleles and genes in the regions where the PCR and sequencing primers had been designed, it was discovered that some of the previously designed primers actually had very close homology to genomic regions of other KIRs. This was true for exon 6 in particular (Figure 9). In this case, new primers were designed for this region (Figure 11), with the forward amplification primer designed in a region upstream of the original primer, and the reverse primer located slightly downstream of the original. However, the sequences generated were still identical to the sequences obtained by the original combination of primers. These uncertainties were the reason for several confirmatory experiments, discussed further in 4.1.3 and 4.1.4. Topo TA cloning was used to sequence the full length of a single strand of DNA to determine whether these variants belonged to a specific KIR2DL2/2DL3 gene/allele, or whether they were a misamplification of different KIR(s).

Table 6. Allele typing results and codon variations combined from exons 4, 5, and 6.

	KIR2DL2 allele 1	KIR2DL2 allele 2	KIR2DL3 allele 1	KIR2DL3 allele 2	Codon variation	Exon	# patients (N=1009)
A.	001/002/005	-	001/003	-			139
			004		206 CCC	5	3
			005		207 TAT	5	52
			006				13
B.	003	001/002/005	-	-	169 CGT	5	23
					206 CCC	5	
					207 TAT	5	
		004			236 TCT	6	6
					237 AAA	6	
C.	003	-	001/003	-			106
			002/007		206 CCC	5	6
			005		207 TAT	5	50
			006				18
D.	003	003	-	-	169 CGT	5	21
					206 CCC	5	
					207 TAT	5	
					236 TCC	6	
					237 GAA	6	
E.	003	001/002/003/005	-	-	37 no CGC	4	35
					206 CCC	5	
					207 TAT	5	
F.	003	-	001/003	-	37 no CGC	4	30
			005		206 CCC	5	18
G.	004	-	001/003	-	206 CCC	5	21
					207 TAT	5	
					237 AAA	6	
H.	-	-	001/002/ 003/007	001/002/ 003/007	206 CCC	5	19
					207 TAT	5	
			001/003	002/007			28
			005	005			131
			006	006			29
			001/003	new			207
			002/007	005new			14
				006	237 AAA	6	3
			004	006			1
			005	006			8
			005	005			19
			006	new			9

Note: Dashes indicate absence of allele.

Exon 6-PCR-F 5'-3' (8,650-8,668)

Primer F	GAC	ART	GGG	CGT	CAC	ATA	C
2DL2	---	**-A-**	---	---	---	---	-
2DL3	---	**-G-**	---	---	---	---	-
2DL1	---	---	---	T--	---	---	A
2DS1	---	---	---	T--	---	---	A
2DS2	---	---	---	---	---	---	A
2DS3	---	---	---	---	---	---	-
2DS4	---	---	---	T--	---	---	A
2DS5	---	---	---	T--	---	---	A
2DP1	---	---	A-C	---	---	---	-
3DL2	---	---	---	-A-	-G-	-C-	-
3DS1	---	---	---	---	---	-C-	-

Exon 6-PCR-R 5'-3' (9,298-9,318)

Primer F2	TTC	TCT	GTT	ACG	GCA	AGG	CTG
2DL2	---	---	---	---	---	---	---
2DL3	---	---	---	---	---	---	---
2DL4	---	---	C--	--C	--A	---	--C
2DL5	---	CT-	C--	--T	---	---	--C
2DS1	---	---	---	---	---	---	--C
2DS2	---	---	---	--A	---	---	--C
2DS3	---	---	---	--A	---	---	--C
2DS4	---	---	---	---	---	---	--C
2DS5	---	---	---	---	---	---	-GC
2DP1	---	---	---	-A-	--A	---	--C
3DL2	---	---	---	---	---	---	--C
3DS1	---	---	---	---	---	---	--C
3DP1	---	---	---	---	---	---	--C

Figure 11. Regions of primer homology for new KIR2DL2/2DL3 PCR primers for exon 6 amplification, designed to be more specific at the 3' annealing end.

Note: Dashed lines indicate base pairs identical to primer. Bold font indicates complete homology with primer. KIRs not listed are assumed to have no significant homology. Original primers are found in Figure 9.

4.1.3 TOPO TA Clone® Sequences from Cloning the 6 kb Exon 4, 5, and 6 Genomic Fragment were Non-Specific

Two patient samples that had variations in the separate exon 4, 5, and 6 typing from groups A and B were then selected for 6 kb co-amplification, cloning and sequencing. Since each vector will only take up a single strand of DNA, this was done to confirm that exon 5 and 6 sequences were on the same genomic strand as exon 4. After PCR amplification, the 6 kb strands that should have contained genomic exons 4, 5, and 6 were inserted into TOPO TA vectors. Samples were then digested with EcoRI to determine if the correct fragments were inserted. The EcoRI digest of the TOPO TA clones® containing the 6 kb insert showed a wide variety of enzyme cutting patterns (Figure 12), while the enzyme was expected to cut only twice on either side of the insert, resulting in two bands being 4 kb (vector) and 6 kb (insert). However, the insert was cut internally, in addition to the expected sites on either side of the insert. Upon examination of the potential EcoRI cut sites within the expected insert, there were several sites that were not exact matches, but could potentially be recognized by EcoRI in sub-optimal reaction conditions. However, the digest was performed according to the manufacturer's recommendations and commercial buffers were used, so it was unlikely that the restriction digest reaction conditions were to blame for the multiple cut sites. Alternatively, misamplification of the insert may have resulted in extra EcoRI sites within the sequence.

Since the sequencing primer sites, T3 and T7, were located on the plasmid, flanking the insert, the sequence of the original PCR primers used to amplify the insert could be seen. When the DNA sequences of the cloned fragments were examined, several

observations were made. First, when the primer sequence was compared to the clone sequence, the forward PCR primer sequence captured by the upstream T3 primer did not match the sequence observed in the clones. Rather, it had the incorrect sequence 5'-ATG GAA GGA CCT GCA CCA GGA GTT AT -3', instead of the expected 5'- ATG GAA GGA CCT GCA CCA GGA GTT AA -3', suggesting that the 3' end did not anneal to the correct sequence. Second, when the sequence was compared to the NCBI Blast database, the clone sequences did not correspond to the original PCR-amplified sequenced clones, but instead showed high homology to predicted KIR3DL7 (95%), KIR2DS3 (95%), and KIR2DS2 (97%) based on the forward sequences. The BLAST search also called up KIR2DL3 (97%) but with different amplification and primer binding than the original exon 4 products. The reverse primer appeared to bind the correct primer sequence, however the amplicon sequence matched a chromosome 19 alternate assembly (98%) containing KIR2DL1 and KIR2DL3. Some sequences contained fragments that did not resemble KIRs at all were BLAST searched and mapped to the X-chromosome. Finally, when the sequencing primers for exons 4, 5 and 6 (Table 4) were used in an attempt to sequence individual exons from the plasmid, no sequence was obtained, suggesting that the insert did not contain their sequence.

These observations indicated that the fragment was incorrectly amplified because the DNA sequence did not match what the KIR specific primers were supposed to amplify. This could be due to polymerase errors, problems with repetitive regions within introns, and incorrect primer binding, possibly to several different KIRs which may have crossed over and then recombined during the PCR reaction. One possibility is that the reverse PCR primer located at the exon 6 end of the amplicon was not specific enough

when compared to the reverse primer that was used to amplify exon 4 separately in 3.2.2. This raised questions as to the utility of this method, since even slight mispriming may lead to the amplification of the wrong KIRs. To see if increasing the amplification stringency could resolve these problems, the primer annealing temperature was increased up to 10 degrees above the original temperature. The sequences were compared between each annealing temperature, but similar results were obtained, thus this did not improve the specificity of the reaction. We concluded that this method for cloning the long 6 kb region containing the three KIR exons of interest was not optimal for detecting the sequence desired. The nature of the KIR gene family, with its multiple homologous regions and many duplications, may be subject to PCR or cloning artifacts, the effects of which are especially evident when a long fragment is desired.

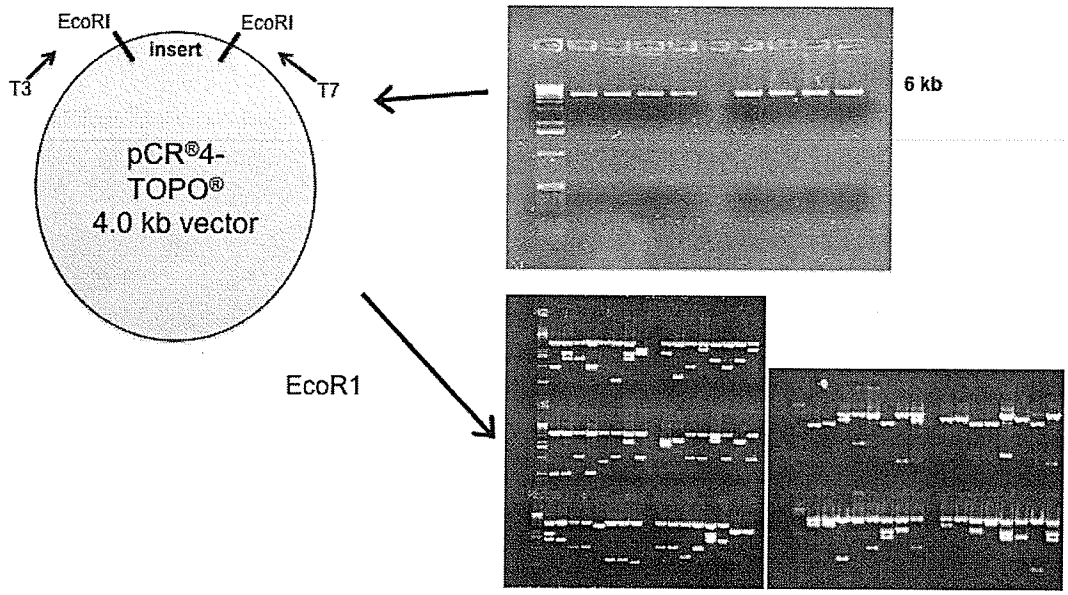

Figure 12. The 6 kb genomic fragment containing KIR2DL2/2DL3 exon 4, 5, and 6 was inserted into a TOPO TA cloning vector, but the EcoRI digest of the plasmids showed unexpected cut patterns, indicating that the insert sequence was incorrect.

4.1.4 Exon 4 and 5 Clones Revealed Incorrect Amplification and Sequencing

Using the same rationale as the exon 4, 5, and 6 co-amplification cloning, we co-amplified, cloned, and sequenced exon 4 and 5 in an attempt to overcome problems associated with the longer 6 kb fragment. The 4 kb PCR-amplified fragments containing exons 4 and 5 were used to obtain 48 clones per patient for the same two patients that were cloned for the 6 kb cloning, and were sequenced using the T3 and T7 primers. However, the forward primer sequences observed was similar to that from 4.1.3, and while the reverse primer appeared to match the expected primer sequence, again the Blast results from the insert sequence did not have complete homology with any known KIR. Also, the sequence obtained from T3 and T7 primers did not have complete homology with the sequences obtained from the PCR-based sequencing approach. We concluded that this method for cloning the long 4 kb region containing exons 4 and 5 was not optimal for detecting the sequence desired.

4.1.5 Exon 4 Clones Revealed Correct Amplification and Sequencing

Based on the codon variants evident after the analysis of exon 5 and 6 sequences, and based on the presence and higher number of gene-typing codons within exon 4, we cloned and sequenced to confirm the accuracy of the PCR sequence data.

To determine if the PCR primers (Table 3) correctly amplified exon 4, a sample of four patients was selected for cloning and sequencing this 560 bp fragment. 48 clones were obtained per patient. Based on the EcoRI restriction pattern, the correct-sized insert was observed when the samples were run on an agarose gel (Figure 13). Upon examination of T3 and T7 sequences for the exon 4 clones, it was observed that the

sequences matched the expected KIR2DL2/2DL3 sequences, including the site where the forward and reverse primers should bind. The sequences generated from these clones matched the exon 4 typing sequences obtained by sequence-based typing. In each set of 48 clones for a given patient, two clonal populations were observed in each heterozygote for KIR2DL2 and KIR2DL3, or for two different alleles of either gene. Homozygotes had identical sequences for all 48 clones. The cloning results indicated that the sequence-based typing method employed for exon 4 was accurate and can be used with confidence for genotyping KIR2DL2 and KIR2DL3.

To confirm the presence of new alleles, this method was then used to generate 48 clones from each of 21 patients that had exon 4 codon variations. The capacity of cloning to separate the sequence of each strand was utilized to confirm the presence of 2 clonal populations and to assign the codon sequence variances to the correct strand, as discussed in 4.1.6.

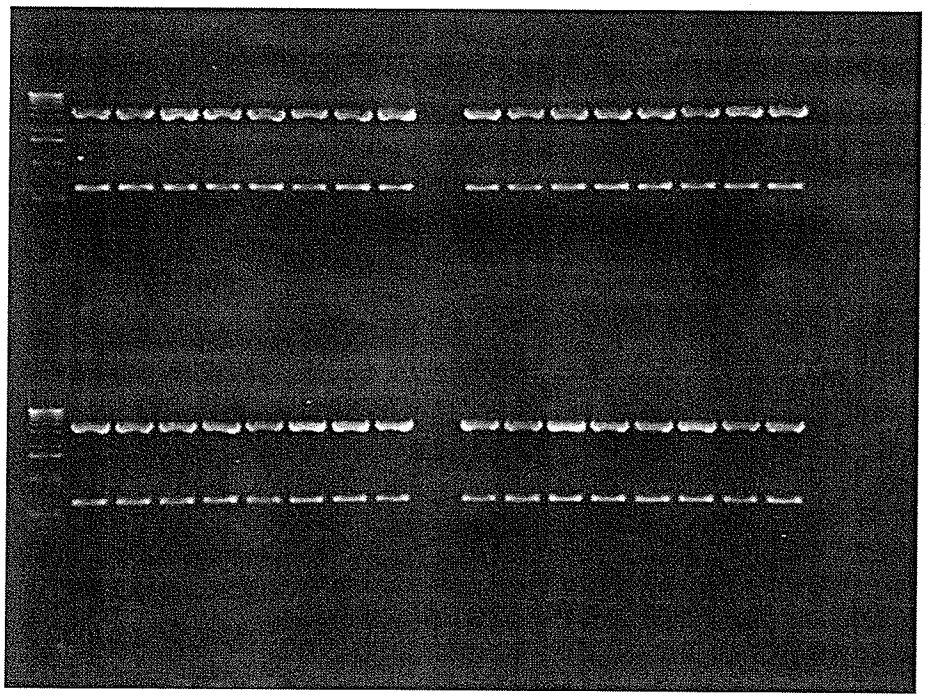

Figure 13. EcoRI digest of plasmids containing the exon 4 insert demonstrates that all inserts were the correct length.

4.1.6 Sequence-Based Typing Based on Exon 4 Sequences

Exon 4 sequences were used to differentiate 2DL2 and 2DL3 from each other at the gene level for 957 women, and to divide KIR2DL2 into two allele groups: 2DL2*001/002/003/005 and 2DL2*004 and 2DL3 into 3 allele groups: 2DL3*001/002/003/007, 2DL3*004/005, and 2DL3*006. While the majority of sequences fit into these five groups, there were just over 100 subjects whose sequences were similar to the known KIR2DL2/2DL3 consensus, but had codon variations and did not seem to match any other known KIRs. Some of these codon variations showed up in multiple subjects, and were classified into 16 groups of distinct variations, listed in Table 7.

To examine the genomic sequence of each co-amplified individual gene or allele separately, exon 4 cloning was performed, with each clone containing a separate DNA strand or allele. Cloning was performed, using 48 clones per patient for two patients (if available) per variant. The plasmids were sequenced using T3 and T7 primers to detect each allele strand separately. Each patient had two clonal populations, each representing a separate allele. As shown in Table 8, each mismatch could be attributed to the patient having one new allele and one known allele, or two new alleles. These were assigned to each of the 16 original variants (Table 8). The T3/T7 sequencing showed that the variants could actually be narrowed down to 8 distinct new sequences or alleles (Table 9), even though they had initially shown up as what appeared to be 16 groups during analysis of the PCR-sequencing approach. This was due to the actual variant strand being paired with different alleles in different patients. This demonstrated the importance of cloning as a tool for separating the sequences of co-amplified strands. The new allele

sequences were submitted to GenBank, and accession numbers were assigned to each. Arbitrary names, based on similarity to known allele groups, were created until official WHO nomenclature is assigned, as indicated in Table 9.

Table 7. Genotype patterns that did not match known KIR database sequences for exon 4, domain 1.

Variant #	Allele 1	Allele 2	Codon variant location	# patients
i.	2DL2*001/002/003/005	2DL2*001/002/003/005	37	6
ii.	2DL2*001/002/003/005	2DL2*001/002/003/005	37, 52 new GAC	16
iii.	2DL2*001/002/003/005	2DL2*001/002/003/005	37, 82	1
iv.	2DL2*001/002/003/005	2DL3*001/002/003/007	71	2
v.	2DL2*001/002/003/005	2DL3*001/002/003/007	96	3
vi.	2DL2*001/002/003/005	2DL3*004/005 new	37, 71, 82 (32)	5
vii.	2DL2*001/002/003/005 new	2DL3*001/002/003/007	56, 82 (37)	10
viii.	2DL2*001/002/003/005 new	2DL3*001/002/003/007	56, 82, 52 new GAC (37)	34
ix.	2DL2*001/002/003/005 new	2DL3*004/005	32, 71, 82 (37)	2
x.	2DL2*001/002/003/005 new	2DL3*004/005	32, 71, 82, 52 new GAC (37)	11
xi.	2DL3*001/002/003/007	2DL3*001/002/003/007	75	1
xii.	2DL3*001/002/003/007	2DL3*004/005 new	56, 71, (32)	10
xiii.	2DL3*001/002/003/007	2DL3*004/005	82, 52 new GAC	1
xiv.	2DL3*004/005	2DL3*004/005 new	32	3
xv.	No match	No match	40, 44, 51, 77	1
xvi.	2DS2	2DS2		1

Note: KIR2DL2/2DL3 exon 4 codons can be found in Figure 8.

Table 8. Cloning results resolved the variants in exon 4.

Variant #	Patient #	New typing result allele 1	New typing result allele 2
i.	1137 2079	2DL2*001/002/003/005	2DL2*001/002/003/005 new-a
ii.	1102	2DL2*001/002/003/005	2DL2*001/002/003/005 new-b
iii.	1643	2DL2*001/002/003/005 new-c	2DL2*001/002/003/005 new-a
iv.	1924	2DL2*001/002/003/005	2DL3*001/002/003/007
v.	1560	2DL2*001/002/003/005 new-d	2DL3*001/002/003/007
vi.	1782 1996	2DL2*001/002/003/005	2DL3*004/005 new-e
vii.	2025 2177	2DL2*001/002/003/005 new-a	2DL3*001/002/003/007
viii.	2044 1346	2DL2*001/002/003/005 new-b	2DL3*001/002/003/007
ix.	1744	2DL2*001/002/003/005 new-a	2DL3*004/005
x.	2030 2150	2DL2*001/002/003/005 new-b	2DL3*004/005
xi.	2156	2DL3*001/002/003/007	2DL3*001/002/003/007 new-f
xii.	2068	2DL3*001/002/003/007	2DL3*004/005 new-e
xiii.	1484	New Variant-g	New Variant-h
xiv.	1640	2DL3*004/005	2DL3*004/005 new-e
xv.	2051	2DL2*001/002/003/005 new-b	2DL3*004/005 new-e
xvi.	1720	2DL2*001/002/003/005 new-b	2DL3*004/005 new-e

Table 9. Summary of new variants identified through cloning.

Variant name	Codon differences	GenBank accession #
2DL2*001/002/003/005 new-a	37-CGC → 37-CCC	FJ188688
2DL2*001/002/003/005 new-b	37-CGC → 37-CCC 52-GAT → **52-GAC**	FJ188689
2DL2*001/002/003/005 new-c	82-AAA → 82-AAG	FJ188690
2DL2*001/002/003/005 new-d	96-GGG → **96-GGA**	FJ188691
2DL3*004/005 new-e	32-CGG → 32-CTG	FJ188692
2DL3*001/002/003/007 new-f	75-GAG → **75-GAA**	FJ188693
2DL3 new variant-g (NV-g)	44-ACA → **44-GCA** 51-TCA → **51-CCA**	FJ188694
2DL3 new variant-h (NV-h)	40-AAA → **40-AAG** 77-CAT → **77-CGT**	FJ188695

Note: Bold underlined text indicates new codons that have never been observed at that specific site for any alleles of either 2DL2 or 2DL3.

4.2 Allele Frequencies in the Pumwani Cohort

Based on the typing results from 957 women (allele count 2N=1914) and taking into account the putative new alleles identified, seven KIR2DL2 allele groupings were observed (Table 10). KIR2DL2 alleles accounted for 33.0% of all alleles identified at the KIR2DL2/2DL3 locus in the Pumwani cohort. The major KIR2DL2 group identified was KIR2DL2*001/002/003/005, which was present at a frequency of 27.0%. In addition, eight KIR2DL3 allele groupings were identified, with the KIR2DL3 alleles accounting for 67.0% of alleles at the KIR2DL2/2DL3 locus. The major KIR2DL3 group identified was KIR2DL3*001/002/003/007, at a frequency of 47.0%.

All the allele frequencies matched Hardy-Weinberg Equilibrium values (Table 11), as determined by χ^2 testing using the program PyPop [297]. This indicated that the Pumwani cohort's KIR frequencies have not likely been influenced by pressure due to factors such as selection, mutations, non-random mating, limited population size, random genetic drift and gene flow.

Table 10. KIR allele and gene frequencies in the Pumwani cohort, based on exon 4 typing results.

KIR2DL2/2DL3	Count	% alleles	1 copy	2 copies
A. 2DL2 Allele	2N=1914			
2DL2 total	631	33.0	433 (0.45)	99 (0.10)
001/002/003/005	516	27.0	386 (0.40)	65 (0.07)
004	30	1.5	30 (0.03)	0 (0)
001/002/003/005 new-a	19	1.0	19 (0.02)	0 (0)
001/002/003/005 new-b	62	3.2	62 (0.06)	0 (0)
001/002/003/005 new-c	1	0.05	1 (0.001)	0 (0)
001/002/003/005 new-d	3	0.16	3 (0.003)	0 (0)
B. 2DL3 Allele	2N=1914		1 copy	2 copies
2DL3 total	1283	67.0	433 (0.45)	425 (0.44)
001/002/003/007	900	47.0	446 (0.47)	227 (0.24)
004/005	278	14.5	236 (0.25)	21 (0.02)
006	82	4.3	67 (0.07)	7 (0.007)
004/005 new-e	20	1.0	20 (0.02)	0 (0)
001/002/003/007new-f	1	0.05	1 (0.001)	0 (0)
New variant-g	1	0.05	1 (0.001)	0 (0)
New variant-h	1	0.05	1 (0.001)	0 (0)
C. Gene	N=957			
2DL2	532	55.6		
2DL3	858	89.7		

Note: The proportion of the total number of people is indicated in parentheses.

Table 11. Hardy-Weinberg Equilibrium testing for KIR2DL2/2DL3 Allele Frequencies.

KIR2DL2/2DL3	Observed	Expected	χ^2	P value
A. KIR2DL2				
Common	N/A	N/A	2.31	0.6788
Lumped genotypes	N/A	N/A	1.43	0.2314
Common+ lumped	N/A	N/A	3.74	0.4419
All homozygotes	490	500.90	0.24	0.6261
All heterozygotes	467	456.10	0.26	0.6097
Common heterozygotes by KIR2DL2 allele				
2DL2/2DL3	433	422.97	0.24	0.6259
2DL2*001/002/003/005	386	376.89	0.22	0.6389
2DL2*001/002/003/005 new-a	19	18.81	0.00	0.9653
2DL2*001/002/003/005 new-b	62	59.99	0.07	0.7954
2DL2*004	30	29.53	0.01	0.9310
Common genotypes				
0 (2DL3/2DL3 homozygotes)	425	430.01	0.06	0.8090
2DL3:2DL2*001/002/003/005	353	345.89	0.15	0.7021
2DL3:2DL2*001/002/003/005 new-a	12	12.74	0.04	0.8366
2DL3:2DL2*001/002/003/005 new-b	46	41.56	0.47	0.4910
2DL3:2DL2*004	19	20.11	0.06	0.8046
2DL2*001/002/003/005:2DL2*001/002/003/005 new-a	6	5.12	0.15	0.6981
2DL2*001/002/003/005:2DL2*001/002/003/005 new-b	16	16.71	0.03	0.8612
2DL2*001/002/003/005:2DL2*004	11	8.09	1.05	0.3058
B. KIR2DL3				
Common	N/A	N/A	9.56	0.1443
Lumped genotypes	N/A	N/A	1.32	0.2497
Common+ lumped	N/A	N/A	10.89	0.0919
All homozygotes	354	337.66	0.79	0.3740
All heterozygotes	603	619.34	0.43	0.5115
Common heterozygotes by KIR2DL3 allele				
2DL2/2DL3	433	422.97	0.24	0.6259
2DL3*001/002/003/007	466	476.80	1.99	0.1584
2DL3*004/005	236	237.62	0.01	0.9162
2DL3*006	68	78.49	1.40	0.2365
2DL3*004/005 new-e	20	19.79	0.00	0.9625
C. Common genotypes				
0 (2DL2/2DL2 homozygotes)	99	104.1	0.24	0.6231
2DL2:2DL3*001/002/003/007	288	296.71	0.26	0.6132
2DL2:2DL3*004/005	109	91.65	3.28	0.0699
2DL2:2DL3*006	29	27.03	0.014	0.7053
2DL2:2DL3*004/005 new-e	7	6.59	0.03	0.8742
2DL3*001/002/003/007:2DL3*001/002/003/007	227	211.60	1.12	0.2897
2DL3*001/002/003/007:2DL3*004/005	116	130.72	1.66	0.1979
2DL3*001/002/003/007:2DL3*006	31	38.56	1.48	0.2235
2DL3*004/005:2DL3*004/005	21	20.19	0.03	0.8568
2DL3*006:2DL3*004/005	8	11.91	1.28	0.2572
2DL3*001/002/003/007:2DL3*004/005 new-e	10	9.40	0.04	0.8460

4.3 Associations of KIR/HLA with HIV-1 Infection and Disease Progression

4.3.1 Crosstabs Analysis Revealed Associations of KIR/HLA with HIV-1 Resistance

To determine if differences existed in KIR allele frequencies between the HIV-1 resistant and positive women, we conducted crosstabs analysis using the χ^2 test. No significant associations with resistance or susceptibility were noted on the basis of individual KIR2DL2/2DL3 alleles, as seen in Table 12, A and B, but when grouped, the new KIR2DL3 (P=0.046) alleles were slightly overrepresented in the HIV-1 resistant group (Table 12, C), as were the new KIR2DL2 and 2DL3 combined (P=0.055).

Further crosstabs analyses were performed, to study differences in KIR genotype frequencies, i.e. individuals with at least one copy of that particular allele or allele group, between the HIV-1 resistant and positive women (Table 13). While the genotypes containing specific KIR2DL2 and KIR2DL3 higher-resolution allele groups did not associate with resistance or susceptibility to HIV-1, a trend for protection was observed for individuals with any KIR2DL3 allele in their genotype (P=0.074, O.R.=2.116, 95% C.I.=0.823-5.437). In contrast, a trend towards HIV-1 susceptibility was noted for KIR2DL2 homozygotes (P=0.082, O.R.=0.483, C.I.=0.188-1.242).

Using the KIR Ligand Calculator [7], we grouped the HLA-C alleles into two allotypes based on amino acid differences: C group 1 (HLA-C1) alleles have serine at position 77 and asparagine at position 80 and ligands for KIR2DL2/2DL3. C group 2 (HLA-C2) has asparagine at position 77 and lysine at position 80, and act as ligands for KIR2DL1. HLA-C1 genotypes (P=0.066, O.R.=1.533, 95% C.I.=0.926-2.538) had a trend towards resistance, while women with HLA-C2 genotypes (P=0.096, O.R.=0.695,

C.I.=0.424-1.139), had a trend toward over-representation in HIV-1 positive women (Table 13).

Since HLA-C acts as a ligand for KIR2DL2/2DL3, KIR/HLA genotypes were also tested for their associations with HIV-1 resistance and susceptibility by crosstabs (Table 14). While none of the allele-based KIR2DL2/2DL3 groups were associated with resistance or susceptibility when their ligands were considered, we did find that women in the Pumwani cohort who were homozygous for both 2DL3 and HLA-C1 were significantly more likely to be HIV-1 resistant (P=0.005, Odds Ratio (O.R.)=2.554, 95% Confidence Interval (95% C.I.)=1.350-4.823). This is in contrast to the findings by Jennes et al in Côte d'Ivoire sex workers [286], who found this genotype to be detrimental. Jennes et al had also found KIR2DL2/2DL3 heterozygotes with C2 to be protected from HIV-1 infection [286], but no such association was observed in the Pumwani cohort (P=0.271, O.R.=1.229, C.I.=0.712-2.121).

Finally, to determine if these associations might be due to other known resistance factors, the association of KIR2DL3/HLA-C1 with HIV-1 resistance was tested against other HLA genotypes known to associate with resistance in the Pumwani cohort, including HLA-A, HLA-B, HLA-C (Luo et al, paper under review), DRB1 [201], and DQB1 genotypes [199]. Table 15 shows the results from binary logistic regression analysis using a forward Wald method, confirming that the KIR2DL3/HLA-C1 association is independent of these other factors.

Table 12. KIR allele associations with HIV-1 resistance and susceptibility.

KIR2DL2/2DL3	HIV-1 resistant 2N=200 (%)	HIV-1 positive 2N=1038 (%)	P value
A. 2DL2 Group			
2DL2*001/002/003/005	48 (24.0)	278 (26.8)	0.421
2DL2*004	3 (1.5)	17 (1.6)	0.591
2DL2*001/002/003/005 new-a	2 (1)	10 (0.96)	0.603
2DL2*001/002/003/005 new-b	5 (2.5)	31 (3.0)	0.460
2DL2*001/002/003/005 new-c	1 (0.5)	0 (0.0)	0.162
2DL2*001/002/003/005 new-d	1 (0.5)	1 (0.10)	0.297
B. 2DL3 Group			
2DL3*001/002/003/007	94 (47.0)	502 (48.4)	0.561
2DL3*004/005	33 (16.5)	144 (13.9)	0.569
2DL3*006	7 (3.5)	42 (4.0)	0.748
2DL3*004/005 new-e	4 (2.0)	12 (1.2)	0.250
2DL3*001/002/003/007 new-f	0 (0)	0 (0)	1
NV-g	1 (0.5)	0 (0)	0.162
NV-h	1 (0.5)	0 (0)	0.162
C. New Alleles			
2DL2 new	9 (4.5)	42 (4.0)	0.070
2DL3 new	6 (3.0)	12 (1.20)	**0.046**
2DL2/2DL3 new combined	15 (7.5)	54 (5.2)	**0.055**

Table 13. KIR2DL2/2DL3 and HLA-C genotypes and their associations with HIV-1 resistance and susceptibility.

KIR2DL2/2DL3, HLA-C	HIV-1 resistant	HIV-1 positive	P value	Odds ratio	95% C.I.[1]
A. 2DL2 group	N=100 (%)	N=519 (%)			
KIR2DL2	55 (55.0)	286 (55.1)	0.535	0.996	0.648-1.531
2DL2*001/002/003/005	45 (45)	245 (47.2)	0.384	0.915	0.595-1.407
2DL2*004	3 (3.0)	17 (3.3)	0.591	0.913	0.263-3.176
2DL2*001/002/003/005 new-a	2 (2.0)	10 (1.9)	0.603	1.039	0.224-4.814
2DL2*001/002/003/005 new-b	5 (5.0)	31 (6.0)	0.460	0.829	0.314-2.185
2DL2*001/002/003/005 new-c	1 (1.0)	0 (0)	0.162	1.010	0.990-1.030
2DL2*001/002/003/005 new-d	1 (1.0)	1 (0.2)	0.297	5.232	0.325-84.351
KIR2DL2hom	5 (5.0)	51 (9.8)	**0.082**	**0.483**	0.188-1.242
B. 2DL3 group	N=100 (%)	N=519 (%)			
KIR2DL3	95 (95.0)	467 (90.0)	**0.074**	**2.116**	0.823-5.437
2DL3*001/002/003/007	74 (74.0)	377 (72.6)	0.442	1.072	0.659-1.744
2DL3*004/005	30 (30.0)	135 (26.0)	0.239	1.219	0.762-1.951
2DL3*006	7 (7.0)	39 (7.5)	0.528	0.926	0.402-2.134
2DL3*004/005 new-e	4 (4.0)	12 (2.3)	0.250	1.760	0.556-5.573
2DL3*001/002/003/007 new-f	0 (0.0)	0 (0)	-	-	-
NV-g	1 (1.0)	0 (0)	0.162	1.010	0.990-1.030
NV-h	1 (1.0)	0 (0)	0.162	1.010	0.990-1.030
KIR2DL3hom	45 (45.0)	233 (44.9)	0.535	1.004	0.653-1.544
KIR2DL2/2DL3het	50 (50.0)	235 (519)	0.224	1.209	0.787-1.855
C. HLA-C allotype	N=114 (%)	N=594 (%)			
C1	72 (63.2)	372 (62.6)	0.502	1.023	0.675-1.550
C2	89 (78.1)	497 (83.7)	**0.096**	**0.695**	0.424-1.139
C1hom	24 (21.1)	88 (14.8)	**0.066**	**1.533**	0.926-2.538
C2hom	213 (35.9)	39 (34.2)	0.412	0.930	0.610-1.418
D. New Alleles	N=100 (%)	N=519 (%)			
2DL2 new all	8 (8.0)	42 (8.1)	0.582	0.988	0.449-2.172
2DL3 new all	5 (5.0)	12 (2.3)	0.123	2.224	0.766-6.457
2DL2/2DL3 new all	13 (13.0)	53 (10.2)	0.252	1.314	0.687-2.513

Note: Odds ratio represents the odds of being HIV-1 resistant if the genotype is present.

Hom=homozygote, het=heterozygote.

[1] C.I. Confidence Interval

Table 14. KIR2DL2/2DL3 and HLA-C and their associations with HIV-1 resistance.

KIR/HLA	HIV-1 resistant N=96 (%)	HIV-1 positive N=482	P value	Odds ratio	95% C.I.[1]
2DL2/C1	32 (34.1)	165 (34.2)	0.483	0.961	0.604-1.528
2DL2/C2	46 (47.9)	228 (47.3)	0.500	1.025	0.661-1.589
2DL3/C1	55 (57.3)	273 (56.6)	0.499	1.027	0.660-1.599
2DL3/C2	66 (68.8)	362 (75.1)	0.122	0.729	0.452-1.177
2DL2*001/002/003/005/C1	27 (28.1)	143 (29.7)	0.433	0.928	0.571-1.508
2DL2*001/002/003/005/C2	37 (38.5)	196 (40.7)	0.394	0.915	0.584-1.434
2DL2*001/002/003/005 new-a/C1	2 (2.1)	4 (0.8)	0.261	2.543	0.459-14.082
2DL2*001/002/003/005 new-a/C2	2 (2.1)	9 (1.9)	0.569	1.118	0.238-5.258
2DL2*001/002/003/005 new-b/C1	1 (1.0)	20 (4.1)	0.110	0.243	0.032-1.834
2DL2*001/002/003/005 new-b/C2	5 (5.2)	22 (4.6)	0.475	1.149	0.424-3.113
2DL2*001/002/003/005 new-c/C1	1 (1.0)	0 (0)	0.166	1.011	0.990-1.031
2DL2*001/002/003/005 new-c/C2	1 (1.0)	0 (0)	0.166	1.011	0.990-1.031
2DL2*001/002/003/005 new-d/C1	1 (1.0)	0 (0)	0.166	1.011	0.990-1.031
2DL2*001/002/003/005 new-d/C2	0 (0)	1 (0.2)	0.834	0.998	0.994-1.002
2DL2*004/C1	1 (1.0)	9 (1.9)	0.485	0.553	0.069-4.4183
2DL2*004/C2	3 (3.1)	13 (2.7)	0.513	1.164	0.325-4.165
2DL3*001/002/003/007/C1	42 (43.8)	217 (45.0)	0.455	0.950	0.611-1.477
2DL3*001/002/003/007/C2	50 (52.1)	288 (59.8)	0.101	0.732	0.472-1.137
2DL3*004/005/C1	16 (16.7)	87 (18.0)	0.438	0.908	0.506-1.630
2DL3*004/005/C2	22 (22.9)	107 (22.2)	0.485	1.042	0.618-1.756
2DL3*006/C1	3 (3.5)	18 (3.7)	0.528	0.832	0.240-2.880
2DL3*006/C2	5 (5.2)	28 (5.8)	0.523	0.891	0.335-2.369
2DL3*001/002/003/007 new-f/C1	0 (0)	0 (0)	-	-	-
2DL3*001/002/003/007 new-f/C2	0 (0)	0 (0)	-	-	-
2DL3*004/005 new-e/C1	3 (3.1)	8 (1.7)	0.270	1.911	0.498-7.339
2DL3*004/005 new-e/C2	1 (1.0)	11 (2.3)	0.381	0.451	0.058-3.533
2DL3-NV-g/C1	1 (1.0)	0 (0)	0.166	1.011	0.990-1.031
2DL3-NV-g/C2	1 (1.0)	0 (0)	0.166	1.011	0.990-1.031
2DL3-NV-h/C1	1 (1.0)	0 (0)	0.166	1.011	0.990-1.031
2DL3-NV-h/C2	1 (1.0)	0 (0)	0.166	1.011	0.990-1.031
2DL2/2DL3het/C1hom	8 (8.3)	32 (6.6)	0.340	1.278	0.570-2.867
2DL2/2DL3het/C2hom	20 (20.8)	85 (17.6)	0.271	1.229	0.712-2.121
2DL2hom/C1hom	0 (0)	4 (0.8)	0.483	0.992	0.984-1.00
2DL2hom/C2hom	2 (2.1)	16 (3.3)	0.402	0.620	0.140-2.740
2DL3hom/C1hom	16 (16.7)	35 (7.3)	**0.005**	**2.554**	1.350-4.832
2DL3hom/C2hom	13 (13.5)	74 (15.4)	0.392	0.864	0.458-1.629

Note: Hom=homozygote, het=heterozygote.

[1] C.I. Confidence Interval

Table 15. Binary logistic regression analysis shows the independence of the association of KIR2DL3/HLA-C1 homozygosity with resistance, compared with other known resistance-associated HLA genotypes in the Pumwani cohort.

HLA Genotype	B[1]	S.E.[2]	Wald	P-value	Exp(B)[3]
A*01	0.604	0.312	3.757	0.053	1.830
B*151701	1.645	0.642	6.560	0.010	5.181
B*4101	2.424	0.721	11.309	0.001	11.286
B*5702	1.119	0.511	4.790	0.0290	3.062
C*070401	0.920	0.484	3.617	0.057	2.508
DRB1*01	0.960	0.313	9.416	0.002	2.612
DRB1*1102	1.275	0.326	15.318	0.000	3.580
DQB1*050301	1.723	0.984	3.068	0.080	5.602
DQB1*0603	1.453	0.494	8.649	0.003	4.275
DQB1*0609	1.290	0.532	5.889	0.015	3.635
KIR2DL3/HLA-C1 homozygous	0.822	0.386	4.543	**0.033**	**2.275**
Constant	-2.624	0.213	151.664	0.000	0.073

[1] Coefficient of the predictor variables

[2] Standard error

[3] Exponent of B, odds ratio

4.3.2 Kaplan Meier Survival Analysis of KIR2DL2/2DL3 and HLA-C Genotypes with Time to Seroconversion

Next, we determined whether various combinations of KIR and HLA showed differences in time to seroconversion in a prospective analysis of HIV-1 negative sex trade workers. Kaplan Meier survival tests with the log rank test were used to show these associations. First, we looked at gene-level associations for KIR2DL2/2DL3. Results from Kaplan Meier survival plots (Table 16, Figure 14, A-D) showed that when considered separately, neither KIR2DL2 nor KIR2DL3 at the gene level contributed significantly to the rate of HIV-1 seroconversion in women of the Pumwani cohort. However, when KIR2DL2/2DL3 allele groups were considered, several genotypes had slower seroconversion, but P values were not significant: KIR2DL3*004/005 new-e (L.R.=1.731, P=0.188) and the new KIR2DL3 alleles (L.R.=2.502, P=0.114) (Figure 15, A and B, respectively) both had trends to slower seroconversion.

On their own, HLA-C1 and HLA-C2 did not show any associations with seroconversion rate (Figure 15). However, when HLA-C genotypes in an individual were studied along with their KIR2DL2/2DL3 genotype, the combination of KIR2DL2/HLA-C2 (Figure 14, F) had a protective effect, as it is associated with a slower rate of seroconversion (L.R.=4.341, P=0.037). Women with the other broad combinations of KIR2DL2/HLA-C1, KIR2DL3/HLA-C1, and KIR2DL3/HLA-C2 did not show any differences in time to seroconversion (Figure 14, E, G and H, respectively). KIR2DL2*004 appeared to have slower seroconversion when paired with its HLA-C1 ligand, but the P value was non-significant (L.R.=1.988, P=0.159), and in women with C2, its effect was diminished (L.R.=1.444, P=0.230) (Figure 15, C and D, respectively).

Likewise, individuals who possessed KIR2DL3*004/005 new-e as well as HLA-C1 had a trend toward slower seroconversion (L.R.=2.601, P=0.107), but when paired with HLA-C2 the same KIR lost its protective association (L.R.=0.328, P=0.567) (Figure 15, E and F, respectively). Furthermore, we determined the effects of homozygosity for KIR and HLA. KIR2DL3*004/005 homozygotes, when compared to those with one or zero copies of the allele, had a trend towards slower seroconversion (L.R.=2.557, P=0.110) (Figure 15, G), and those with two copies of KIR2DL3 and/or HLA-C2 were more likely to seroconvert than women with just one or no copies (L.R.=3.827, P=0.050) (Figure 15). This prompted us to test this genotype combination with resistance, and while a greater portion of HIV-1 resistant women had this genotype compared to HIV-1 positive (P=0.006), the odds ratio was inconclusive (O.R.=0.954, C.I.=0.938-0.971).

Additionally, women who were homozygous and heterozygous for KIR2DL2 and KIR2DL3 at the gene level were identified, as were women homozygous and heterozygous for HLA-C1 and HLA-C2 classifications. Each of these KIR2DL2/2DL3 and HLA-C combinations were analyzed, and no significant associations were noted. Women who were homozygous for both KIR2DL3 and HLA-C1 (L.R.=1.142, P=0.285) which had been strongly associated with HIV-1 resistance, had slightly slower time to seroconversion but this was not significant.

For those genotypes that demonstrated modest trends towards slower time to seroconversion, it should be noted that the sample sizes were small (<15), and it would be difficult to predict the effect if a larger group of individuals with that genotype were studied. Thus, these results should be treated as exploratory at best.

Table 16. Associations of KIR2DL2/2DL3 and HLA-C with time to seroconversion.

KIR/HLA	# Copies	Total N	N events	Censored (%)	Log rank	P value
A. 2DL2 group						
2DL2	0	207	67	140 (67.6)	0.661	0.416
	1	256	71	185 (72.3)		
2DL2*001/002/003/005	0	252	77	175 (69.4)	0.000	0.986
	1	211	61	150 (71.1)		
2DL2*004	0	451	136	315	1.410	0.235
	1	12	2	10		
2DL2*001/002/003/005 new-a	0	452	136	316 (69.9)	0.669	0.414
	1	11	2	9 (81.8)		
2DL2*001/002/003/005 new-b	0	428	127	301 (70.3)	0.028	0.868
	1	35	11	24 (68.6)		
2DL2*001/002/003/005 new-c	0	462	138	324 (70.1)	0.688	0.407
	1	1	0	1 (100)		
2DL2*001/002/003/005 new-d	0	461	138	323 (70.1)	0.601	0.438
	1	2	0	2 (100)		
2DL2 new (all)	0	415	125	290 (69.9)	0.194	0.659
	1	48	13	35 (72.9)		
B. 2DL3 group						
2DL3	0	49	12	37 (75.5)	0.102	0.749
	1	414	126	288 (69.6)		
2DL3*001/002/003/007	0	146	37	109 (74.7)	2.268	0.132
	1	317	101	216 (68.1)		
2DL3*004/005	0	332	98	234 (70.5)	0.097	0.755
	1	131	40	91 (69.5)		
2DL3*006	0	427	128	299	0.153	0.695
	1	36	10	26		
2DL3*004/005 new-e	0	454	137	317 (69.8)	1.731	0.188
	1	9	1	8 (88.9)		
2DL3*001/002/003/007 new-f	0	462	138	324 (70.1)	0.109	0.741
	1	1	0	1 (100.0)		
NV-g	0	462	138	324 (70.1)	0.688	0.407
	1	1	0	1 (100)		
NV-h	0	462	138	324 (70.1)	0.688	0.407
	1	1	0	1 (100)		
2DL3 new (all)	0	452	137	315 (69.7)	2.502	0.114
	1	11	1	10 (90.9)		
C. HLA-C allotype						
C1	0	168	56	112 (66.7)	0.383	0.536
	1	322	99	223 (69.3)		
C2	0	96	34	62 (64.6)	0.036	0.849
	1	394	121	273 (69.3)		

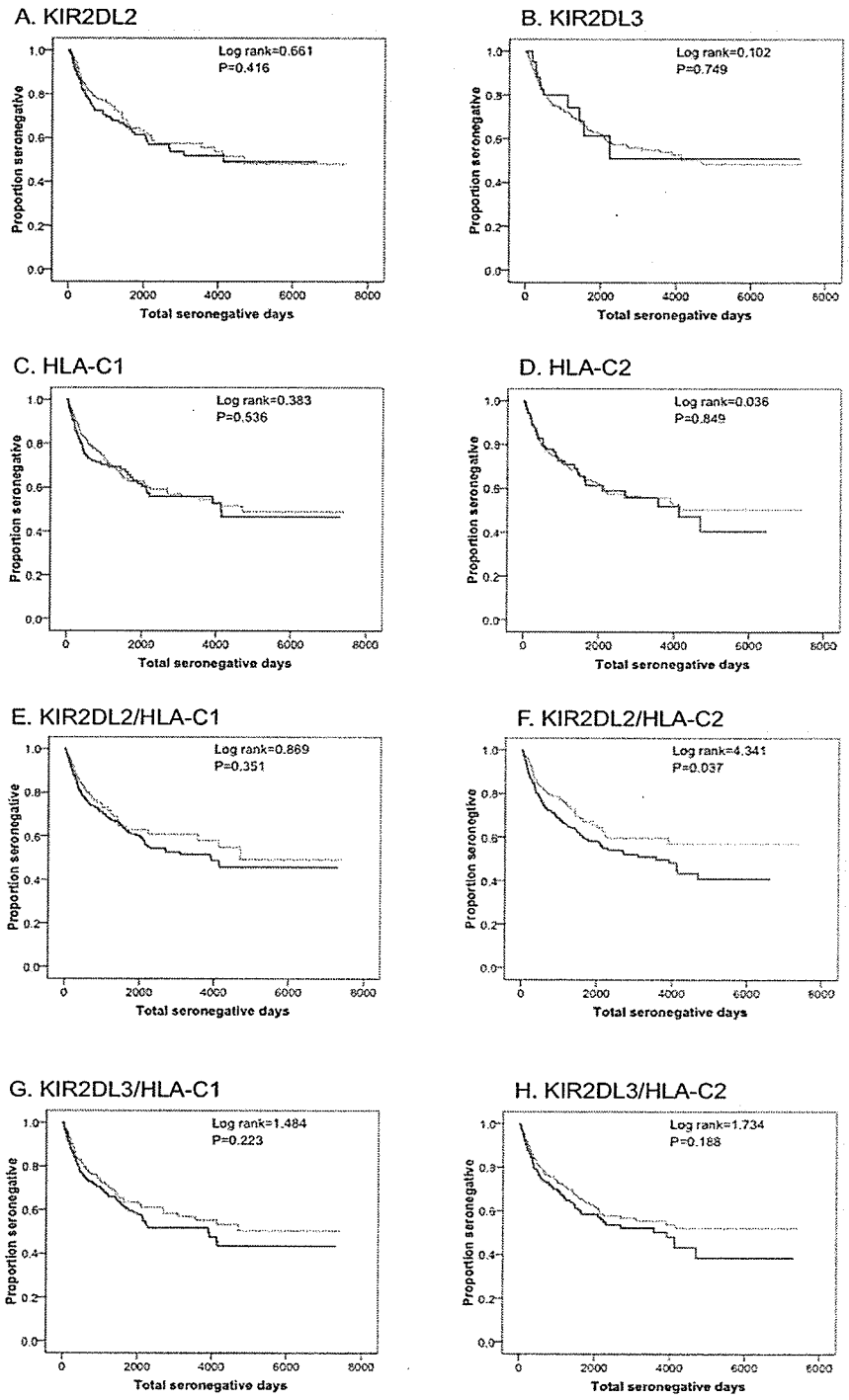

Figure 14. Kaplan-Meier survival analysis shows time to seroconversion for KIR2DL2/2DL3 genes and HLA-C genotypes.

Note: with gene=dotted green line, without=solid blue line.

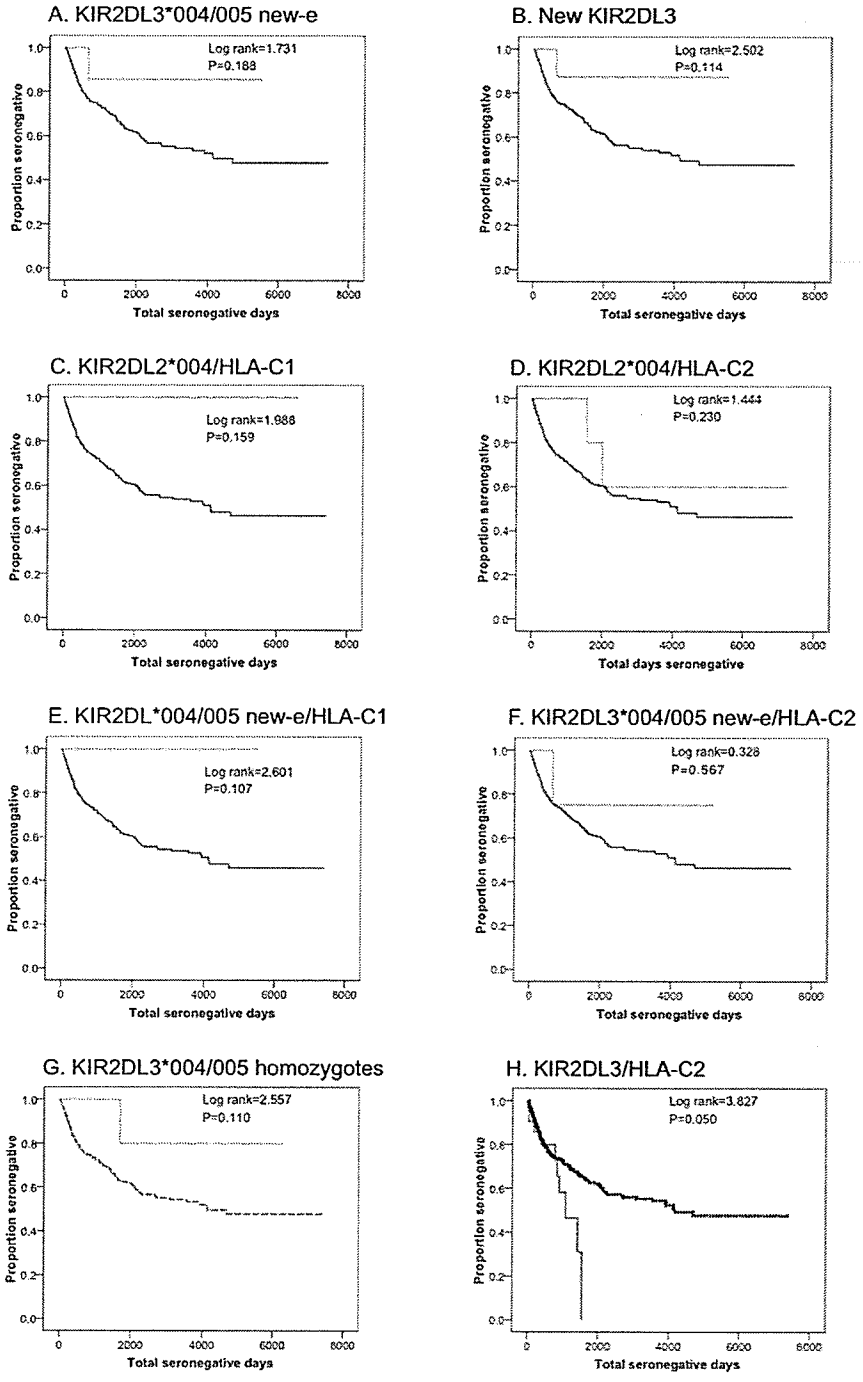

Figure 15. Kaplan-Meier analysis shows time to seroconversion for KIR/HLA.

Note: A-F: with genotype=dotted green line, without=blue solid line; G: 2 copies of allele=dotted green line, without/1 copy=broken blue line; H: 1 or 2 copies=black dotted line, no copies=blue solid line

4.3.3 Associations of KIR and HLA with HIV-1 Disease Progression

The infection and destruction of $CD4^+$ T cells are the hallmarks of HIV-1 infection as the disease progresses to AIDS. To determine a role for KIRs in HIV-1 disease progression, the effects of KIR2DL2/2DL3 and HLA-C genotypes were examined by Kaplan-Meier analysis, in terms of their effect on $CD4^+$ T cell levels: decline to below CD4 400 (Table 17) or 200 (Table 18). First we studied the associations between KIR and disease progression at the gene level, and found that individuals who were homozygous for KIR2DL2 had a trend of slower decline to CD4 below 400 (Log rank=3.526, P=0.060), but no effect was seen on decline to CD4 below 200 (L.R.=1.659, P=0.822) (Figure 16, A). In contrast, the KIR2DL2 genotype was associated with increased susceptibility to HIV-1, but homozygosity had no effect on susceptibility to HIV-1. The KIR2DL3 genotype has a trend with rapid decline to below CD4 400 (L.R.=3.526, P=0.060), while no effect was seen on decline to CD4 below 200 (L.R.=0.051, P=0.822) (Figure 16, B). Conversely, KIR2DL3 homozygosity had actually had a protective trend in the HIV-1 resistance study, but no effects were seen in those with only one copy of KIR2DL3. Women with the new KIR2DL2 genotypes had a trend towards faster decline to CD4 below 400 (L.R.=2.559, P=0.110), but had no effect on decline to CD4 below 200 (L.R.=1.659, P=0.198) (Figure 16, C). In contrast, the new KIR2DL2 alleles had a protective effect in the resistance study.

When the impact of KIR genotypes was studied, KIR2DL2*001/002/003/005 new-b had no impact on the time to CD4 below 400 (L.R.=1.301, P=0.254) but significantly shortened the time to CD4 below 200 (L.R.=6.041, P=0.014) (Figure 17, A). This genotype had no association with resistance or susceptibility to HIV-1.

KIR2DL3*004/005 was trending to rapid time to CD4 counts below 400 (L.R.=3.631, P=0.057), but had no impact on time to CD4 below 200 (L.R.=0.710, P=0.399) (Figure 17, B). However, no associations were observed between KIR2DL3*004/005 and HIV-1 resistance/susceptibility.

In contrast, women with KIR2DL3*006 trended toward a slightly protective effect, with increased times to both CD4 below 400 (L.R.=2.997, P=0.083) and below 200 (L.R.=3.005, P=0.083) (Figure 17, C). However, this genotype had no impact on HIV-1 resistance or susceptibility. The results from the associations with disease progression suggest that genotypes associatied with HIV resistance do not necessarily have protective associations with slower disease progression. In fact, the opposite effect was even observed for several of them.

Table 17. Associations of KIR genotypes with HIV-1 disease progression, measured by Kaplan-Meier analysis of number of days until CD4 drops below 400.

KIR/HLA	# Copies	Total N	N events	Censored (%)	Log rank	P value
A. 2DL2 group						
2DL2	0	253	196	57 (22.5)	0.194	0.660
	1	318	224	94 (29.6)		
2DL2*001/002/003/005	0	293	228	65 (22.2)	2.142	0.143
	1	278	192	86 (30.9)		
2DL2*004	0	556	410	146 (26.3)	0.994	0.319
	1	15	10	5 (33.3)		
2DL2*001/002/003/005 new-a	0	563	413	150 (26.6)	1.098	0.295
	1	8	7	1 (12.5)		
2DL2*001/002/003/005 new-b	0	536	395	141 (26.3)	1.301	0.254
	1	35	25	10 (28.6)		
2DL2*001/002/003/005 new-c	0	571	420	151 (26.4)	-	-
	1	0	-	-		
2DL2*001/002/003/005 new-d	0	570	419	151 (26.5)	0.534	0.465
	1	1	1	0 (0)		
2DL2 new (all)	0	527	387	140 (26.6)	2.559	0.110
	1	44	33	11 (25.0)		
B. 2DL3 group						
2DL3	0	56	35	21 (37.5)	**3.526**	**0.060**
	1	515	385	130 (25.2)		
2DL3*001/002/003/007	0	161	116	45 (28.0)	0.001	0.978
	1	410	304	106 (35.9)		
2DL3*004/005	0	418	297	121 (28.9)	**3.631**	**0.057**
	1	153	123	30 (19.6)		
2DL3*006	0	528	394	134 (25.4)	**2.997**	**0.083**
	1	43	26	17 (39.5)		
2DL3*004/005 new-e	0	560	411	149 (26.6)	0.081	0.775
	1	11	9	2 (18.2)		
2DL3*001/002/003/007 new-f	0	571	420	151 (26.4)	-	-
	1	0	-	-		
NV-g	0	571	420	151 (26.4)	-	-
	1	-	-	-		
NV-h	0	571	420	151 (26.4)	-	-
	1	-	-	-		
2DL3 new (all)	0	560	411	149 (26.6)	0.081	0.775
	1	11	9	2 (18.2)		
C. HLA-C allotype						
C1	0	240	181	59 (24.6)	0.161	0.689
	1	410	297	113 (27.6)		
C2	0	105	76	29 (27.6)	0.001	0.981
	1	545	402	143 (26.2)		

Table 18. Associations of KIR genotypes with HIV-1 disease progression, measured by Kaplan-Meier analysis of number of days until CD4 drops below 200.

KIR/HLA	# Copies	Total N	N events	Censored (%)	Log rank	P value
A. 2DL2 group						
2DL2	0	253	129	124 (49.0)	0.016	0.898
	1	318	154	164 (51.6)		
2DL2*001/002/003/005	0	293	151	142 (48.5)	0.201	0.654
	1	278	132	146 (52.5)		
2DL2*004	0	556	277	279 (50.2)	0.667	0.414
	1	15	6	9 (60.0)		
2DL2*001/002/003/005 new-a	0	563	281	282 (50.1)	2.524	0.112
	1	8	2	6 (75.0)		
2DL2*001/002/003/005 new-b	0	536	262	274 (51.1)	**6.041**	**0.014**
	1	35	21	14 (40.0)		
2DL2*001/002/003/005 new-c	0	571	283	288 (50.4)	-	-
	1	0	-	-		
2DL2*001/002/003/005 new-d	0	570	282	288	1.282	0.258
	1	1	1	0		
2DL2 new (all)	0	527	259	268 (50.9)	1.659	0.198
	1	44	24	20 (45.5)		
B. 2DL3 group						
2DL3	0	57	27	30 (52.6)	0.051	0.822
	1	514	256	258 (50.2)		
2DL3*001/002/003/007	0	161	81	80 (49.7)	1.450	0.229
	1	410	202	208 (50.7)		
2DL3*004/005	0	418	205	213 (51.0)	0.710	0.399
	1	153	78	75 (49.0)		
2DL3*006	0	528	267	261 (49.4)	**3.005**	**0.083**
	1	43	16	27 (62.8)		
2DL3*004/005 new-e	0	560	277	283 (50.5)	0.002	0.969
	1	11	6	5 (45.5)		
2DL3*001/002/003/007 new-f	0	571	283	288 (50.4)	-	-
	1	0	-	-		
NV-g	0	571	283	288 (50.4)	-	-
	1	0	-	-		
NV-h	0	571	283	288 (50.4)	-	-
	1	0	-	-		
2DL3 new (all)	0	560	277	283 (50.5)	0.002	0.969
	1	11	6	5 (45.5)		
D. HLA-C allotype						
C1	0	240	126	114 (47.5)	0.061	0.804
	1	410	196	214 (52.2)		
C2	0	105	46	59 (56.2)	1.039	0.308
	1	545	276	269 (49.2)		

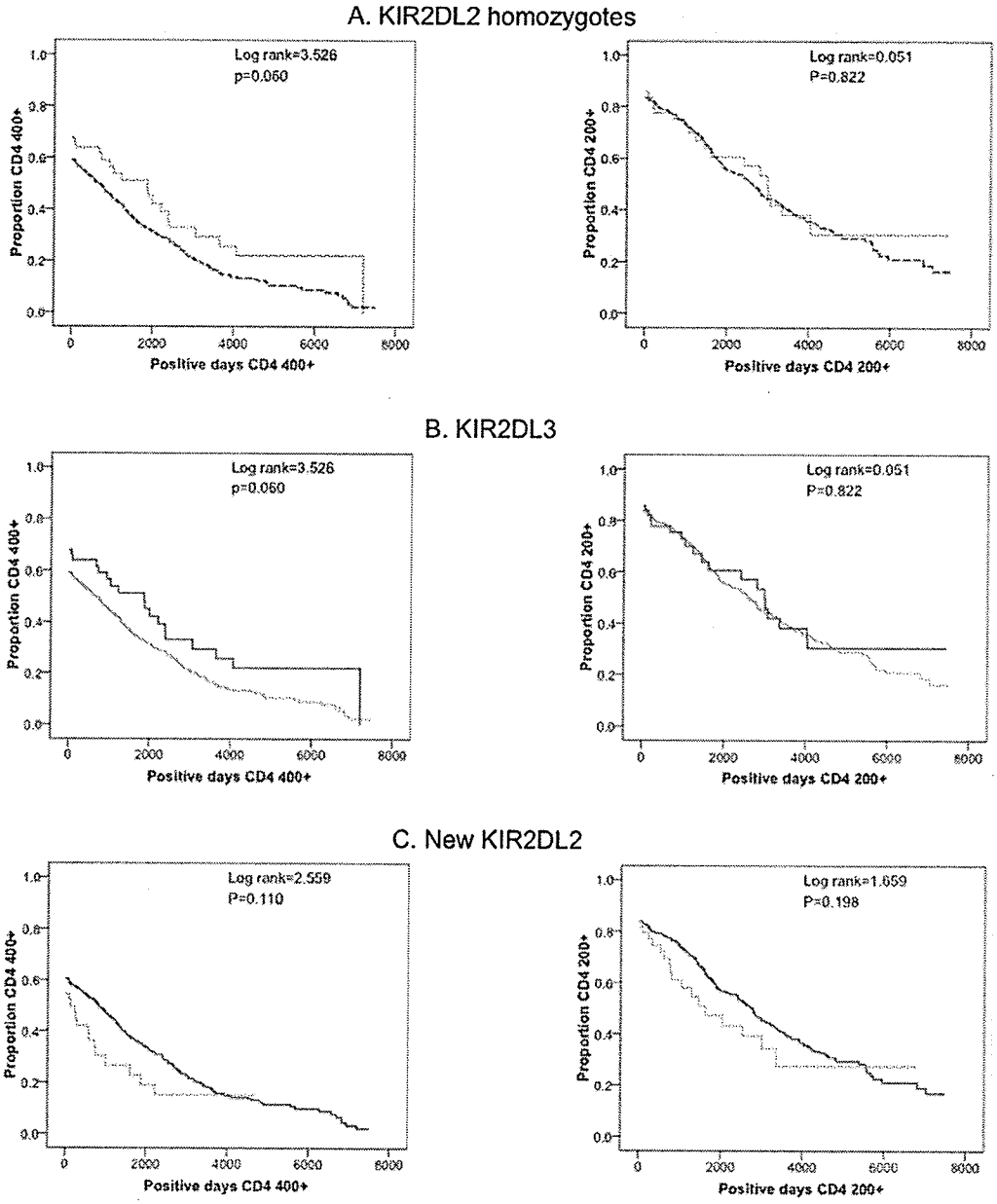

Figure 16. Associations of KIR2DL2/2DL3 with HIV-1 disease progression, measured by CD4 decline to levels below 400 (left) or 200 (right).

Note: A: 2 copies of gene=dotted green line, without or 1 copy=broken blue line.

B-C: with genotype=dotted green line, without=solid blue line.

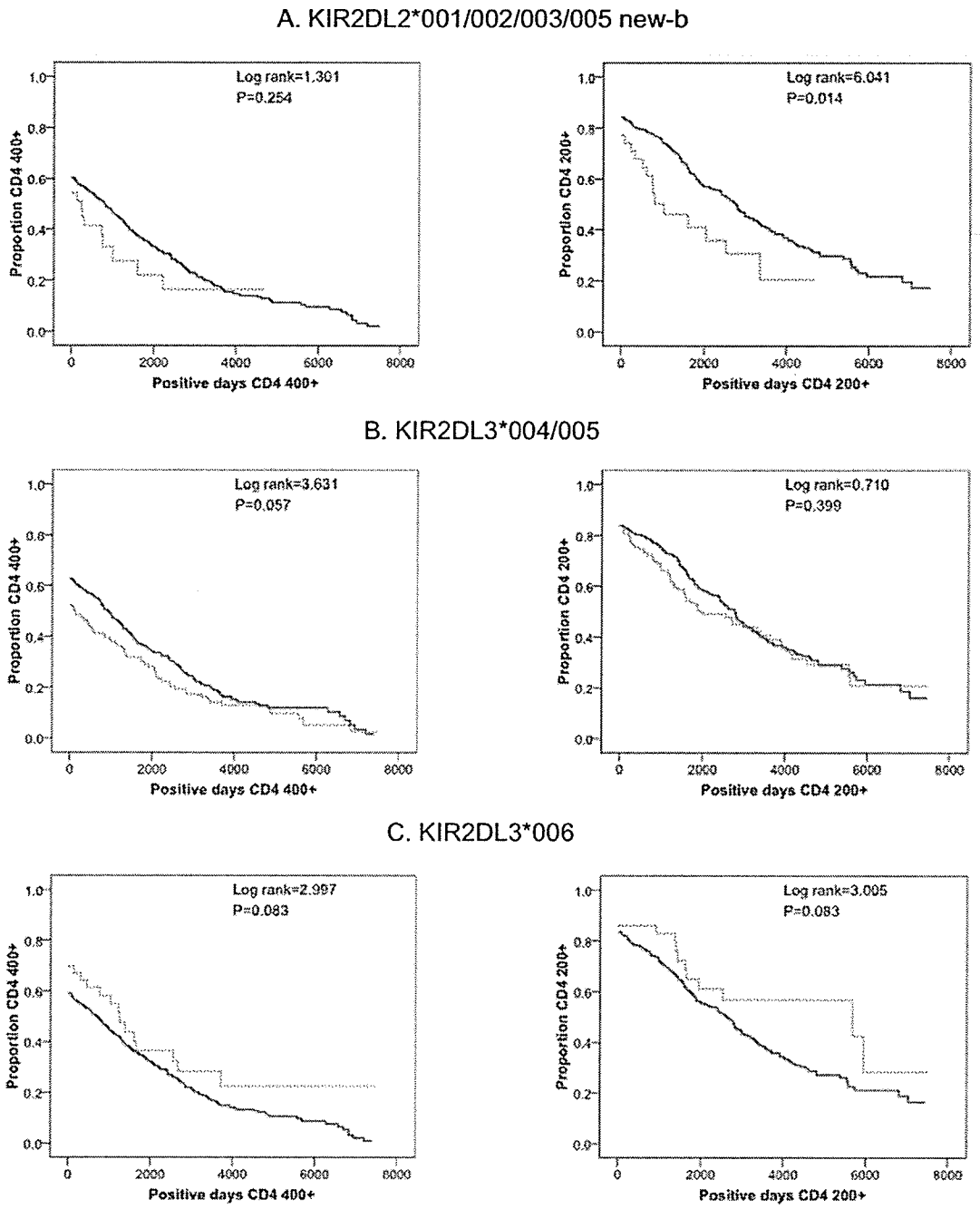

Figure 17. Associations of KIR2DL2/2DL3 genotypes with HIV-1 disease progression, measured by CD4 decline to levels below 400 (left) or 200 (right).

Note: with genotype=dotted green line, without=solid blue line.

When we combined the effects of an individual's KIR2DL2/2DL3 genotype and HLA-C allotype, several key associations with HIV-1 disease progression were identified. The decline to CD4 below 400 in subjects with KIR2DL2*001/002/003/005 new-b was not affected significantly: KIR2DL2*001/002/003/005 new-b/HLA-C1 (L.R.=0.965, P=0.326), and KIR2DL2*001/002/003/005 new-b/HLA-C2 (L.R.=1.404, P=0.236). However, KIR2DL2*001/002/003/005 new-b had been associated with drastically faster drops in CD4 counts to below 200 on its own, and this detrimental effect was also observed whether the subject had HLA-C1 (L.R.=7.995, P=0.005) or HLA-C2 (L.R.=9.016, P=0.003) (Figure 18, A and B, respectively). In contrast, a protective trend was observed in subjects with KIR2DL2*004/HLA-C1 for both time to CD4 below 400 (L.R.=1.715, P=0.190) and 200 (L.R.=2.00, P=0.157) (Figure 18, C). Individuals who were homozygous for both KIR2DL2 and HLA-C1 also had significantly slower time to CD4 decline below 400 (LR=5.857, P=0.016) and a trend to time slower time below 200 (L.R.=2.996, P=0.083).

The KIR2DL3*006 genotype, which was associated with slower disease progression when considered independently (Figure 17, C), had a somewhat weakened effect in women with HLA-C1 in the decline to CD4 below 400 (L.R.=2.058, P=0.151) as well as in the decline to CD4 below 200 (L.R.=0.891, P=0.345) (Figure 19, A). However, when paired with HLA-C2, KIR2DL3*006 had an effect similar to its independent associations of decreasing the time to CD4 below 400 (L.R.=2.972, P=0.085), and trend of decreasing time to CD4 below 200 (L.R.=2.016, P=0.156) (Figure 19, B).

None of the KIR/HLA combined genotypes that were associated with different rates of disease progression had any significant associations with resistance or susceptibility to HIV-1, and the other genotypes that associated with resistance or slower seroconversion, including KIR2DL3/HLA-C1, HLA-C1 homozygotes, and KIR2DL2/HLA-C2, were associated with varied disease progression rate. This, coupled with the opposite effects of KIR2DL2/2DL3 genes on HIV-1 resistance vs. disease progression, reveals one of the limitations in the sequence-based analysis of KIRs: the associations do not reveal any functional reasons for the associations. While the sequence-based typing method is an excellent tool for studying KIRs at the population level, the analysis is exploratory and we need to determine functional mechanisms for these associations. While future experiments will be needed and further work is required to characterize their expression, frequency on NK cells, signaling strength, and full haplotype effects, we can still make predictions and formulate hypotheses based on functional data that have already been published.

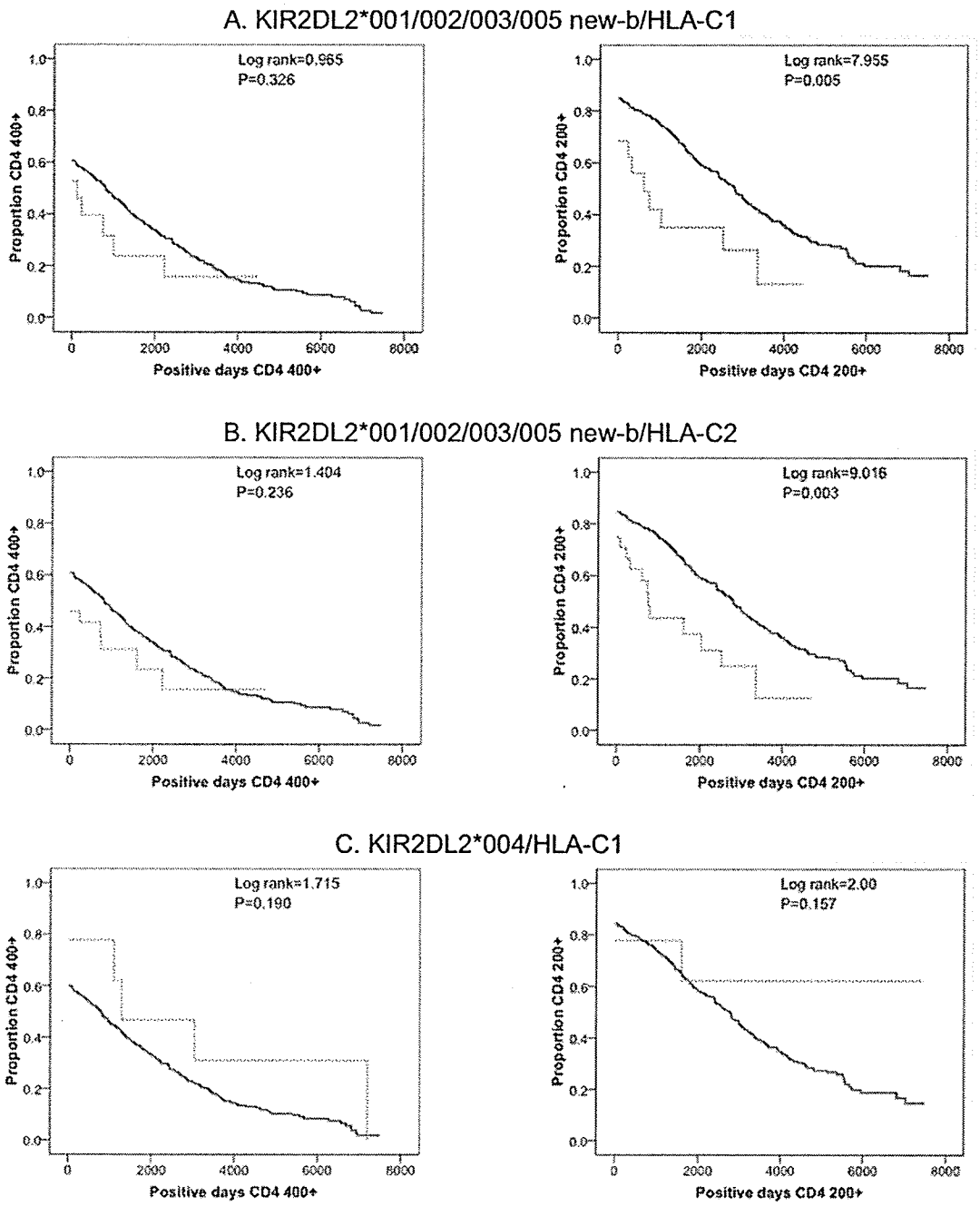

Figure 18. Associations of KIR2DL2/2DL3 and HLA-C combinations with HIV-1 disease progression, measured by CD4 decline to levels below 400 (left) or 200 (right). Note: with genotype=dotted green line, without=solid blue line.

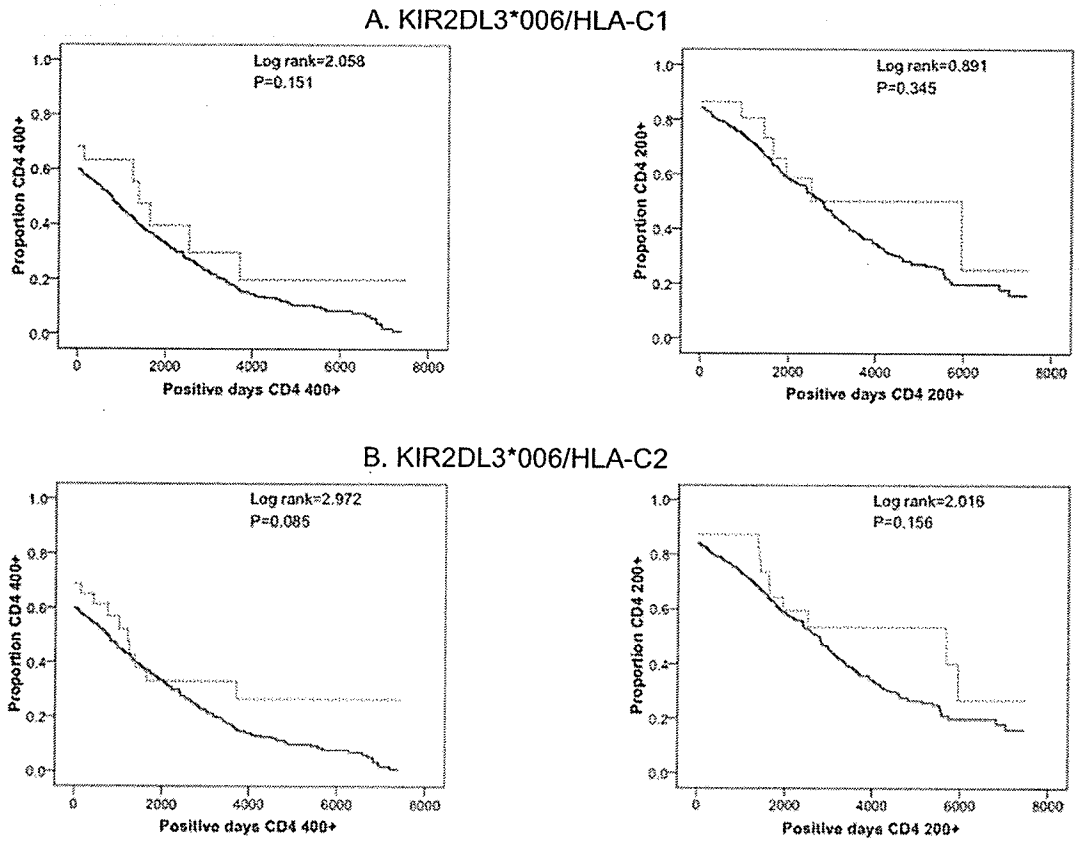

Figure 19. Associations of KIR2DL3*006 and HLA-C1/HLA-C2 with HIV-1 disease progression, measured by CD4 decline to levels below 400 (left) or 200 (right).

Note: with genotype=dotted green line, without=solid blue line.

Section 5: Discussion

The HIV/AIDS pandemic can be considered from several viewpoints. On one hand, HIV-1 prevalence has been sustained globally despite improvements in treatment and prevention, and every vaccine and microbicide tested thus far has failed. Based on this, it is reasonable to assume that left unchecked, the end is nowhere in sight for this massive public health threat. However, from another point of view, there is a great amount of optimism in the fight against HIV/AIDS: several regions have seen declines in new infections due to successful education campaigns, and one of the greatest sources of hope for a vaccine lies within the rare individuals who are naturally resistant to HIV-1, or those who are infected but may be able to remain healthy by controlling their HIV-1 without treatment.

While a preventative vaccine is critical to completely halt the spread of HIV-1, a therapeutic vaccine that could allow an infected person to control their infection would also be beneficial. The women in the Pumwani sex worker cohort in Nairobi, Kenya provide one of the best study populations for observing natural protection against HIV-1. We have learned a great deal about the mechanisms that may contribute to protection against HIV-1, but since this protection is likely multi-faceted; an in depth understanding of each factor mediating resistance will be essential.

Natural killer cells are important in the innate immune response against several viruses including HIV-1, and are mediated by killer cell immunoglobulin-like receptors (KIRs). Specific HLA class I molecules, which are better known for presenting HIV-1 antigens to $CD8^+$ T cells, are the major ligand for these receptors. KIRs have been shown

to be associated with autoimmune diseases, miscarriages, cancer, and some infectious diseases, including HIV-1. However, there are several limitations with the methods available thus far to genotype KIRs. These observations led to the two hypotheses tested in this thesis: that KIR2DL2/2DL3 genes can be genotyped using a sequence-based genotyping method, to a higher level of resolution than other available methods, and that certain specific KIR-HLA combinations are associated with resistance or susceptibility to HIV-1 infection and disease progression, through their role in mediating NK activity against HIV-1 infection.

5.1 Advantages and Limitations of Sequence-based KIR Typing

A sequence-based typing protocol was developed for typing KIR2DL2/2DL3 that for the most part, met the objectives, in that it was less labour-intensive and required less template than SSP and SSOP methods currently available, and it was able to identify new alleles. Based on exon 4 results, KIR2DL2/2DL3 could be typed not only to the gene level with just one amplification, but allele groupings could also be detected in a single sequencing reaction.

However, the need to clone to back up the PCR based sequencing approach also complicates this system. The main factor that prevented the highest resolution of allele detection, however, was that specificity problems existed with the primers for exons 5 and 6. These may have potentially been due to mutations or variation within the introns, as the sequences are not as well classified as those for exons, or it could have simply been due to primer binding dynamics permitting non-homologous binding that could not be overcome by increasing the annealing temperature or modifying reaction conditions.

Consequently, designing primers in this "unknown" region may produce unexpected results, such as non-specific amplification of other KIRs with similar intron sequences. Additionally, if any mutations were present in this location, the sequence of the desired KIR gene would be missed. The limited number of polymorphisms surrounding the regions of interest also reduced the number of potential primer sites. Then again, there is a good possibility that at least some of the variant observed could be attributed to novel alleles. However, the inability to successfully clone full-length amplicons along the whole allele length remains a problem for this approach. An alternative approach could use mRNA or cDNA sequencing, which would eliminate the intron sequences, but this would require extremely high quality samples to maintain the integrity of the RNA. New sequencing techniques will be needed to capture the full repertoire of KIR exons and genes in individuals in population studies.

Overall, the sequence-based typing method developed in this study is effective for typing KIR2DL2/2DL3 genes as well as allele groups that can be distinguished by exon 4 polymorphisms, but not the others. As well, the discovery of new alleles is a testament to the vast diversity in the KIR locus, and justifies sequence-based typing as a valuable tool for uncovering this diversity.

5.2 KIR Allele and Gene Frequencies Compared to Other Ethnic Groups

All of the KIR2DL2/2DL3 alleles and genotypes identified in the Pumwani cohort were in Hardy-Weinberg equilibrium, suggesting that this locus has not been subject to selective pressure in recent generations. While allele frequencies were not yet available for most populations, the gene frequencies, or percentages of some individuals possessing

the given gene within a population, could be compared with those of some other populations worldwide [303]. KIR2DL2, which is possessed by 55.6% of women in the Pumwani cohort (Table 10) , was present at a higher frequency than in Japanese (16%), Chinese Han (17%), Vietnamese (45%), Thai (42%), Irish Caucasian (47.4-45.5%) and Mexican populations (34-43%), and lower frequency than Australian aborigines (79%), Trinidad (64%), and Palestinian populations (62%) but at very similar frequencies compared to Argentinean Caucasians (56%), other Africans (52%), and Greeks (50%). KIR2DL3 was present in 89.7% of women in the Pumwani cohort (Table 10), which is higher than Australian aborigines (67%) and Vietnamese populations (66%), and somewhat lower than Japanese (100%), Thai (97%), Mexican (100%) and Chinese Han populations (99%), but very similar to other populations, including Argentinean Caucasians (87%), other Africans (85%), Trinidad (83%), Palestinians (85%), and Greeks (88%). These results show that KIR frequencies can vary between different ethnic groups, perhaps due to different pathogenic selective forces experienced by each population over time. The agreement of the Pumwani frequencies with other African populations also further supports that our typing system was accurate. Even more insight into the ethnic distribution of KIR may be gained once allele-level typing results become available for other populations, and once full KIR haplotypes are classified in the Pumwani cohort. This information will also be important in disease, vaccine, or functional studies that implicate HLA as well as innate and adaptive immunity.

5.3 KIR2DL2/2DL3 and HLA-C are Synergistically Associated with HIV-1 Resistance, Susceptibility and Seroconversion

The KIR alleles identified in this study were not associated with altered susceptibility to HIV-1 on their own. When pooled, the new alleles identified for both KIR2DL2 and KIR2DL3 were associated with HIV-1 resistance, but when considered at the genotype level, no associations were seen. The small sample size for these genotypes makes it difficult to identify any true associations at this level. Resolving this would require functional studies to test these alleles, expanding the sample size, or by further studies in other cohorts.

While KIR allele group genotypes were not associated with HIV-1 resistance, several interesting associations were observed when considering the broader KIR gene groupings and HLA-C allotypes. Individuals who were homozygous for KIR2DL2 were more likely to be HIV-1 positive than those with no KIR2DL2, but simply having one copy of KIR2DL2 was not detrimental. KIR2DL2 is a strongly inhibitory receptor thought to usually be present on haplotype B, which is variable in both gene and allele content, but usually contains mainly activating receptors. Traditionally, this haplotype is considered to be "activating", based on the presence of activating receptors. However, it is thought that other activating NK receptors in the LRC may be more important and dominant in NK activation than the activating KIRs, and that the main role of KIRs may lie in their inhibitory abilities. This was hypothesized because individuals exist who are homozygous for the generally "inhibitory" haplotype A, which has one activating receptor, KIR2DS4, but even this often exists as a null non-expressed variant. Thus, individuals homozygous for haplotype A often contain no functional activating KIRs, but

still have normal NK activation. It is also important to consider the expression and frequency of the KIRs in a haplotype, as well as the strength of their binding affinity and signaling abilities. For these reasons, the traditional definition of haplotype A being inhibitory and haplotype B being activating based solely on the amount of inhibitory or activating receptor genes present may not be as clear cut as previously though, as certain KIRs may play more important roles in NK activity than others, depending on the circumstances. We also studied the association of HLA-C1 and HLA-C2, due to the importance of their interactions with KIR ligands. Women with the HLA-C2 genotype had a trend towards being more susceptible to infection as well, but in this case homozygosity had no effect on susceptibility.

Individually, KIR2DL3, which is normally associated with Haplotype A, had a slightly protective effect, but KIR2DL3 homozygotes had no associations with resistance. HLA-C1 homozygotes had a trend towards being overrepresented in HIV-1 resistant women as well, but even more intriguing was the observation that in individuals who were homozygous for both KIR2DL3 and HLA-C1, there was a very significant protective association noted. The KIR2DL3/HLA-C1 homozygous genotype was present in 16.7% of resistant women but only 7.3% of HIV-1 positive women. This suggests a synergistic or epistatic interaction between these KIR and HLA genes, and that KIR2DL3/HLA-C1 homozygosity may have a role in HIV-1 resistance in the Pumwani cohort. These loci are unlinked and the groups were not pooled, therefore this was not simply an artificial amplification of significance, since the individuals tested were required to have homozygosity for both genes. Therefore, the protective effect seems to be epistatic. The association was also shown to be independent of other resistance-

associated HLA genotypes in the Pumwani cohort. In contrast, Jennes et al [286] actually found this genotype to be associated with increased susceptibility to HIV-1, in a small sample of sex workers in Côte d'Ivoire, but with a much smaller sample of patients (N=20), with the reasoning that the NK inhibition mediated by this receptor-ligand combination would result in decreased antiviral activity. However, due to their small sample size, the validity of their findings may be questioned.

Although our observation seems counterintuitive because of the apparent inhibitory effect of KIR2DL3 and HLA-C1, recent discoveries about the binding of these ligands shows that this combination is unexpectedly associated with a lower threshold for NK activation, and thus a higher liklihood of being overridden by activating receptors. Several studies have shown that KIR2DL1/HLA-C2 and KIR2DL2/HLA-C2 as well, have a stronger inhibitory signaling response than do KIR2DL3/HLA-C1 [304, 305]. The binding interaction between KIR2DL3/HLA-C1 has also been shown to be weaker but more specific than KIR2DL2 [288], and thus can be overridden by other activating receptors on the NK cell upon binding their appropriate ligands, resulting in observed faster and higher levels of IFN-γ release as well as consequent destruction of the target cell [288, 304, 305]. If this response is also observed in the response to HIV-1, and if this response can eliminate the virus before seroconversion, it would provide strong support for a functional role of KIR2DL3/HLA-C1 homozygosity in the innate immune response to HIV-1, which could contribute to HIV-1 resistance in the Pumwani cohort. Furthermore, KIR2DL3/HLA-C1 homozygosity has also been implicated in the spontaneous clearance of Hepatitis C virus [270], adding further weight to this antiviral

model. A recent study also showed an expansion of NK cells expressing KIR2DL3 in HIV-1 exposed uninfected intravenous drug users with this genotype [306, 307].

Factors that may contribute to the strength of the KIR/HLA interaction include allelic variation, expression levels of a given KIR [249], and peptides in or distal to the HLA binding groove [288]. While KIR2DL3 is thought to confer weaker inhibitory effects than KIR2DL2, it is usually located on the predominantly inhibitory Haplotype A, which also contains KIR2DL1. Thus, in a patient who possesses the KIR2DL1 ligand, HLA-C2, would have a strong inhibitory effect and prevent killing of infected cells, but conversely, HLA-C1 homozygotes would have no ligand, and therefore the target cell could be killed. KIR2DL2, like KIR2DL1, is thought to also have stronger inhibitory effects that KIR2DL3. Since stronger inhibitory signals result in decreased NK activity, and potentially less antiviral activity, this may help explain why KIR2DL2 homozygotes had a trend towards increased susceptibility to HIV-1. Since KIR2DL2 is located on haplotype B, which can potentially contain KIR2DL1 as well, the presence of these two potentially strongly inhibiting KIRs may could outweigh the effects of the activating KIRs on this haplotype depending on their expression levels, and may result in even further strong inhibition in these individuals. However, this association was only evident at the gene level and was not impacted by the individual's HLA-C allotype. Without knowing the individual's full KIR repertoire and level of expression for each KIR, the proposed mechanisms remain purely speculative.

Previously reported associations between KIR2DL2/2DL3 and HIV-1 resistance were not mirrored in the Pumwani cohort. While Jennes et al [286] showed an association of individuals heterozygous for KIR2DL2/2DL3 and lacking their HLA-C1

ligands (thus HLA-C2 homozygotes) with HIV-1 resistance, no such effect was observed in our study. One explanation for this could be that the small sample size (21 exposed seronegative and 20 HIV-1 positive) in the Jennes et al study was not sufficient for associations to have statistical power. Another potential reason for the disparity between the two studies could be due to population-level differences in the presence of other KIR genes in the haplotypes, perhaps due to the presence of KIR2DL1 which is usually present with KIR2DL3 on haplotype A but can also variably be present with KIR2DL2 on haplotype B, which can still bind to C2 and elicit a strong inhibitory effect, or other inhibitory KIRs for which their ligands were present. Additionally, differences between the populations may also exist at the allele level, if allele variants that were common in one population but not the other were to result in functional differences. It is difficult to speculate or predict the effect of other KIRs in the haplotype or allele differences at this time, without knowing which are present in resistant women from either population. To study this, new high-throughput typing technologies for the other KIRs should be developed to determine the full haplotypes and expression patterns for all of the KIR genes in an individual's genome.

When the longitudinal parameter of time to seroconversion was studied, neither KIR2DL2 nor KIR2DL3 at the gene level were associated with an altered rate of seroconversion, nor were HLA-C1 or HLA-C2. While individuals with KIR2DL3*004/005 new-e, new KIR2DL3 genotypes, and KIR2DL3*004/005 homozygotes had trends towards slower seroconversion, sample sizes were not large enough to show significance. When the KIR2DL3*004/005 new-e group was divided into those with HLA-C1 and those HLA-2, a greater protective effect was seen with C1

than C2. Similarly, protective trends were also seen with KIR2DL2*004 in patients with HLA-C1, as none of them seroconverted, but it lost some of its protective effect in individuals with HLA-C2 genotypes. The frequencies of these genotypes were also overrepresented in the HIV-1 resistant women, but the differences were not significant. Further studies in larger populations may resolve whether these observations are actually biologically relevant or due to small sample size. Somewhat contradictory to these observations of modestly protective HLA-C1 pairings to be more protective, however, was the significantly protective association of the broader KIR2DL2/HLA-C2 genotypes with increased time to seroconversion. The KIR2DL2/HLA-C2 association had larger sample sizes, and suggests that the differential HLA-C1/HLA-C2 results may have either been attributed to small sample sizes, or small functional differences may exist between alleles that are not common to that particular KIR gene. However, no protective effect was seen in individuals who were homozygous for KIR2DL2/HLA-C2, nor was the KIR2DL2/HLA-C2 genotype associated with HIV-1 resistance, suggesting that the protective effect of this genotype on seroconversion may involve a more complex mechanism than is obvious by simply its presence or absence, or may depend on other unforeseen factors such as expression or haplotype effects. Since these minor differences between the two types of analysis did exist, the results should be considered exploratory, and larger samples or functional testing would be necessary to further clarify these associations.

Another interesting observation was that individuals with either 1 or 2 copies of KIR2DL3 and/or HLA-C2, in any combination, seroconverted significantly slower than those individuals with neither of them. However, homozygosity for either or both had no

effect, nor did homozygosity for KIR2DL2/HLA-C1 have a negative effect, so it was difficult to suggest any sort of functional significance from this association.

Similar to the protective effects noted for KIR2DL3/HLA-C1 homozygosity in terms of resistance, women with this genotype also had a trend toward slower seroconversion, although it was modest in comparison. This further supports the view that the weak inhibition mediated by KIR2DL3 alleles may be contributing to anti-HIV-1 innate defense by allowing NK cells to become activated by the stronger activating receptors.

5.4 Inhibitory KIRs are Associated with Slower HIV-1 Disease Progression

If left untreated, HIV-1 positive individuals will progress to AIDS at variable rates. While the virus does play a role in this pathogenesis, the host immune system is the major variable in an individual's ability to control viral reproduction and consequently, to maintain healthy CD4 counts. An HIV-1 negative healthy individual would generally have a CD4 count of at least 800, measured in number of $CD4^+$ T cells per mm^3. Since HIV-1 infects and destroys these cells, which are critical in the adaptive immune response, CD4 counts drop as infection progresses. As a patient's CD4 count drops, it becomes less likely that their immune system will be able to defend against infections, and AIDS-defining illnesses typically appear when patients reach the threshold of CD4 counts below 200. Since the women in this study were treatment-naïve at the time of study, any differences in CD4 decline could be attributed to their own immune systems and were not influenced by antiretroviral drugs. PEPFAR-supported antiretroviral treatment starts when these women's CD4 counts drop below 200. Longitudinal analysis

was performed to measure time to CD4 counts below 200 as an indicator of progression to AIDS, and time to CD4 below 400 as another checkpoint of disease progression.

Women who were homozygous for the hypothetically more inhibitory KIR2DL2 showed slower disease progression as measured by time to CD4 below 400, and conversely women with KIR2DL3 genotypes had the inverse association, with faster progression to counts below 400. However, these genotypes did not appear to have an effect on the patient's progression to AIDS. This was an interesting role reversal in terms of protection, when compared to HIV-1 resistance, where we found that the theoretically less inhibitory KIR2DL3/HLA-C1 homozygous combination was associated with protection against infection. The observations suggest that the mechanisms of protection mediated by NKs are different for HIV-1 resistance, which was associated with potentially less KIR-mediated inhibition and thus lower thresholds for NK activation, and for HIV-1 disease progression, which was associated with potentially more inhibition and thus less NK activity.

As well, individuals with KIR2DL2*001/002/003/005 new-b alleles progressed to AIDS at a much faster rate than those without, and the less common KIR2DL2*001/002/003/005 new-a also showed a detrimental decline to CD4 below 400. When grouped, women with any of the new KIR2DL2 alleles identified in this study had a trend towards faster progression to CD4 counts below 400. This was intriguing, because the new-a and new-b alleles, which made up the majority of the new KIR2DL2 alleles group (81 out of 85 alleles), both have a major amino acid change in common: amino acid site 16, which corresponds to codon 37, is normally arginine in 2DL2, but in both 2DL2*001/002/003/005 new-a and 2DL2*001/002/003/005 new-b, this has mutated

to become proline, which is normally present in KIR2DL3. This switch of protection being attributed to lowered inhibition in HIV-1 resistance, to increased inhibition in disease progression may seem counterintuitive, but it makes sense for several reasons.

In HIV-1 positive individuals, faster progression to AIDS is strongly correlated with persistent immune activation, as measured by inflammation and increased $CD8^+$ T cell activation [308], therefore the stronger inhibition of NKs mediated by KIR2DL2 could potentially lessen this effect, and conversely, activated NKs could contribute to the potentially detrimental effects of immune activation: cytokine production, cytotoxicity, and stimulation of T cells. Stimulation of T cells would make more targets for HIV-1 to infect. Therefore, if increased NK inhibition could lower overall immune activation, this may result in slowing the spread of chronic HIV-1 infection, but greater NK activation may in turn speed up disease progression. A recent study showed, via mutagenesis experiments, that KIR2DL2 is actually able to bind HLA-C2 in addition to HLA-C1, which strongly inhibits NK cytotoxicity. Thus, strong inhibition could then be expected in KIR2DL2 homozygotes regardless of their C1 or C2 ligands. Logically then, we would expect that the slower CD4 decline to 400 observed with KIR2DL2 homozygotes in the Pumwani cohort might be related to the increased inhibition associated with this genotype. The arginine at amino acid site 16 in D1 of KIR2DL2 has been identified as the site (along with 148 in D2) that confers its binding ability to C2, and is predicted to change the angle between the two domains [288]. However, this is also the site of the mutation to proline that distinguishes the KIR2DL2*001/002/003/005 new-a and new-b alleles, from the rest of the KIR2DL2 alleles. KIR2DL3, which is less inhibitory and can only bind to HLA-C1, also has a proline at site 16, and therefore this mutation in the

KIR2DL2*001/002/003/005 new-a and new-b alleles makes them more KIR2DL3-like and may limit their inhibitory potential. This could help explain why those women with the KIR2DL2*001/002/003/005 new-a and new-b alleles rapidly declined to AIDS, in contrast to other KIR2DL2 alleles that would still bind both C1 and C2 and potentially result in high levels of inhibition.

An alternate explanation for the more inhibitory KIR2DL2 being associated with delayed HIV-1 progression is that receptors with higher binding strength to their target ligands, such as KIR2DL2, may have a lower threshold of activation and a stronger NK response if the ligand is lost, such as through downregulation of HLA-C on the target cells by HIV-1. However, HIV-1 Nef protein is able to downregulate HLA-A, and –B, but not HLA-C, so this is less of a viable theory for KIR2DL2/2DL3 [309]. Therefore, while this mechanism is possible, we predicted it may have less of an effect on the action of KIR2DL2/2DL3 than it may for KIRs that bind downregulated HLA ligands. To test this, NK cells expressing KIR2DL2 could be used in cytotoxicity assays in which infected cells would either express the HLA-C ligand or not express HLA-C. Based on the amount of activation or inhibition seen, a mechanism could be elucidated.

5.5 A Model of Inhibition and Activation for KIRs in HIV-1 Infection

While the theory that a KIR/HLA combination favouring less inhibition and lower activation thresholds is supported by current literature, the idea proposed in this study that less inhibition may contribute to less immune activation and thus slower disease progression is somewhat contradictory to other studies in some aspects but agrees in other aspects [275, 282]. Martin et al [282] looked at the KIR3DS1/3DL1 locus, and

found that the activating receptor KIR3DS1 in association with its putative ligand, HLA-B Bw4-80I, was associated with slower progression to AIDS, with the rationale that this KIR strongly binds its putative HLA ligand and triggers NK activation, resulting in destruction of infected cells. The same group also showed that cell lines expressing this KIR were able to inhibit HIV-1 replication in cells expressing HLA-B Bw4-80I . However, the mechanism for this has remained elusive, as binding studies have failed to show any interaction between this receptor-ligand pair [280]. To complicate matters, the same group also found that certain highly expressed inhibitory KIR3DL1 alleles, located at the same locus but different haplotypes as KIR3DS1, along with HLA-B Bw4-80I were also associated with slower disease progression [282]. Boulet et al then showed that this KIR/HLA combination also lowers the risk of infection in exposed individuals [284]. The rationale is that the loss or downregulation of the inhibitory receptor's ligand by HIV-1 would result in especially strong activation. HIV-1 Nef protein downregulates HLA-A, and –B, but not HLA-C, which are the major ligands for inhibitory KIRs cells [309]. Incredibly, KIR3DL1*004, which is not even expressed at the NK cell surface, was the most associated with slower disease progression [282]. To make sense of how the observations in the Pumwani cohort fit with these associations as well as their complicated functional implications, further studies will need to be carried out, taking into account whole-haplotype effects for both KIR and HLA, more conclusive proof of ligand binding, differences in signalling strength, and potential signaling differences conferred by the cytoplasmic tail.

The differential associations of KIRs in resistance vs. disease progression may stem from differences in their functional mechanisms: NKs play a more direct defensive role

in preventing infection as one of the first lines of innate defense against viruses, but they may be more important as moderators in the adaptive response or contribute to immune activation in chronic HIV-1 infection. Their influences may even differ when early or late stages of disease are considered. A model developed based on the rationale provided in 5.3 and 5.4 is summarized in Table 19.

Table 19. The potential contributions of KIR and HLA genes towards HIV-1 resistance and disease progression.

KIR2DL2/2DL3	HLA-C	Resistance/ Susceptibility	Disease Progression	Proposed Contribution by KIRs	NK Activity
KIR2DL3 homozygous	HLA-C1 homozygous (C1 or C2 for disease progression)	Resistance	Faster (KIR2DL3 genotype)	Less inhibition by weakly inhibiting KIR2DL3	More activation
KIR2DL2 homozygous	HLA-C1 or HLA-C2	Susceptibility	Slower	More inhibition by strongly inhibiting KIR2DL2	Less activation
KIR2DL2 with position 16 Arg→Pro	HLA-C1 or HLA-C2	-	Faster	Less inhibition – KIR2DL2 becomes functionally more like KIR2DL3	More activation

Section 6: Conclusions and Future Directions

For the purpose of molecular epidemiology studies, the novel sequence-based typing method reported here provided a sufficient level of resolution to detect significant associations of KIRs with HIV-1 resistance and disease progression, while improving

upon many of the limitations of SSP and SSOP typing for exon 4. If a higher level of typing resolution for both KIR and HLA associations were desired, then larger sample sizes would likely be necessary, so that the number of individuals in each group would provide enough power for statistical calculations. This study also supports recent concepts that KIR2DL2 and KIR2DL3 are alleles of the same gene [310] because everyone who was negative for 2DL3 had 2DL2 and vice versa. Importantly, this method identified eight novel allele variants, which would have been missed by any other PCR or probe based method. The further characterization of these will be important for future NK/KIR studies.

KIR and HLA are encoded on separate chromosomes and are thus not physically linked, but we have shown that they are synergistic and associated with HIV-1 resistance and progression to AIDS. This is the first epidemiological report of KIR2DL3/HLA-C1 homozygotes being associated with HIV-1 resistance, despite being implicated in protection from other viruses, as supported by functional data from in vitro influenza A virus (IAV) studies as well as epidemiological associations for HCV. This provides further evidence for the idea that NK responses depend on both KIR and HLA in synergistic manner. Thus, studies that focus on only one or the other may be missing important associations. The association of KIR2DL3/HLA-C1 homozygotes with resistance was also shown to be independent of other HLA genotypes. This is also the first study that suggests that inhibition of NK cells due to KIRs may actually slow down HIV-1 disease progression by contributing to an overall environment of decreased activation. Thus, it seems that KIR2DL2 and KIR2DL3 with their ligands probably play a different role in protection from establishing infection, or resistance, vs. fighting active

infection, or disease progression. This study also makes the point that the classical definition of the individual receptor or haplotype as either inhibitory or activating should not be the sole factor taken into account when considering its actual biological interactions and activity, because strength of interactions, relative signal strength, mutations, and presence or absence of ligands are all extremely important as well. Taking KIR into account may even help explain some of the previously identified HLA associations.

Despite these exciting associations shown between KIRs and HIV-1, many questions remain about their functional significance. As well, further efforts should be directed at developing different sequencing approaches to characterize the rest of the KIR2DL2/3DL3 exons, as well as the other KIR genes, which undoubtedly contribute to the balancing act of receptors in the activation/inhibition of NKs. Higher resolution allele typing may be valuable in future functional studies to determine differences in the specific function and/or expression of individual alleles. Pyrosequencing may be a viable option, if future generations of this technology are able to capture larger fragments of sequences, but current protocols are limited in that they produce overlap sequences that are probably too short to assemble into accurate contigs for a gene family with as many homologous repetitive regions as KIR.

It will also be crucial to determine the expression of KIRs and their ligands, as well as their frequency of expression or number of NK cells expressing them, using methods such as real-time PCR, or by flow cytometry if specific antibodies are developed, since KIR2DL2 and KIR2DL3 cannot currently be distinguished. Also, while the known ligands for KIR2DL2 and KIR2DL3 are HLA-C1, in vitro studies have shown that they

may also be able to bind certain HLA-C2 allotypes [288] or even HLA-B. Thus, alternative ligands for KIRs should also be explored.

Phenotypic and functional studies are needed to validate these genetic findings, using in vitro assays measuring cell-mediated cytotoxicity via NK activation markers such as CD69 or chromium release assays, inhibition of HIV-1 replication, and cytokine production. In addition to the surface expression on NKs and ligand-binding, less conventional roles for KIRs should also be considered, as exemplified by the non-expressed 2DL4 which has been implicated in HIV-1 resistance by an endosomal mechanism in conjunction with soluble HLA-G ligand [311], and determining the role of KIRs expressed on T cells should be explored as well.

In closing, while resistance-associated KIR genotypes were not found in every resistant individual, this was not surprising, because to date, no single or exclusive resistance-associated factor has been common to every resistant individual. However, KIRs are clearly an important piece of the puzzle, and further work to determine their function in more detail is warranted. These findings support the concept that resistance is due to a number of protective factors acting in concert. Understanding the full complement of these factors and how they interact will surely be invaluable when developing new therapies, microbicides, and vaccines.

Section 7: Publications from this Study

1. Identification of four novel KIR2DL2 and two novel KIR2DL3 alleles in an East African population (in preparation).
2. A novel sequence-based typing method for KIR2DL2/2DL3 genotyping (in preparation).
3. Killer cell immunoglobulin-like receptors and their associations with HIV-1 resistance and disease progression (in preparation).

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Section 9: Abbreviations Used

ADCC	Antibody-dependant Cellular Cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cell
APOBEC3G	Apolipoprotein B mRNA-Editing Enzyme-Catalytic Polypeptide-Like 3G
ARV	AIDS-Associated Retrovirus
CDC	Centres for Disease Control and Protection
CSW	Commercial Sex Worker
CTL	Cytotoxic $CD8^+$ T Cells
DC	Dendritic Cell
ddH20	Distilled Deionized Water
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphate
EtBr	Ethidium Bromide
EU	Exposed Uninfected
gp	Glycoprotein
GRID	Gay-Related Immune Deficiency
HAART	Highly Active Antiretroviral Therapy
HCV	Hepatitis C Virus
HGNC	HUGO Genome Nomenclature Committee

HIV resistant	HIV-1 resistant women
HIV susceptible	HIV-1 susceptible women
HIV-1	Human Immunodeficiency Virus Type 1
HIV-2	Human Immunodeficiency Virus Type 2
HLA	Human Leukocyte Antigen
HPV	Human Papilloma Virus
HTLV	Human T-cell Leukemia Viruses
IDU	Injection Drug User
IFN	Interferon
IL	Interleukin
IN	Integrase
IRF-1	Interferon Regulatory Factor-1
ITAM	Immunoreceptor Tyrosine-based Activating Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
KIR	Killer cell Immunoglobulin-Like Receptor
LAV	Lymphadenopathy-Associated Virus
LAIR	Leukocyte-Associated Inhibitory Receptor
LB	Luria-Bertani
LD	Linkage Disequilibrium
LILR	Leukocyte Immunoglobulin-Like Receptor
LTNP	Long Term Non-Progressors
MHC	Major Histocompatibility Complex
ML cohort	Nairobi sex worker cohort ("Malaya": Swahili for Pprostitute)

mDC	Myeloid Dendritic Cell
PAMPS	Pathogen-Associated Patterns
PBMC	Peripheral Blood Mononuclear Cells
PCP	Pnemocystis Pneumonia, was originally abbreviation for *Pneumocystis carinii*
PCR	Polymerase Chain Reaction
pDC	Plasmacytoid Dendritic Cell
PEPFAR	President's Emergency Plan for AIDS Relief
PRR	Pattern Recognition Receptor
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
SDF-1	Stromal Derived Factor 1
SHIV	Simian HIV
SNP	Single Nucleotide Polymorphism
SSOP	Sequence Specific Oligonucleotide Probe
SSP	Sequence Specific Primers
STI	Sexually Transmitted Infection
TB	Tuberculosis
TBE	Tris-Borate EDTA
Th1	Type 1helper T Immune Response
Th2	Type 2 helper T Immune Response
TLR	Toll-Like Receptor
Trim-5α	Tripartite Motif 5-alpha

µl	microlitre
µM	micromolar
Vif	Viral Infectivity Factor
WHO	World Health Organization