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Sarah Connolly Date

Host Factors that Modulate Risk of Heterosexual HIV-1 Acquisition and Disease Progression

By

Sarah Connolly
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Immunology and Molecular Pathogenesis

_____
Eric Hunter, PhD
Advisor

_____ _____
Susan Allen, MD, MPH
Committee Member

Paul Rota, PhD
Committee Member

_____ _____
Cheryl Day, PhD
Committee Member

Sean Stowell, MD, PhD
Committee Member

Accepted:

_____
Lisa A. Tedesco, PhD
Dean of the James T. Laney School of Graduate Studies

_____
Date

Host Factors that Modulate Risk of Heterosexual HIV-1 Acquisition and Disease Progression

By

Sarah Connolly
BS, University of Florida, 2012
MPH, Emory University, 2020

Advisor: Eric Hunter, PhD

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Abstract

Host Factors that Modulate Risk of Heterosexual HIV-1 Acquisition and Disease Progression
By Sarah Connolly

The Human Immunodeficiency Virus (HIV-1) currently infects 37.9 million people and is responsible for a total of 32 million deaths since the first cases were reported in 1981. In the absence of a cure or preventive vaccine, highly effective antiretroviral therapy and public health approaches to prevent new infections are the best means for overcoming the epidemic. Understanding how various host factors like Fc-gamma receptor (FcγR) genotype, human leukocyte antigen (HLA) haplotype, and other sexually transmitted infections (STI) modulate the risk of HIV-1 acquisition can improve prevention strategies and identify targets for an HIV-1 vaccine.

Single-nucleotide polymorphisms at the FcγRIIA and FcγRIIIA loci result in distinct genetic variants with different binding affinities for IgG antibodies. We examined two heterosexual transmission cohorts with different subtypes of HIV-1, subtype A and subtype C, and found no associations between FcγR genotype and HIV-1 acquisition or disease progression.

In a cohort of serodiscordant Zambian couples, we explored how HIV-1 adapts to host HLA pressure across the entire subtype C proteome. We found that individuals infected by a virus where the proteins Pol or Vif were already adapted to their HLA alleles experienced higher viral loads and faster CD4 decline.

Finally, owing to the fact that STIs are a known risk factor for HIV-1 acquisition, we sought to understand sociodemographic and laboratory risk factors for the bacterial STIs chlamydia (CT) and gonorrhea (NG) in a population of Zambian women at high-risk for HIV-1 infection. Through multivariate logistic regression modeling, we identified and compared associations between CT and NG with city, age, literacy, education, unprotected sex, contraception, and co-infections with other STIs. We found that risk factors were different for CT and NG, and that signs and symptoms were not associated with either infection.

The human immune system is highly variable both within and between individuals, and co-infections by other pathogens add further complexity. HIV-1 is constantly engaged in a delicate balance with numerous host factors in order to evade immune detection and maximize survival. Characterizing these host factors provides insight that might enable humans to tip the balance in our favor.

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Chapter I: Introduction

Introduction

The modern pandemic of Human Immunodeficiency Virus-1 (HIV-1) originated from a cross-species transmission of a similar virus, known as Simian Immunodeficiency Virus (SIV), found in non-human primates. Sequence analysis and molecular clock estimation suggest that transmission might have occurred sometime in the early twentieth century(1). However, HIV-1 did not enter the global spotlight until June of 1981 when the U.S. Centers for Disease Control and Prevention (CDC) published a report detailing a rare outbreak of *Pneumocystis carninii* pneumonia (PCP) in five gay-identifying men living in Los Angeles, California(2). PCP typically only presents in immunosuppressed patients, so it was a further surprise when just a few months later another article was published documenting eight cases of the rare Kaposi's sarcoma in young homosexual men in New York City(3). This publication was the first to suggest that the pathogenesis of this condition may be related to a sexual exposure(3).

As HIV-1 continued to spread, reports both domestically and internationally continued to describe cases of opportunistic infections seen in men, without an obvious underlying cause(4-6). By the end of 1982 the condition had gained the name Acquired Immune Deficiency Syndrome (AIDS)(7) and had been observed in other populations as well, such as drug users(8), Haitians(9), hemophiliacs(10), and even heterosexual women(11) and infants(12). Finally, two groups independently discovered the retrovirus responsible for this mysterious immunosuppression in 1983(13) and 1984(14), and surveillance and prevention measures were underway.

Today the conversation about HIV-1 and AIDS is much more optimistic. The virus and human immune responses against it have been, and continue to be, characterized. Advances in anti-retroviral drug therapy have limited negative side effects and have made tremendous strides in controlling viral loads and slowing disease progression, significantly extending the life expectancy of those infected by the virus. Vaccines have been produced and tested, with varying degrees of efficacy. Prevention science is developing new methods and strategies targeted to specific populations in order to reduce the number of incident infections and increase awareness of HIV-1 risk and promote regular testing. The future of HIV-1 research continues to be bright with new vaccine strategies being developed, improved model animal systems, and steps towards a functional cure and preventive vaccine.

Global HIV-1 Epidemiology

HIV entered the human population during at least two separate zoonotic transmissions from non-human primates leading to the two distinct circulating forms of HIV, HIV-1 and HIV-2(15). HIV-1 is responsible for the majority of the HIV epidemic globally and is further sub-divided into groups and sub-types(15-17). HIV-2 remains relatively geographically contained in West Africa(18). The groups of HIV-1 consist of M (main), O (outlier), N (non-M/non-O), and P where M is the group of HIV-1 responsible for the majority of the pandemic and infects the greatest number of people worldwide(15-17). Within HIV-1 group M, viral sequences can be categorized phylogenetically into a number of subtypes, sometimes called clades. These subtypes are A, B, C, D, F, G, H, J, K, and numerous circulating recombinant forms (CRFs), which are combinations of the subtypes listed(15-17). These subtypes vary in geographic distribution,

however subtype C predominates in southern Africa and the Middle East, ultimately accounting for nearly 50% of HIV-1 infections worldwide.

One of the concerns in the field at this time is whether or not a vaccine would be able to protect against multiple subtypes of virus. The enormous diversity, ability to recombine between subtypes, and high mutation rate are among the challenges to creating a broad and effective vaccine. Much of the research is centered in the developed nations of Europe and the Americas where subtype B is the predominant circulating subtype, however this subtype only accounts for approximately 11% of the cases worldwide(15). This highlights the need to focus research efforts towards subtypes of greatest disease burden, to establish broad correlates of immune protection, and to identify viral targets that are conserved across many, if not all, subtypes that would be difficult for the virus to escape by mutation.

As of 2018, the global prevalence of HIV-1 was 0.8% with approximately 37.9 million people living with HIV-1 or AIDS worldwide(19). In the same year, there were 1.7 million new infections and 770,000 deaths(19). Cumulatively, since the beginning of the HIV-1 epidemic a total of nearly 75 million individuals have been infected and the disease has claimed the lives of 32 million people(20). However, despite these large numbers, significant progress has been made. The number of individuals receiving antiretroviral therapy (ART) continues to rise, the amount of AIDS related deaths has dropped dramatically in the last decade, and the number of new infections is also decreasing, most notably in children where between the years 2010 and 2015 the incidence of new infections reduced by 50%(21).

While the overall picture of the global HIV-1 epidemic is improving, some regions are still suffering. The greatest burden lies in sub-Saharan Africa where the HIV-1 prevalence is 3.9%(19), compared to the global 0.8%, and in some regions the rate of new infections is

actually increasing(21). In many areas, women are disproportionately affected, sometimes accounting for nearly 60% of those infected(21). Fortunately, the rate of mother-to-child transmission has decreased, but now heterosexual transmission accounts for more than 80% of new infections(22). These statistics demand scientific and public health attention and stand as testimony to the desperate need for a preventive vaccine against HIV-1.

HIV Vaccine Trials

To date, there have been numerous HIV vaccine trials(23), but four of these are most frequently discussed: AIDSVAX (2 parts), STEP, and RV144. None of these trials have been tremendously successful, but they have served to guide the field closer to defining correlates of protection against HIV-1, especially the RV144 trial, which showed modest protection despite the absence of neutralizing antibodies or robustly protective T-cell responses(24). Overall, the goals of each trial were similar: to safely induce lasting, protective immunity (either cellular, humoral, or both) against HIV-1 in order to prevent incident infections; to result in reduced viral load, higher CD4 counts, and dampened markers of HIV-1 disease progression in vaccinated individuals that do become infected; and provide protection against a broad diversity of HIV-1 viral variants across subtypes.

The AIDSVAX phase 3 clinical trials took place in 1998-2000 and were in two parts, Vax003 among injection-drug users (IDUs) in Thailand and Vax004 among men who have sex with men (MSM) and high-risk heterosexual women in North America and The Netherlands(25). These trials tested the effectiveness of an alum adjuvanted recombinant HIV-1 envelope glycoprotein gp120 (rgp120) subunit vaccine (created from two subtype B strains for Vax004, and one subtype B and one subtype E strain for Vax003) to reduce the acquisition of HIV-1(25,

26). Although this study observed a robust immune response in most vaccinees, and the incidence of infection inversely correlated with peak antibody levels, this vaccine was unable to successfully prevent against HIV-1 infection or delay markers of HIV-1 disease progression(26, 27).

The STEP study was a phase 2 proof-of-concept study that began in 2004 and examined the ability of an adenovirus vector, Ad5, to act as a carrier for *gag*, *pol*, and *nef* genes and establish protective cellular immunity. The primary endpoint of this study was reduction in the incidence of HIV-1 infections, but also the researchers were interested in whether vaccinated individuals who did become infected would experience lower viral loads and thus subdued disease progression(28). Ultimately, the vaccine proved to be ineffective and the study was stopped prematurely. However, post-hoc analyses actually found that there was an increase of incident HIV-1 infections among men in the vaccinated group(28), which was a shocking finding for the HIV research community. Differences were thought to be based on pre-existing immunity to the Ad5 vector, gender, and circumcision status, and brought into question the feasibility of using a vectored vaccine as well as targeting a vaccine to generate a cellular, rather than humoral, immune response.

The RV144 trial in Thailand from 2003-2006 used a prime-boost vaccination strategy, and resulted in the most success seen from an HIV vaccine trial to-date. The prime was a canarypox vector carrying the HIV-1 genes *env*, *gag*, and *pro*. The boost used the rgp120 from the Vax003 trial that was bivalent for subtypes B and E. The incidence rate in the vaccinated group was 31% lower than in the placebo group, a modest but encouraging finding(24). Because of the lack of neutralizing antibodies elicited, failure to reduce viral load or increase CD4 count in vaccinees that became infected, and overall limited understanding of the immune mechanisms

behind this observed protection, the field has continued trying to decipher the true correlate of protection from the RV144 trial. Many scientists argue that rather than exhibiting a neutralizing effect, the antibodies are facilitating effector functions, for example in the form of antibody-dependent cellular cytotoxicity (ADCC)(29).

The results of these trials are mixed, with some trials exhibiting no effect, some appearing to increase the risk of infection, and others demonstrating modest protection. The variability in outcomes, and often to the surprise of scientists, has reinforced the incompleteness of our understanding of the immune mechanisms responsible for controlling and preventing HIV-1 infections. There is much work remaining to uncover and measure the multiple factors responsible for these results, and how best to induce the desired immune profile with a safe vaccine.

Strategy for Reducing Transmission in Heterosexual Couples

As researchers continue to make progress towards developing a vaccine and improving other preventive biomedical tools (such as gels, films, and vaginal rings) the duty to prevent new infections lies largely in the hands of public health professionals to raise awareness, educate, test, and link individuals to care in the communities around the world that need it most. A few existing strategies include structural and behavioral interventions such as increasing access to counseling, testing, and medical care, and providing male and female condoms. Other approaches utilize currently available tools to reduce the risk of acquiring or transmitting the virus through using ART in high-risk uninfected groups, known as Pre-Exposure Prophylaxis (PrEP)(30), voluntary circumcision programs, or initiating treatment in infected individuals as

soon as infection is detected which reduces viral load and prevents further transmission, known as Treatment as Prevention (TasP)(31).

Another example of a successful intervention has been the development and implementation of Couples' Voluntary Counseling and Testing (CVCT). Dr. Susan Allen and the Rwanda-Zambia HIV Research Group (RZHRG) pioneered CVCT through Project San Francisco (PSF) beginning in 1986 in Kigali, Rwanda. In 1994, the effort expanded to include Lusaka, Zambia through the Zambia-Emory HIV Research Project (ZEHRP). This method of counseling couples together increases awareness of HIV-1 serodiscordance, increases condom use(32), and ultimately reduces transmission between serodiscordant couples to 7-8% per year, contrasted to 20-25% transmission without counseling(33). Counseling couples together has been critical for raising awareness about the possibility of discordance, which represents over 20% of couples in Zambia(34), and encouraging more consistent use of condoms by educating and discussing this method of prevention with both partners simultaneously. As a result of CVCT's dramatic success, it has become the standard of antenatal care in Rwanda and has captured the attention of numerous other countries that now look to Rwanda for training and technical assistance in this public health best practice(35).

HIV Disease Progression

After becoming infected with HIV-1, many individuals experience symptoms of acute retroviral syndrome, which are similar to those of the flu or mononucleosis and typically onset about one to two weeks post-infection. Patients at this stage present with symptoms such as fever, sore throat, and malaise, among others, which resolve after several days to weeks(36-38). Following this brief period of visible disease, the virus enters latency and the infection begins the

largely asymptomatic chronic phase that persists for several years. The diagnosis of AIDS, or stage 3 HIV infection, is primarily characterized by severe immunosuppression, measured by a $CD4^+$ T cell count less than 200 cells per microliter of blood(39).

In addition to the numerous viral factors that affect HIV disease progression, many host factors may influence the progression of disease as well, such as expression levels of co-receptor and innate antiviral defense proteins, and variations in genetic sequences of chemokines and other host restriction factor genes(40). Some ways to prolong the chronic phase and delay reaching the stage of AIDS involves maintaining a healthy lifestyle, but most importantly consistently taking ART. Not only has ART been shown to reduce the progression of HIV and maintain higher $CD4^+$ T cell counts, but by significantly lowering the viral load ART dramatically reduces the likelihood of transmitting the virus to someone else and is the foundation of the TasP concept(41).

HIV Viral Characteristics & Life Cycle

HIV-1 is part of the virus family *Retroviridae* and of the genus *Lentivirus*. Lentiviruses are characterized by the long incubation period before onset of disease, as is typical of clinical HIV-1 infection. When HIV-1 was first isolated, scientists mistakenly believed it belonged to the recently discovered family of human T-cell leukemia viruses (HTLV), although this new virus was distinct from previously discovered viruses in this family it was thought to be a novel HTLV virus, HTLV-III (13, 14).

HIV-1 contains two copies of a single-stranded, positive-sense RNA genome that codes for structural, essential replicative, and accessory regulatory proteins. Each virion is packaged with the enzymes protease, integrase, and reverse transcriptase, and is enveloped, meaning each

virion is surrounded by a phospholipid bilayer derived from the plasma membrane of the previously infected host cell during the viral budding process. The viral life cycle involves the following steps: attachment, entry, reverse transcription, integration, replication, assembly, and egress. Each of these steps is met with a response from the host's immune defenses, and antiretroviral drugs have also been targeted to intervene at many of these steps. The next sections will cover each step of the viral life cycle, the version of host defense employed during that stage, and will discuss strategies the virus has developed to overcome these host responses. HIV infection is very much an arms race between the virus and the immune system, and science has also entered this race.

Viral Attachment and DC-SIGN

The first step of the viral lifecycle is attachment, which begins with the HIV-1 surface glycoprotein, a trimer of gp120 molecules, binding to its receptor, CD4, on the surface of an immune cell. CD4 is mainly expressed on helper T-lymphocytes but can also be found on the surface of numerous other immune cells such as macrophages, NK cells, and even neutrophils(42). However, the main targets for HIV-1 infection are T-helper cells and macrophages. Recent research has shown that gp120 has specific affinity to the integrin $\alpha_4\beta_7$ and on certain T cells this integrin is in a complex with CD4. In their study, these T cells appeared more highly susceptible to infection, which raises questions about the role of $\alpha_4\beta_7$ in viral attachment(43). Furthermore, while these cell subsets may be more susceptible, it has been shown that blocking $\alpha_4\beta_7$ does not inhibit infection or viral replication, suggesting that this receptor is not essential to the virus life cycle(44). Most recently however, *in vitro* experiments report that most HIV virions and gp120 glycoproteins appear to be poor ligands for the $\alpha_4\beta_7$

integrin(45), and so the case remains open for the exact role and extent to which this molecule influences HIV infection and cellular susceptibility.

Another important manner in which virions attach to cells is by the C-type mannose binding lectin present on the surface of dendritic cells (DCs), known as dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin, or more simply, DC-SIGN. The first hints of this interaction were observations showing that DCs pulsed with virus increased the infection of T cells, but did not themselves become infected(46, 47). Further studies began to demonstrate that the virions were transported on the surface of mature DCs or internalized in special intracellular compartments distinct from the endolyosomal pathway, and were then infecting the T cells in *trans* at the virological synapse(48, 49). This was in contrast in immature DCs which could become infected and then infect T cells *de novo* by the production of new virus(48, 49). Soon it was discovered that DC-SIGN was the DC surface protein responsible for the capture of HIV virions(50, 51), and that it resulted in the rapid viral internalization into low pH nonlysosomal compartments which preserved the viral infectivity and enabled the *trans*-enhancement of T cell infection(52).

This is an interesting strategy of the virus because DCs are among the first cells present at the site of infection and are migratory in nature. HIV exploits this feature to rapidly traffic itself to the secondary lymphoid organs where it will encounter vast numbers of its preferred target, $CD4^+$ T cells. Furthermore, DCs are the perfect cell to hijack for this purpose because as professional antigen presenting cells (APCs), they frequently interact closely with $CD4^+$ helper T cells at an immunological synapse to initiate an immune response. DCs also provide the necessary signals to activate the T cell, which makes the T cell more susceptible to HIV infection and results in more efficient replication of the virus. Finally, because the virion is not

internalized in the endolysosomal pathway, its antigens are not processed and loaded in the MHC class II molecules on the DC, and because the DC itself is not becoming infected the antigens are neither loaded onto MHC class I molecules. Without MHC presentation by the DC, the T cell does not encounter the antigen, and the initiation of an immune response through antigen presentation is avoided. Thus, the virus has effectively evolved to use the DC-SIGN receptor to safely hide itself in specialized compartments until it reaches the lymph node where the DC does the work of *trans*-infecting T cells through a virological synapse.

Attachment via FcγRs

Another mechanism of *trans*-infection similar to DC-SIGN, which we explored, is through the attachment of virus-antibody immune complexes (ICs) to Fc-gamma receptors (FcγRs) and the subsequent trafficking of virions in this manner to secondary lymphoid organs. In general, FcγRs are found on the surface of a variety of immune cells, bind to the constant Fc-region of gamma-immunoglobulins (IgG) and direct cellular effector responses. In this way, FcγRs are a critical bridge between the humoral and cellular immune responses. Within the isotype IgG, there are four subclasses, IgG1, IgG2, IgG3, and IgG4, which differ slightly in the Fc-region and have different binding affinities for FcγRs(53). Among the relevant locations that IgG antibodies are found include the serum, cervicovaginal secretions, seminal secretions, and rectal secretions(54).

There are seven known FcγRs in humans: FcγRI (CD64), FcγRIIA (CD32a), FcγRIIC (CD32c), and FcγRIIIA (CD16a) are activating FcγRs; FcγRIIB (CD32b) is inhibitory. FcγRn is a neonatal FcγR, which is involved in transporting and recycling immunoglobulins(55), and FcγRIIIB (CD16b) is a glycosylphosphatidylinositol (GPI) anchored protein which mediates

phagocytosis only in the presence of co-expressed complement receptor type 3 (CR3, CD11b)(56-58). The activating receptors either contain an immunoreceptor tyrosine-based activation motif (ITAM), or associate with another protein subunit called the FcR-common γ chain (FcRγ) which contains this motif. The single inhibitory receptor contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its intracellular domain. The ITAM or ITIM modulates, either positively or negatively respectively, the effector function of the cell following engagement of the FcγR. These diverse functions could include antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular virus inhibition (ADCVI), antibody-dependent cellular phagocytosis (ADCP), cytokine production, degranulation, antigen presentation, B-cell activation, and complement-dependent cytotoxicity, as well as modulation of antibody half-life and biodistribution throughout the body(59, 60).

FcγRI is considered a high-affinity activating FcγR because it requires the binding of only one monomeric IgG to initiate function. FcγRIIA and FcγRIIIA are low-affinity activating receptors meaning they require the cross-linking of multiple FcγRs on the surface of a single cell before triggering a cellular response(61). FcγRs can be cross-linked by the binding of an immune complex (IC) to the cell surface, as may be the case with HIV virions bound in complexes by host antibodies. Since most cells simultaneously express activating and inhibitory receptors, it is the balance of these signals that sets the threshold for cellular function. Other factors in the immune system can modulate this response, such as complement proteins and cytokines, by altering the cellular expression of activating or inhibitory receptors on the surface of a cell(62). For example, in a state of inflammation cytokines would be produced in response to the immunological insult, then these cytokines would act on other cells to upregulate the expression of activating receptors in order to escalate the local immune response. During the dampening

phase of the immune response, immunomodulatory cytokines would then act on these cells to again alter the FcγR expression profile, perhaps this time increasing the expression of inhibitory receptors or decreasing the expression of activating receptors.

Diversity at the Human FcγR Locus

Most of the genes for the human FcγRs are clustered nearby one another on chromosome 1(63). One mechanism that creates diversity due to the gene proximity at this locus is copy number variation (CNV). CNV of the FcγR genes occurs during Nonallelic Homologous Recombination (NAHR) between segmental duplications(64). CNV is observed in FcγRIIIA, FcγRIIIB, and FcγRIIC, but not in FcγRIIA or FcγRIIB(65, 66). In studies of CNV at FcγRIIIA, more than 90% of the subjects expressed only two copies(65, 67). CNV is an important inter-person difference because reduced copy number at FcγRIIIA and FcγRIIIB has been shown to be associated with systemic autoimmunity and systemic lupus erythematosus (SLE)(68-71). It has also been shown to be a risk factor for sarcoidosis susceptibility(72), and there is some debate about which locus, FcγRIIIA or FcγRIIIB, plays a role in CNV associated with rheumatoid arthritis(71, 73, 74).

Furthermore, three of the FcγRs have polymorphic variants that differ among individuals and confer altered binding affinity for IgG. FcγRIIA has two allele variants that differ by a single nucleotide polymorphism (SNP) resulting in an amino acid substitution at position 131; the allele for histidine at this position (131H) results in a higher affinity for IgG1 and IgG2 than the allele coding for arginine (131R)(55, 61, 75). Interestingly, the prevalence of FcγRIIA alleles has also been shown to vary between ethnic populations(76). FcγRIIIA also has two allele variants differing by a SNP that causes an amino acid substitution at position 158; the allele encoding

valine at this position (158V) results in a higher affinity for IgG1, IgG2, IgG3, and IgG4 than the allele for phenylalanine (158F)(55, 61, 77). Finally, FcγRIIIB has two variants that result in different glycosylation patterns(61, 78). These polymorphisms create genetic diversity among the population at these loci and could potentially affect the way an individual responds to an infectious insult or a vaccine.

Polymorphisms in FcγRIIIA assumed greater importance following the Vax004 HIV vaccine trial where it was observed that individuals possessing the homozygous high affinity FcγRIIIA genotype, 158VV, became infected more quickly after vaccination than individuals with the homozygous low affinity genotype, 158FF, or those receiving the placebo vaccine; in this study no effect was observed based on FcγRIIA genotype (79). To explore the potential cause for this, the researchers then investigated the cellular effector functions of individuals with the various genotypes. In the case of both FcγRIIA and FcγRIIIA, individuals with homozygous high affinity genotypes, 131HH and 158VV respectively, exhibited higher levels of ADCVI than homozygous low affinity genotypes, 131RR and 158FF respectively. However, despite this increase in effector function, a Cox proportional hazard model resulted in higher hazard ratios for HIV infection in those with high affinity genotypes following vaccination in the Vax004 trial(80). Finally, FcγRIIA genotype has been linked to HIV-1 disease progression where individuals with the homozygous low affinity genotype, 131RR, experience a faster decline in CD4 count and faster progression to AIDS than those with homozygous high affinity or heterozygous genotypes(81). Both of these studies were conducted in cohorts of predominantly men who have sex with men and are therefore limited in their ability to inform the role of FcγR genotype in women or in the context of heterosexual transmission.

Viral Entry

After the initial engagement of gp120 with CD4, a conformational change occurs in the envelope protein complex that exposes another site on gp120 that is then able to bind to a chemokine receptor on the target cell, either CCR5 or CXCR4. This two-receptor binding strategy results in a more stable attachment of the virion to the cell surface. Binding of the co-receptor by gp120 induces conformational changes in gp41, a second HIV-1 glycoprotein trimer, such that its fusion peptide moves to penetrate the membrane of the cell and begin the fusion process bringing the cell membrane together with the viral envelope. Once fusion is complete the viral capsid is free to enter the cytoplasm of the host cell, where during the next step of the lifecycle, reverse transcription, the capsid is disassembled.

Several host factors are at play following viral entry but preceding reverse transcription. One of these is a ubiquitin E3 ligase, tripartite motif protein 5α (TRIM5α), which binds to the HIV-1 capsid and enforces cross-species restriction and is responsible to some degree for viral tropism. It has been shown that human TRIM5α is unable to restrict HIV-1, but that Rhesus monkey TRIM5α (RhTRIM5α) and other Old World Monkey TRIM proteins are able to restrict infection by HIV-1(82, 83). The complete mechanism through which TRIM5α restricts the virus is unclear, however it appears to act by accelerating the disassembly and degradation of the viral capsid protein (CA) in the cytosol in a proteasome independent manner. This premature degradation inhibits the replication cycle of the newly entered virus(83, 84).

Another intracellular mechanism that responds to HIV-1 CA is Cyclophilin A (CypA), which can be found in DCs and induces a type-I interferon anti-viral state following detection of intracellular HIV-1(85). It has been shown that CypA is a cofactor that can assist TRIM5α in restricting HIV-1 in non-human primate cells, but that this interaction is not always necessary for

TRIM5α to function(86, 87). Furthermore, studies have demonstrated that in human cells CypA regulates the infectivity of HIV-1, actually making it more infectious, and that it is packaged into virions exiting a cell(88-90). Indeed, recent experiments suggest that in human cells CypA may actually protect the HIV-1 capsid from TRIM5α(91). Thus, the impact and role of CypA varies from one species to another and likely plays a role in viral host selection and tropism.

Reverse Transcription

Following viral entry, the next step in the HIV-1 lifecycle is reverse transcription, which takes place in the host cell cytoplasm. HIV-1 virions contain a unique enzyme called reverse transcriptase. This enzyme has the ability to convert positive-sense RNA into a double-stranded DNA copy. This is the opposite of traditional molecular biology dogma where DNA is transcribed into RNA, which is then translated into protein. This property of generating DNA from an RNA template is what lends the name "reverse" transcriptase to the enzyme. One important characteristic of this process is that reverse transcriptase is error-prone because it lacks the proofreading capability of typical DNA polymerases. The frequent errors, approximately one per genome per replication cycle, result in numerous mutations, allowing the virus to quickly evolve to evade immune pressure, develop resistance to antiretroviral drugs, diversify into a quasi-species, and become an elusive vaccine target. Since the virion is packaged with two copies of the RNA genome, if a break in the RNA is encountered while reverse transcribing the first copy the reverse transcriptase enzyme can exercise copy choice and switch to the other strand of RNA to continue synthesizing the single continuous cDNA copy.

If two genetically different HIV virions, such as viruses of different subtypes, infect the same host cell, virions containing an RNA from each can be produced and then recombination

can occur during reverse transcription. Therefore, in addition to the error-prone reverse transcriptase, recombination is another way of generating HIV viral diversity and yet another obstacle to developing a broadly effective HIV-1 vaccine.

One example of a host factor that interferes with HIV-1 replication during the reverse transcription step is a family of proteins called apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G/B/D/F/H, or APOBEC(92). APOBEC proteins are single-stranded DNA cytidine deaminases that are packaged into the virion and work during reverse transcription after entry into a new target cell. They function by removing the amine group from cytosines in the negative-stranded DNA copy (cDNA), thus converting this nucleotide to a uracil. Then during positive strand synthesis, this U is read as a thymine and encodes for an adenine instead of the original guanine in the new DNA strand. The result of this is multiple G to A mutations, which causes the cell to recognize this transcript as aberrant and leads to its degradation. Alternatively, if it is not degraded and successfully integrates into the host genome, it will fail to produce effective, virus-producing, transcripts because of its hypermutated state, with introduced missense mutations and premature stop codons(92).

Another potential mechanism by which APOBEC restricts reverse transcription is by directly interfering with the reverse transcriptase enzyme. It is speculated that APOBEC proteins bind to the viral RNA itself and physically block the processivity of the enzyme so that the cDNA cannot be completely synthesized(93). In an attempt to evade the restriction of APOBEC by its various mechanisms, HIV-1 encodes Viral Infectivity Factor (Vif) to antagonize APOBEC. Vif targets APOBEC proteins for degradation in the proteasome before they can be incorporated into new virions(92).

SAM domain and HD domain-containing protein 1, SAMHD1, is another host factor that intervenes during reverse transcription. SAMHD1 is thought to function by depleting dNTPs, reducing the amount available to below a critical level necessary for reverse transcriptase to synthesize cDNA(92). HIV-1 does not encode a viral protein to antagonize the action of SAMHD1, however HIV-2 responds with an accessory protein, Vpx, which degrades SAMHD1.

Finally, there is an innate immune sensor capable of early detection of the reverse transcribed genome. This is an intracellular pattern recognition receptor (PRR) located in the cytosol called cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase (cGAS). cGAS leads to the production of cGAMP which interacts with the adaptor protein Stimulator of Interferon Genes (STING) and initiates an antiviral immune response in the form of type-I interferons and cytokine production(94).

Integration

The end product of reverse transcription is a linear double-stranded DNA in which regions at the 5' and 3' ends of the RNA genome have been duplicated to generate long terminal repeat (LTR) regions. This longer-than-genome-length double-stranded DNA copy is associated with a few capsid proteins and the viral integrase enzyme to form the pre-integration complex (PIC). This is transported through the nuclear pore into the cell nucleus where the viral integrase mediates covalent joining of the viral genome with the host chromosomal DNA. Once integrated, this viral-encoded segment of the host genome is referred to as the provirus.

A host factor that is suspected of interfering with nuclear transport is myovirus resistance protein 2 (MxB). This restriction factor is induced by interferon and likely acts in the stage

between reverse transcription and integration through interactions with the viral capsid. MxB suppresses the nuclear accumulation and chromosomal integration of the viral cDNA(95, 96).

The provirus, which because of its LTRs, encodes the necessary signals to program RNA synthesis by the host cell RNA polymerase can now be actively transcribed or in some instances remain transcriptionally silent. It is this latter latent state that allows HIV-1 to remain dormant for many years, and also which makes curing this infection extremely difficult because infected cells are not always producing virus and can therefore escape immune detection during latency.

Replication

Within the Long Terminal Repeat Regions (LTR) of the provirus lie many important regulatory and host-mimicking sites to aid in initiation of transcription and to help disguise the HIV-1 proviral transcripts as host RNA, which encourages the cell to manufacture HIV virions and not trigger an immune response(97). These include promoter and enhancer elements, a region that programs the addition of a 5' cap on the newly transcribed RNA, a 3' polyadenylation signal, and a binding site for the host transcription factor NFk-B, which plays an important role in initiating proviral transcription in activated immune cells. Starting from the proviral DNA genome, the replication process follows the canonical molecular biological pathway of being transcribed into RNA, which is transported to the cytoplasm, and then translated into protein. While full-length RNA transcripts can function both as mRNA and new RNA genomes, some are processed and spliced into subgenomic mRNAs.

The accessory proteins of HIV-1 have various different functions throughout the viral life cycle, however two essential regulatory elements are especially important during this stage. The first protein is HIV trans-activator of transcription (Tat), a regulatory factor that is one of the first

proteins made and binds to the trans-activation response element (TAR) region of newly synthesized RNA, and helps to stabilize the RNA polymerase II complex and improve processivity to generate full-length RNA transcripts(97). The second important early protein is the regulator of expression of virion proteins (Rev), which binds to the Rev response element (RRE) on the mRNA and assists in the transport of long mRNA transcripts from the nucleus to the cytoplasm, thereby avoiding the RNA splicing machinery(97).

Assembly & Egress

Once the viral proteins are formed, they must be transported through the cell and assemble into new virus particles that can exit and move onto a new target. The method of egress varies depending on the cell type that is infected. In the case of T lymphocytes, the new virions assemble at the plasma membrane and bud from the host cell. In the case of macrophage infection, virions assemble on membranes of, and collect in, a virus-containing compartment (VCC) that is an interior extension of the plasma membrane(98). The structural protein Gag promotes the initiation of the assembly process after it is transported to the cell plasma membrane, localizing in regions of cholesterol-enriched microdomains, called lipid rafts(99). The Gag polypeptide is directed to tightly associate with the inner leaflet of the plasma membrane in the lipid rafts via a myristyl switch; this involves the exposure of an N-terminal myristic acid moiety following interaction with phosphoinositol residues in the membrane. Multimerization of the Gag protein brings multiple myristic moieties into close proximity and anchors the assembling polypeptides to the membrane(100).

Viral glycoproteins, anchored by gp41 in the plasma membrane, also collect at the assembly site and viral RNA is packaged into the forming virion. To exit the cell, the virion then

buds from the cell surface of the T lymphocyte and carries the plasma membrane of this cell as its envelope. The terminal budding and excision process is mediated by hijacked host machinery called the endosomal sorting complexes required for transport, or ESCRT(101). In an infected macrophage, virus may bud into interior extensions of the plasma membrane allowing later transmission to occur at a virologic synapse between the macrophage and a new target $CD4^+$ T cell(102). This method of cell-to-cell transmission from macrophages to T cells is especially relevant in the context of cell-dense secondary lymphoid organs where there is a large population of target cells available.

One interesting aspect of HIV-1 budding is that the newly budded virion is not immediately infectious. In order to become infectious, the particle must undergo maturation during which time the Gag polyprotein must be cleaved by the protease enzyme that is packaged in the budding virion(101). Once the cleavage of this protein occurs the viral matrix, capsid, and nucleocapsid then rearrange into their mature form, resulting in a virion capable of infecting a new host cell.

Human cells have developed a way to disrupt viral egress through the use of a host factor called tetherin, also known as BST-2 or CD317. This host factor is an interferon-stimulated protein that is upregulated during an anti-viral host state. It functions by literally tethering the virions to the host cell surface so that they cannot be released from the cell and infect new host cells(103). In macrophages, tetherin localizes to the VCC and is likely the protein that is responsible for tethering the virions into this intracellular compartment(104). To overcome this host defense, HIV-1 viral protein U (Vpu) is an HIV-1 accessory protein that antagonizes tetherin so that the virions can regain the ability to exit the cell(105, 106).

Another related function of Vpu is its interaction with CD4 in the endoplasmic reticulum of infected cells where it triggers CD4 degradation(107, 108). Similarly, the HIV-1 accessory protein, Negative Factor (Nef) also is able to downregulate the cellular expression of CD4(109). This could be advantageous to the virus in several ways; first it may prevent a lethal form of superinfection in a single cell and prevent recombination in a way that may yield a defective virion. Second, it could alter cellular activation patterns by removing this key immune receptor from the surface. And finally, because during the budding process the newly formed virion may interact with its receptor, CD4, on the surface of the infected cell and in this way CD4 would function similarly to tetherin by inhibiting the egress of virions and limiting their ability to find and infect a naïve target cell. It would be of no benefit for the virus to re-infect an already infected cell, therefore degrading and downregulating the expression of CD4 is a strategy of the virus to improve its release from cells.

Yet another host restriction factor that plays a role in the transmission of virions is the host transmembrane protein SERINC5(110). This protein localizes on the host cell plasma membrane and gets incorporated into newly formed virions. Once inside the virion this host factor can impair the ability of that virion to penetrate and infect a new host cell. The virus combats this factor again through the utilization of Nef, which acts by preventing the incorporation of this protein into new virions and increases the particle infectivity of the virus(111).

Defensins

Small host antimicrobial molecules called defensins have been shown to protect against HIV-1 infection. Defensins are produced by epithelial cells and leukocytes and are cysteine-rich

cationic peptides that are an important innate immune defense against many bacterial, viral, and fungal infections. Two types of defensins have been shown to participate in HIV-1 antiviral defense, α-defensins and β–defensins. Interestingly, defensins have a dual-action approach by targeting both the virion and the target cell directly and their expression does not require the replication of HIV-1(112).

Human α-defensins have lectin activity, allowing them to bind to carbohydrates on HIV gp120 and CD4 with high affinity(113). This activity is strongly attenuated by the presence of serum through the competing interactions with other positively charged serum proteins. However, in the absence of serum, such as at the mucosal surfaces, these defensins could be critical for binding to virions and disrupting the viral envelope or binding to the surface glycoproteins(112).

Defensins can also alter the host cell response by signaling through cell-surface receptors, which can interrupt the transcription of viral RNA or block the PIC from entering the nucleus(112). The α-defensins can also downmodulate the surface expression of CD4 in $CD4^+$ T cells(114). In this way defensins are inhibiting the earliest events of HIV-1 infection by downregulating the expression of the critical receptor, by disrupting the viral envelope, and by binding to both gp120 and CD4 perhaps interfering with their interaction with each other.

Human β–defensins inhibit HIV-1 infection in the intracellular compartment in a dose-dependent manner by inhibiting the accumulation of early reverse transcribed DNA products(115). A host-cell modification by β–defensins is the downmodulation of HIV-1 co-receptor, CXCR4, in T cells(116). It is speculated that due to the high presence of β–defensins in the oral cavity, this could be a reason for the low transmission of HIV-1 via the oral route(116).

Similar to the FcγRs, the loci for the defensins are polymorphic and vary between individuals. They also are known to have CNV, which alters the amount of RNA copies of each gene, but has not been definitively linked to altered levels of protein expression(112). A SNP in the untranslated region of the human β–defensin gene was associated with risk of perinatal HIV-1 transmission from mothers to children in an Italian pediatric cohort(117). Finally, both α- and β–defensins are abundant in breast milk and have been shown to be associated with reduced risk of intrapartum and postnatal HIV-1 transmission(118-120).

MHC and CTL Escape

Another important set of host factors that could play a role at many different stages of the viral replication cycle, and that varies between individuals, is the human major histocompability complex (MHC). The MHC locus is a highly variable region of the human genome and is responsible for encoding the human leukocyte antigen (HLA) proteins. There are two classes of MHC proteins that participate in antigen presentation, class I and class II, and each specialize in presenting peptides from different antigenic sources to T cells. MHC class I molecules present short peptides, 8-10 amino acids in length, derived from intracellular sources. These peptides, which bind into a groove on the surface of the HLA molecule, can be cellular, self, proteins that are trafficked to the cell surface for monitoring of normal cellular activity by other components of the human immune system. $CD8^+$ T cells, also known as cytotoxic T lymphocytes (CTLs), and Natural Killer (NK) cells are key to this function. Alternatively, peptides can be derived from the proteins of intracellular pathogens, such as viruses, that have invaded the body. They are presented on MHC class I molecules to alert the immune system to foreign pathogens and initiate a cascade of immune responses.

The other type of MHC, MHC class II, presents slightly longer peptides, 15-24 amino acids in length, and displays these for $CD4^+$ T cells. The source of antigen for MHC II presentation is extracellular and only certain types of cells, known as professional antigen-presenting cells (APCs), carry out this form of antigen sampling and presentation. These professional APC cell types are macrophages, DCs, and B-cells and they sample external antigens from the local milieu, process them, and present them to the helper T cells.

HLA alleles are inherited and expressed in a co-dominant fashion, meaning that each allele that is inherited (one maternal and one paternal) is expressed equally on the surface of cells as MHC molecules. There are 3 genes for MHC I: HLA-A, HLA-B, and HLA-C; of which, HLA-B is the most polymorphic. There are also 3 genes for MHC II: HLA-DP, HLA-DQ, and HLA-DR. In the event that an individual inherits a different allele from each parent, co-dominant expression dictates that they will then express a total of 12 different MHC molecules on the surface of their cells.

HLA alleles, especially those encoding MHC class I, are important in the context of HIV-1 infection because viruses are intracellular pathogens. Once the virus enters the cell, the cell can begin to process viral peptides and display them on the surface, alerting and activating the rest of the immune system. Certain MHC I alleles, such as HLA-B*1302, HLA-B*2705, and HLA-B*5701 to name a few, have been associated with less severe HIV-1 disease progression due to their improved ability to present HIV-1 peptides(121). On the other hand, there are MHC I alleles that are associated with faster HIV-1 disease progression and higher set-point viral load, such as HLA-B*5802, HLA-B*1801, and HLA-B*3502(121). The variation between alleles most often results in different amino acid residues being present in the peptide-binding pocket; even slight changes in amino acids in this region alter the specificity with which certain viral

peptides will be bound. Protective alleles are likely more efficient at presenting HIV-1 peptides, or are able to present peptides of higher importance such that a targeted immune response against those proteins exerts a stronger selective pressure. Those alleles associated with higher viral load set point and faster disease progression likely carry polymorphisms that result in lower binding efficiency for key HIV-1 peptides.

In effort to evade this aspect of the host immune response, the HIV-1 accessory protein Nef, downregulates the surface expression of MHC I. The degree of downregulation depends on the subtype of HIV-1, however the goal is the same – to avoid presentation of viral antigens to the immune system(122). While the advantage of not presenting viral antigens via MHC I reduces the chance of detection and killing by CTLs, the immune system utilizes NK cells to patrol for cells failing to express the appropriate amount of MHC, an indicator of viral interference that also results in killing of the cell. To strategically avoid killing by both CTLs and NK cells, evidence suggests that HIV-1 Nef selectively downregulates certain MHC molecules, such as HLA-A and HLA-B which may be better at displaying peptides of HIV-1 origin, but preserves expression of HLA-C so that the immune system's NK cells do not recognize the cells as infected(123).

Transmission of HLA-Adapted Virus

On a population level, HIV encounters an enormous variety of HLA haplotypes that continually exert selection pressure on the virus, so that variants that have reduced susceptibility to the immune response and higher viral load thrive and can be successfully transmitted to the next host. While within a given host, these escape mutations may be beneficial to the virus by improving its ability to evade the host's immune response, after transmission to a new host with

a different HLA haplotype, these same adaptations instead may impart a fitness cost to viral replication(124). In fact, individuals infected by a virus with many escape mutations in Gag epitopes that are not recognized by the new host's HLA alleles, have lower plasma viral loads(125-127). This highlights the delicate balance that exists between replicating and evading the host immune response, which in some cases forces the virus to sacrifice replication capacity in order to survive in the current immune environment.

In instances where the escape mutation carries a high fitness cost, the polymorphism often reverts to the original pre-escape sequence upon transmission to a new host lacking the restrictive HLA allele(128). However, not all escape mutations have a fitness cost, and these risk being maintained in the viral sequence and transmitted to subsequent hosts(128). Throughout time, and over many transmission events within a regional population, the viral genome will accumulate these low-fitness-cost HLA-escape polymorphisms which will eventually become fixed in the circulating consensus sequence(129). This fixation is apparent when mutations in effective subdominant CTL epitopes appear in the consensus sequence of viruses circulating in regions with high frequencies of the restrictive HLA allele(130). One example are the subtype C consensus-encoded escapes from HLA-B*1503, which is a common HLA allele in regions where subtype C HIV-1 circulates, but not in regions where subtype B is the primary circulating variant(131). The degree of HLA adaptation varies between cohorts and regions, even within the same HIV subtype(132).

In a study of HLA class I driven escape in Zambia, 77.3% of the escape polymorphisms identified corresponded to some HLA class I allele present in the Zambian study population, while only 25.8% of the escape mutations were associated with the HLA class I alleles in the current host(126). The archiving of escape mutations in the circulating consensus sequence

presents a problem for newly infected individuals. In this same study, a median of 18.8% of transmitted polymorphisms were already adapted to the new host's HLA class I alleles from the moment of transmission(126). The consequence of this preadaptation was higher early set point viral load and more rapid CD4 decline, compared to individuals who were infected by a less preadapted virus(126, 132).

Antibodies Against HIV-1

Perhaps one of the most discussed host factors the immune system has in fighting HIV-1 is anti-HIV-1 antibodies. The production of antibodies is ongoing throughout infection in a continuous attempt to keep up with the ever-changing virus. This molecular arms race is perpetuated by numerous factors; in addition to the error-prone reverse transcriptase and the possibility of recombinant variants, another factor that makes HIV-1 a difficult target to a stable antibody population is the glycosylation of the envelope glycoproteins. HIV-1 gp120 is very heavily modified by sugars which can vary in position to hide regions of the protein from antibody responses. The envelope glycoprotein coding regions are among the most variable in the HIV-1 genome, allowing the virus to avoid the immune pressure of antibodies.

Despite the inability of most individuals to mount an antibody response that clears HIV-1, broadly neutralizing antibodies (bnAbs) do exist. These special antibodies recognize conserved epitopes on the viral spike that cannot be mutated by the virus without resulting in severe fitness costs. One example of such a region is the CD4 binding site, since without this very specific region the virus would be unable to interact with its receptor and enter a new host cell. The first class of bnAbs that were isolated bind the CD4 binding site and is represented by VRC01. Some unique characteristics of VRC0-class bnAbs are a short light-chain

complementarity-determining region 3 (LCDR3) and a heavy chain with significant somatic mutations such that it can structurally resemble the CD4 molecule(133, 134). Other bnAbs possess other rare features, such as a long heavy chain CDR3 that is capable of reaching through the gp120 glycan shield to interact with amino acids in the variable loop(133).

Overall, the required presence of unique antibody characteristics makes bnAbs uncommon early enough in infection. In a study following the evolution of bnAbs in a patient, the researchers found that the unmutated common ancestor of the antibody bound strongly to the transmitted founder (TF) virus, and furthermore that viral diversification preceded the generation of neutralization breadth in a manner of co-evolution(135). This explains how even if an individual does eventually manage to generate bnAbs, they are still unable to clear the virus due in large part to continued viral diversification. It is the goal of vaccine researchers to develop a preventive vaccine that will elicit the generation of bnAbs that are both broad acting, across many subtypes of HIV-1, and also highly potent. This is being attempted through numerous strategies, some of which include using germline-targeted immunogens to stimulate and guide the affinity maturation of bnAbs(134), and others which focus on engineering an Fc-region that will induce better cellular effector functions such as ADCC(136).

Finally, antibodies are an important host factor to consider because studies of TF viruses have demonstrated that these viruses are more sensitive to antibody neutralization than other members of the viral quasi-species that did not transmit(137, 138). This could present a promising target for vaccine strategies if ancestral viral variants are more susceptible to antibody neutralization.

Other Sexually Transmitted Infections as Risk Factors for HIV Acquisition

Sexually transmitted infections (STI) caused by other pathogens such as the bacteria *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG)(139-142), the parasite *Trichomonas vaginalis* (TV)(143, 144), the virus Herpes Simplex Virus Type 2 (HSV-2)(145-147), and even perturbances in the healthy vaginal microbiota, such as bacterial vaginosis(139, 148) are demonstrated risk factors for HIV acquisition, and therefore another relevant host factor to consider. In addition to sharing the same route of transmission, STIs can predispose an individual to HIV infection by inducing changes in the genital tract that increase inflammation, cause ulceration of the genital mucosa, and recruit more HIV target cells to the site of transmission. Infections by CT and NG are also associated with increased viral shedding in women infected with HIV(149), which increases her male partners' exposure to virus and subsequently their risk of HIV acquisition. In men, infections such as those that cause genital ulcers or inflammation, reduce the selection bias imposed on virions during female-male heterosexual HIV transmission(150). Overall, in Zambian HIV-1 serodiscordant couples, the population attributable risk of genital ulceration and inflammation in either or both partners was 63% for male-to-female HIV transmission, and 80% for female-to-male HIV transmission(142).

In 2016, HSV-2 was attributable for 37.1% of incident HIV-1 infections in the WHO African region(147). In effort to address this key risk factor, several clinical trials aimed to reduce the risk of HIV infection by providing treatment for HSV-2 infections with acyclovir, a medication which reduces outbreaks and speeds healing of ulcers caused by HSV-2, but does not cure the infection itself(151, 152). Unfortunately, these clinical trials were unsuccessful in reducing HIV transmissions. Researchers later discovered that even after HSV-2 ulcers resolve, an enriched population of $CD4^+$ T cells and DCs expressing DC-SIGN persist at the former site

of ulceration for up to 8 weeks following healing(153). This increase in HIV target cells, even after the HSV-2 outbreak has cleared, may help explain why HSV-2 is such a critical risk factor for HIV acquisition, and why the resolution of ulcers via treatment with acyclovir was unsuccessful. However, many of the other STIs, including CT, NG, and TV, are curable and treating these infections may result in a decreased risk of HIV acquisition.

Additionally, these infections are important aside from being HIV risk factors because if left untreated they can lead to a variety of damaging reproductive complications such as pelvic inflammatory disease, ectopic pregnancy, miscarriage, preterm birth, and infertility(154-157). In order to prevent these negative health consequences and lower the incidence of HIV attributable to other STIs, the best approach would be to diagnose and treat cases of curable STIs.

One common, but problematic, strategy is syndromic management, where STIs are screened for based on signs and symptoms(158). However in the majority of cases, and especially in women, STIs are asymptomatic making detection and treatment extremely challenging(141, 159). Another approach is mass drug administration in high-risk populations(160, 161), but unnecessary exposure to antibiotics can have undesired consequences. Recently, NG has received much attention for the rise of antimicrobial resistant strains, which complicate control efforts and raise concerns about antibiotic stewardship(162). Further complicating control efforts are complex sexual networks in high-risk populations and limited resources to provide partner referral services, which together lead to high rates of re-infection following treatment. A better understanding of the risk factors for STIs could improve case detection, diagnosis, and treatment, and ultimately reduce the impact of these STIs as host-related risk factors for HIV acquisition.

Summary

Overall, this introduction followed the viral life cycle, exploring the numerous host factors responsible for defending against HIV-1 at each step, and how in some cases variations in these host defenses can alter the risk of HIV-1 acquisition. However, the virus does not simply allow itself to be restricted by these host factors, so this chapter also discussed some of the viral strategies for escaping these immune defense mechanisms.

From the complexities of HIV-1 epidemiology to the extensive intra-host viral diversity, creating a successful vaccine against HIV-1 is a global health challenge. Of utmost importance to a vaccine development is safety, therefore considering host factors such as FcγR genotype variation is critical to avoid undesirable outcomes such as increased rates of infection among certain groups, as was observed in the Vax004 trial. Studying host factors will also lend powerful insights into effective and ineffective strategies for combating HIV-1 infection; it is wise to learn as much as possible about these immune defenses so that vaccine strategies can target or mimic the most promising avenues of viral prevention and control.

Chapter II: Fc-gamma receptor IIA and IIIA variants in two African cohorts: Lack of consistent impact on heterosexual HIV acquisition, viral control, and disease progression

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Abstract

Human Fc-gamma receptors (FcγRs) FcγRIIA and FcγRIIIA contain amino acid variants with both high and low affinities for IgG that modulate antibody-mediated effector functions. Recent HIV vaccine trials suggested that these FcγR variants can influence susceptibility to HIV infection, which prompted us to fully assess the role of FcγR variants on HIV acquisition, viral control, and disease progression in two longitudinal heterosexual transmission cohorts with HIV subtypes A and C as the major circulating viruses. For 836 participants, molecular genotyping resolved genetic variations encoding the FcγRIIA (131H/R) and FcγRIIIA (158V/F) single nucleotide polymorphisms. Kaplan-Meier curves, Cox proportional hazards models, and linear regression models did not reveal any clear or consistent FcγR association with time to HIV acquisition, viral load in early infection, or extent of CD4+ T-cell decline over time after infection. Overall, previous epidemiological findings on FcγR variants and vaccine efficacy are not readily applicable to heterosexual HIV transmission.

Introduction

In the human immune system, Fc receptors bridge the cellular and humoral immune responses and are important in antibody protection against HIV infection(1). Collections of these receptors are found on the surface of most immune cells and act by binding the constant region of immunoglobulins and subsequently directing cellular functions. Those that bind IgG antibodies are known as Fc-gamma receptors (FcγRs), and there are seven known FcγRs in humans: FcγRI (CD64), FcγRIIA (CD32a), FcγRIIB (CD32b), FcγRIIC (CD32c), FcγRIIIA (CD16a), FcγRIIIB (CD16b), and FcRn(2).

This study focuses specifically on the role that two Fc-gamma receptors, FcγRIIA (CD32a) and FcγRIIIA (CD16a), play in HIV transmission, viral load set point, and disease progression. Both FcγRIIA and FcγRIIIA are low-affinity activating receptors, meaning their signaling necessitates cross-linking of multiple FcγRs on the surface of a single cell before triggering a cellular response(3). FcγRs can be cross-linked by the binding of an immune complex (IC) to the cell surface. FcγRIIA is found on all myeloid cells, including monocytes, macrophages, neutrophils, basophils, eosinophils, mast cells, and dendritic cells (DCs), and plays a role largely in phagocytosis(4, 5). FcγRIIIA acts to trigger antibody-dependent cellular cytotoxicity (ADCC) with its presence on natural killer (NK) cells, monocytes, and macrophages(4, 5).

There is diversity among these two receptors due to the presence of single nucleotide polymorphisms (SNPs) that contribute to differential binding affinity for various subclasses of IgG. FcγRIIA has two allele variants that differ by a single SNP that toggles the amino acid at position 131from arginine (131R) to histidine (131H), resulting in a higher affinity for IgG1 and IgG2(3, 4, 6). Interestingly, the prevalence of FcγRIIA alleles has been shown to vary between

ethnic populations(7), but has never before been described for Rwanda or Zambia. FcγRIIIA also has two allele variants differing by a SNP that results in amino acid toggling at position 158; the codon for valine at this position (158V) results in a higher affinity for IgG1, IgG2, IgG3, and IgG4 than the codon for phenylalanine (158F)(3, 4, 8).

The impact of FcγRs on HIV infection has been implicated in vaccine efficacy, HIV disease progression, and cellular effector function in response to virus, but a firm consensus has yet to be reached. In the Vax004 recombinant gp120 vaccine trial, which consisted of North American men who have sex with men (MSM), FcγRIIA genotype showed no effect, but vaccinated individuals with the homozygous high affinity FcγRIIIA VV genotype had a greater rate of infection compared to controls and vaccinated individuals with VF and FF genotypes(9).

In the Multicenter AIDS Cohort Study (MACS) of North American MSM, it was observed that individuals with the homozygous low affinity FcγRIIA RR genotype progressed to AIDS at a more rapid rate than those with HR or HH genotypes, and that FcγRIIIA genotype did not play a role(10). Somewhat similarly, another study found double homozygous low affinity individuals RR/FF progressed to disease faster, but also that high affinity FcγRIIIA VV was not associated with natural viral suppression(11). However, in a cohort of female sex workers (FSW) in Kenya no association was observed between FcγRIIA or FcγRIIIA genotypes and disease progression(12).

The question of whether FcγR genotype impacts transmission has been explored largely in the context of mother-to-child transmission (MTCT). In 2004, Brouwer reported that in a Kenyan cohort the homozygous high affinity FcγRIIA HH genotype was more prevalent among HIV-infected infants in a dose-dependent relationship with HR and RR genotypes, and that maternal genotype did not play a role in transmission(13). A recent study in South Africa

contrasted this finding and reported that maternal genotype was important and showed that FcγRIIA RR genotype was associated with increased odds of transmission and that the FcγRIIIA V allele was associated with reduced MTCT risk(14). In another cohort of Kenyan mother-infant pairs, infant genotype was not associated with infection or disease progression, however maternal genotype, specifically FcγRIIIA VF heterozygosity, was associated with increased risk of MTCT during the early breastfeeding period(15). Finally, a study that included multiple modes of transmission (sexual and intravenous drug use), found an association between the high affinity FcγRIIIA VV genotype and presence of HIV infection, but the association was weakly significant and included a small study population(11).

These existing studies were mostly conducted in all-male or all-female cohorts, and have resulted in conflicting data regarding the effects of FcγRIIA and FcγRIIIA on both HIV-1 transmission and disease progression. Due to known sexual dimorphism in immune responses(16), it is important to examine this question in both men and women. Furthermore, it is still unclear what role these receptors may play in heterosexual transmission, which accounts for more than 80% of transmission events in Africa(17). The cohorts studied here address both of these concerns, as well as compare FcγR impact with respect to two different subtypes of HIV, which has not been studied previously. We compare subtype A, which is the primary circulating variant in Rwanda, and subtype C, the primary circulating variant in Zambia which is responsible for the greatest burden of HIV infections globally(18). Overall, the results of these studies in over 800 participants suggest that FcγRIIA and FcγRIIIA genotypes have no consistent, meaningful impact on transmission, viral control, or disease progression in either men or women in two subtype-distinct countries.

Methods

Cohort and subject selection: This project was conducted in partnership with the Rwanda Zambia HIV Research Group (RZHRG), a historical longitudinal cohort of ART-naïve, heterosexual, HIV-1 serodiscordant-couples which began sample collection in 1995 in Rwanda and Zambia. Public health messaging and HIV prevention efforts such as Couples' Voluntary Counseling and Testing (CVCT), condom provision, contraception, screening, and treatment for sexually transmitted infections were provided to all participants in Zambia and Rwanda(19).

For this nested case-control study design, HIV+ men and women (163 incident and 54 chronic infections from Zambia, 114 incident and 107 chronic infections from Rwanda) and HIV- men and women (186 from Zambia, 212 from Rwanda) were selected from a larger cohort of over 2,000 serodiscordant and transmitting couples in each country. Participants were enrolled into the original cohort study based on elevated risk of heterosexual HIV transmission as members of a cohabiting HIV serodiscordant partnership, therefore seroconverters and uninfected individuals both experienced similar exposure and risk factors for HIV acquisition.

HIV-uninfected participants were followed and HIV tested at regular intervals; either every three months or every one month, and in the event of seroconversion were invited to participate in the International AIDS Vaccine Initiative's (IAVI) Early HIV Infection cohort, Protocol C, which began enrollment in 2006(20, 21). Protocol C also enabled longitudinal follow up in HIV-infected individuals to monitor disease progression through the regular collection of samples for viral load and CD4 testing; in this study population, approximately 58% of the HIV-infected subjects were enrolled in Protocol C.

The primary circulating HIV-1 variant in Zambia is subtype C, and is the subtype that represents 97% of the HIV-infected Zambians in this study (Table 1A). Approximately 55% of

the HIV-1 infected Zambian group was enrolled in IAVI's Protocol C and was followed longitudinally to monitor HIV disease progression through regular viral load and CD4 count measurements.

In Rwanda, subtype A is the major circulating variant and accounts for approximately 84% of the HIV-infected individuals in this group (Table 1A). In the Rwandan group, 62% of HIV-1 infected participants were enrolled in IAVI's Protocol C and subsequently followed to assess HIV disease progression.

Given the high percentage of subtype A viruses in Rwanda and subtype C in Zambia, for this study, country is used as a proxy comparison for virus subtype. A total of 836 participants were included (Table 1B).

DNA extraction: Genomic DNA previously extracted from participant samples was used if available, or freshly extracted from buffy coats stored in RNALater using the Qiagen DNeasy Blood & Tissue Kit in 96-well format (cat. 69581), or Qiagen QIAmpDNA Blood Mini Kit (cat. 51104). RNALater was removed prior to extraction by gently mixing 500µl of buffy coat with 1mL PBS -/- and centrifuging at 1,500 x g for 5 minutes, the supernatant was removed and the pellet was resuspended in PBS without calcium and magnesium (PBS -/-) to follow Qiagen kit manufacturer protocol.

Real-Time PCR for FcγR Genotyping: Taqman SNP Genotyping assays were used to genotype samples on the Applied Biosystems 7900 Real-Time system; FCGR3A assay ID C_25815666_10 (ThermoFisher Scientific, Waltham, MA) was used to detect SNP ID rs396991, and FCGR2A assay ID C_9077561_20 (ThermoFisher Scientific, Waltham, MA) was used to

detect SNP ID rs1801274. Reactions were prepared using TaqMan Genotyping Master Mix, TaqMan probe, and 2.25ul of genomic DNA, to a final volume of 5ul, and were run in triplicate for each SNP on a 384-well plate. Positive controls for each genotype and no-template controls were tested in triplicate during each run.

Previously extracted genomic DNA samples were of varying quality and concentration resulting in differing signal strengths, but an equal ability to identify the SNP present. In our hands, there were no discrepant genotyping results. In the few cases where not all of the triplicate samples amplified, the genotype was determined based on the well(s) that were positive. In the few cases where Taqman genotyping was not initially successful for any of the triplicate runs, the segment containing the SNP was re-amplified using ThermoFisher Scientific Sanger Sequencing Primer Pairs (Hs00293327_CE for FCGR2A and Hs00395867_CE for FCGR3A) in 35 cycles of PCR with Q5 enzyme and re-tested as described above. In nearly all cases the genotype was determined following this additional round of amplification.

Verification by Sanger sequencing was performed for 36 samples, 6 for each genotype, using the Sanger Sequencing Primer Pairs mentioned above and Taqman SNP results matched 100%. Segments of DNA containing the region of interest from individuals confirmed by Sanger sequencing were PCR-amplified, purified using the Promega PCR clean-up kit, and used as positive controls for future experiments.

Covariates: HIV subtypes A and C were separated using cohort country as a proxy, Rwanda consisting mainly of individuals infected with subtype A viruses, and Zambia representing subtype C viruses. Biological sex was coded as a categorical variable. Donor viral load is the viral load (VL) of the chronically-infected partner nearest to the time of transmission and was

coded as a three-level categorical variable ($<10^4$, 10^4-10^5, and $>10^5$ copies/mL) based on thresholds previously shown to be biologically significant to transmission(22, 23). Set point viral load was defined as the geometric mean of all VL measurements (requiring at least two) for a given individual collected between 30 and 365 days post estimated date of infection. *In vitro* viral replicative capacity (RC) of the transmitted founder virus was available for 122 acutely HIV-infected Zambian individuals(24). HLA haplotype for the alleles B.42, B.57, B.5801, and B.81 was determined as previously described(25).

Exposures of interest: FcγRIIA and FcγRIIIA genotypes were categorized separately based on the affinity for IgG resulting from the SNP of interest, either high affinity (HH or VV, respectively), heterozygous (HR or VF, respectively), or low affinity (RR or FF, respectively). In order to characterize the combined effect of these two receptors in a given individual, high affinity FcγR exposure levels were also created using a scoring system where homozygous low affinity (RR, FF) genotypes were worth 0, heterozygous (HR, VF) worth 1, and homozygous high affinity (HH, VV) worth 2. The sum of their score at both loci yields the exposure level. Thus, levels range from 0 to 4 where individuals lacking any high affinity receptors were assigned to level 0, and those homozygous for high affinity alleles at both loci were assigned to level 4.

Statistical Analyses: Prevalence of FcγRIIA and FcγRIIIA genotypes by country, sex, and HIV infection status groups were analyzed using 2x3 and 4x3 chi-square tests. A Mantel-Haenszel test for trend was used to compare odds of FcγRs affinity exposure level by HIV status, stratified by country; level 1 was used as the reference group because it contained the largest sample size.

Time-to-infection (TTI) from study enrollment was evaluated by Kaplan-Meier survival analysis and the log-rank test for trend in a combination of approximately equal numbers of individuals with HIV incident infections and longitudinally followed HIV- partners from the same cohort. Analyses followed stratification by country, sex, and FcγR genotype or high affinity exposure level. Two-way comparisons combining affinity groups were also tested (High/Het vs. Low, High/Low vs. Het, High vs. Het/Low). Individuals who were lost to follow-up after a seronegative test result were right-censored from the Kaplan-Meier survival analysis; Rwandans were censored after 36 months due to small sample size following that time point. Adjusted Cox proportional hazard models for associations between TTI and exposure level controlled for donor viral load and sex. The full model initially contained both potential covariates and was reduced to those reported using the R StepAIC model selection function in both directions.

Log set point viral loads stratified by country and genotype were compared using the Kruskal-Wallis rank sum test (nonparametric one-way ANOVA) with Dunn test for pairwise comparisons and Sidak correction for multiple comparisons. Adjusted linear regression models for associations between FcγR exposure level and log set point viral load were built where full models initially contained all potential covariates (sex, HLA alleles B.81, B.42, B.5801, B.57 and RC for Zambia) then were reduced to those reported using the R StepAIC model selection function in both directions.

CD4 decline from initial infection was assessed using Kaplan-Meier survival analysis to endpoints <400, <350, <300, and <200 cells/μl stratified by country, sex, and FcγR genotype or high affinity exposure level then compared using a log-rank test for trend. Two-way comparisons combining affinity groups were also tested (high/het vs. low, high/low vs. het, high vs. het/low).

Adjusted Cox proportional hazard models initially controlling for sex, HLA alleles B.42, B.57, B.5801, and B.81, set point viral load, and in Zambia, *in vitro* viral replicative capacity, underwent bidirectional model selection using the R StepAIC function. The covariates remaining after model selection are reported.

Results

FcγRIIA and IIIA genotype distribution by HIV status in Rwanda and Zambia

To determine whether FcγR polymorphisms influenced susceptibility to HIV-1 infection, we genotyped 836 individuals and compared the proportion of FcγRIIA and FcγRIIIA genotypes across sex and infection status within each country and observed that the distribution did not vary significantly by any of the criteria studied (Figure 1). Fewer individuals with FcγRIIIA homozygous high affinity (158VV) genotype are noted across all groups in both populations. Chi-square tests comparing the distribution of each genotype by sex and HIV infection status, within each country, revealed no significant differences. Additional analyses compared HIV infection status and genotype across country, genotype among infected individuals stratified by sex, and infection status with presence of at least one high affinity allele, none of which demonstrated any statistical association.

In order to determine whether particular combinations of high and low affinity alleles of both receptors might influence infection status, we assigned a score of 0 for individuals homozygous for both low affinity alleles (RR + FF) and a score of 4 for individuals homozygous for both high affinity alleles (HH + VV), with scores of 1-3 representing the remaining combinations. Comparing high affinity exposure level stratified by country, HIV infected individuals had more than twice the odds of being homozygous for both high affinity FcγR receptor alleles (versus having just one high affinity FcγR receptor allele) compared to uninfected individuals (Table 2; Zambia: OR = 2.98, 95% CI = (0.60, 14.86); Rwanda: OR = 2.06, 95% CI = (0.71, 5.96)), although the sample size for those with exposure level 4 is small in both countries and the ORs were not statistically significant. A Mantel-Haenszel test for trend showed no evidence of a linear association between the odds of high affinity FcγR exposure level

and HIV positivity (Zambia: Extended Mantel-Haenszel chi square for linear trend = 0.13, p-value = 0.72; Rwanda: Extended Mantel-Haenszel chi square for linear trend = 0.67, p-value = 0.41).

Time-to-infection and FcγRIIA or FcγRIIIA genotype

Despite FcγR genotype not being associated with HIV infection status in Rwanda or Zambia, it is possible that individuals with a certain genotype are predisposed to becoming infected more quickly when exposed. The longitudinal design of the cohort provides us with time-to-infection (TTI) data, based on date of study enrollment. A Kaplan-Meier survival analysis and a log-rank test for trend were used to compare TTI between genotypes stratified by country (Figure 2). The log-rank tests for trend, as shown in Figure 2A-D, demonstrated no statistically significant association between TTI and either FcγR locus in Rwanda or Zambia. However, when stratified by sex and combining high/het genotypes vs. low, it was observed that Rwandan men possessing at least one high affinity allele of FcγRIIIA (VF or VV) tended to become infected more quickly than Rwandan men with a homozygous low affinity FcγRIIIA genotype (FF), log-rank p=0.04 (Supplemental Figure 1). All other comparisons were not statistically significant at alpha=0.05.

TTI was also examined by the level of exposure to high-affinity FcγRs and no significant dose-dependent association was observed (Figure 3, A-B). Comparing individuals with exposure level 4 to all other exposure levels combined, Rwandans with exposure level 4 became infected more quickly than Rwandans with other exposure levels (Zambia log-rank p = 0.09, Rwanda log-rank p = 0.04; Supplemental Figure 2) however the sample size of individuals with level 4 is much smaller than the other groups combined (7 vs. 322 in Zambia, 7 vs. 244 in Rwanda). While

this is consistent with the increased odds of exposure level 4 among HIV+ cases (Table 2), as noted, there were fewer individuals with this exposure level compared to other groups, and statistically there was no trend for a dose-dependent decrease in TTI as number of high affinity FcγRs increased.

A Cox proportional hazard model was used to examine TTI adjusting for potential covariates; the maximum model considered biological sex and donor VL. For Zambia, sex was dropped during model selection, but low donor VL was associated with a reduced rate of infection, while high donor VL (>100,000 copies/mL) was associated with an increased rate. Although there was again no trend between presence of high affinity alleles and TTI in either country, Zambian individuals with exposure level 4 versus 1 had a statistically significantly increased hazard ratio (Figure 3C). In contrast, in Rwanda only high donor VL was significantly associated with an increased hazard ratio, with at most a trend ($p=0.121$) towards exposure level 4 versus 1 impacting the hazard ratio (Figure 3D).

Set point viral load and FcγR genotype

Set point viral load is a measure of host control of viral replication and was assessed in relation to FcγR genotype using the Kruskal-Wallis rank sum test and Dunn test pairwise comparison with Sidak correction for multiple comparisons, which detected no significant differences (Figure 4).

Adjusted linear regression models assessed set point viral load as a continuous variable and showed no trend by exposure level. However, Rwandan individuals with exposure level 0 had significantly lower set point viral loads than those with level 1, $p = 0.02$ (Table 3).

CD4 decline to <350 cells/µl and FcγRIIA or FcγRIIIA genotype

One measure of HIV disease progression is CD4 decline, which is an indicator of immunosuppression. For this study, time from initial infection to CD4 count less than 350 cells/µl was compared between FcγR genotypes by country using Kaplan-Meier survival analysis and the log-rank test for trend (Figure 5). These analyses indicated no statistically significant relationship between FcγR genotype and disease progression. Similar results were obtained where the end-point utilized CD4 counts of 400, 300, and 200 cells/µl.

In Zambia, there was no association between exposure level and time-to-CD4 decline to <350 cells/µl (Figure 6A) or any other CD4 count endpoint (400, 300, or 200 cells/µl) in a Cox proportional hazard regression model. In Rwanda for time-to-CD4 decline to 350, the group of individuals with exposure level 2 had a statistically significantly lower adjusted hazard ratio (aHR = 0.46, p-value = 0.03) when compared to individuals with exposure level 1 (Figure 6B). Individuals with exposure level 0 had the same hazard ratio as those with exposure level 2, but this was not statistically significant (aHR = 0.46, p-value = 0.09). For time-to-CD4 decline to less than 300 cells/µl in Rwanda, delayed time to event was observed in individuals with exposure level 0 (aHR = 0.19, p-value = 0.01) and exposure level 2 (aHR = 0.39, p-value = 0.03), relative to those with exposure level 1 (Supplemental Figure 3). However, no effect of exposure level was observed for CD4 decline to 400 or 200 cells/µl in individuals from this country. Set point viral load and HLA alleles B.57 (Zambia) and B.5801 (Rwanda) were also associated with time-to-CD4 decline to 350.

Discussion

Data from previous studies have examined the role of FcγRIIA and FcγRIIIA genotype in all-male or all-female cohorts relating to HIV vaccine efficacy(9), disease progression(10, 12), and cellular effector function(26). The majority of these studies consider risk groups distinct from heterosexual co-habiting couples, which is the most common mode of HIV transmission worldwide(17). Furthermore, the design of these studies has limited them to addressing either acquisition or disease progression, but not both in the same study population. The RZHRG cohorts, in which acutely infected individuals were identified for longitudinal follow-up, enabled the current study to uniquely address these questions in a setting where we can control for potential sex differences as well as explore viral subtype differences, which has not previously been considered. We were thus able to examine the effects of FcγRIIA and FcγRIIIA genotype on the heterosexual HIV transmission event, viral control, and disease progression within the same study population. Additionally, because the cohorts consist of serodiscordant couples, uninfected individuals had similar risk factors as those who became acutely-infected, providing an internal control.

<u>FcγRIIA and FcγRIIIA genotype distributions in Rwandan and Zambian populations are similar to others worldwide, and not associated with HIV infection status</u>

The distribution of FcγRIIA and FcγRIIIA alleles in either HIV-uninfected or infected individuals has not been previously described for Rwanda or Zambia, and are consistent with those in other countries reported in the literature(11, 15, 27, 28). In a Tanzanian cohort of 174 HIV-uninfected and 99 HIV-infected individuals, similar FcγRIIA and FcγRIIIA genotype distributions were observed among HIV-uninfected individuals, with no difference by biological

sex, FcγRIIA (p = 0.76) or FcγRIIIA (p = 0.88). However, for HIV-infected individuals, differences were observed by biological sex at both loci, FcγRIIA (p = 0.003) and FcγRIIIA (p = 0.014), with significantly more HIV-infected men having at least one high affinity allele (H or V) and significantly more HIV-infected women possessing the homozygous low affinity genotype (RR or FF) (Kijak, unpublished).

The current study included a larger sample size than the Tanzanian study, 836 individuals equally representing men and women, and found no association between the distribution of particular FcγRIIA and FcγRIIIA genotypes and HIV-1 infection status by biological sex. Interestingly, the odds of having homozygous high affinity alleles at both loci (HH and VV), versus exposure level 1, were more than two times greater among infected individuals compared to uninfected individuals in both Zambia and Rwanda, although this result was not statistically significant. Moreover, it is difficult to interpret this finding biologically given the small number of individuals in exposure category 4 and because we did not observe any linear trend in odds ratio as FcγR exposure level increased.

Time to infection may be associated with highest level of exposure to high affinity FcγRs

In the same Tanzanian cohort described above, men with at least one high affinity allele at both the FcγRIIA and FcγRIIIA loci acquired HIV at a faster rate in a Kaplan-Meier survival analysis than men with a homozygous low affinity genotype (RR or FF) at one locus (n = 95, log-rank, p = 0.004) (Kijak, unpublished). The opposite was true for women, where those with at least one locus encoding the homozygous low affinity genotype (RR or FF) became infected more rapidly, (n = 169, log-rank, p = 0.045) (Kijak, unpublished). This suggested a potential biological mechanism by which women with low affinity FcγRs were more susceptible to

acquiring HIV infection, whereas men with high affinity FcγRs may be at a greater risk. A similar finding was reported in the Vax004 trial where vaccinated men with the homozygous high affinity FcγRIIIA VV genotype acquired HIV at a faster rate than vaccinated men with at least one low affinity FcγR(9).

In the current study however, FcγRIIA or FcγRIIIA genotype was not consistently associated with TTI, despite the numerous ways in which it was examined. Although, in one analysis, when stratified by sex and high affinity and heterozygous alleles combined, Rwandan men with at least one high affinity allele at FcγRIIIA (VF or VV) became infected more rapidly than Rwandan men with the homozygous low affinity FcγRIIIA genotype (FF) (log-rank, $p = 0.041$). Furthermore, Kaplan-Meier survival analysis by exposure level showed the highest level in Rwanda (VV and HH) was associated with a more rapid TTI than all other levels combined ($p = 0.037$). However, the number of individuals in this group was small (n=7) and exposure level 4 was not significant compared to exposure level 1 in a Cox proportional hazard model which included sex and donor VL (aHR = 2.34, $p = 0.121$). Nevertheless, in Zambia, a Cox proportional hazard model that controlled for donor VL indicated that TTI was significantly shorter for those with exposure level 4 than those with exposure level 1 (aHR=2.89, $p = 0.027$).

Although together these findings seem to indicate that high affinity FcγRs may confer a greater risk for HIV acquisition, we failed to observe a linear decrease in TTI by increased exposure level to high affinity FcγRs, as might be anticipated if high affinity FcγRs were a true risk factor for HIV acquisition. It is possible that rather than a dose-dependent effect of FcγRs on risk, there is instead a biological threshold effect where only those with the highest exposure to high affinity FcγRs are at elevated risk. Alternatively, since FcγRs interact with antibodies to direct immune cell function, it is possible that their effect is more pronounced in situations, such

as in a vaccinated individual, where large amounts of pre-existing antibodies to HIV are present, which could explain the findings following the Vax004 HIV vaccine trial and why little to no effect is observed in HIV-antigen-naïve individuals in serodiscordant partnerships. Nevertheless, these results lack definitive consistency and only capture a small number of individuals with the highest exposure level due to the fact that the homozygous high affinity FcγRIIIA VV genotype is present in less than 10% of the Rwandan and Zambian populations. Moreover, in order to fall into exposure level 4 these individuals must also fall within the approximately 22% of the population who possess the homozygous high affinity FcγRIIA HH genotype.

Markers of HIV disease progression are not associated with FcγRIIA and FcγRIIIA genotypes

Early set point viral load(29, 30) and CD4 decline(31) are clinical indicators of host viral control and HIV disease progression(32). Previous studies have indicated that homozygous low affinity FcγR genotypes were associated with faster disease progression(10),(11), possibly due to diminished cellular effector function in response to HIV infection as it has been shown to be more robust in individuals with high affinity FcγR genotypes(26). Since FcγRs perform their function via contact with antibodies, one might expect that FcγR genotype would be more strongly associated with disease progression than TTI due to the greater abundance of pathogen-specific antibodies present. Although we observed no significant association between any specific genotype and set-point viral load, when we consider exposure to combinations of both alleles, we observed that Rwandan individuals with 0 high affinity FcγR alleles experienced a statistically significantly lower set point viral load compared to individuals with only 1 high affinity FcγR allele suggesting that fewer high affinity FcγR alleles may be beneficial. However when examining CD4 decline in a Cox proportional hazard model that took into account

protective HLA alleles, Rwandan individuals with exposure level 2 experienced a slower decline to CD4 count <350 cells/μl. These results are not in agreement with the hypothesis that fewer high affinity FcγRs corresponds to more rapid HIV disease progression.

Interestingly, in Zambia no association was observed between FcγR genotype and set point viral load or time-to-CD4 decline (to <400, 350, 300, or 200 CD4 cells/μl). In both countries, there was no dose-response relating number of high affinity FcγRs and CD4 decline. This lack of linear trend, and only observing statistically significant associations in Rwanda which contradict the existing literature, make it challenging to draw conclusions about the effect of FcγR genotype on early set point viral load and rate of CD4 decline.

Conclusion

The lack of consistent effect of different FcγR genotypes on susceptibility to HIV acquisition, viral control, or disease progression in the current study, is largely in agreement with several studies suggesting that FcγRIIA(9, 11, 12, 15) and FcγRIIIA(10, 12) do not play a significant role in these processes. Some of the results presented here indicate that maximum exposure to both homozygous high affinity FcγRIIA and FcγRIIIA genotypes could increase rate of HIV acquisition, with the caveat that those with exposure level 4 represented a very small group of individuals in both Rwanda and Zambia. Athough the relevance of this finding remains to be confirmed, one could envisage a mechanism whereby during heterosexual transmission a transmitted virus is coated in donor antibodies that interact more strongly with high affinity FcγRs in the recipient, thus resulting in more rapid acquisition. The observation that transmitted viruses are more efficiently neutralized by donor antibodies compared to non-transmitted variants(33, 34) could be interpreted as support for this hypothesis, since increased binding of the transmitted virus to antibody could result in better immune complex formation.

Overall, in the more than 800 individuals in Rwanda and Zambia that were examined in this study, we failed to observe a consistent association between FcγRIIA or FcγRIIIA genotype and risk of HIV infection, viral control, or disease progression. Nevertheless, FcγRs may be important in settings with large amounts of previously generated anti-viral antibodies, such as in a vaccine trial.

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A.	Subtype A	Subtype C	Subtype D	Recombinant	Not Subtyped	Total
Zambia	4 (2%)	199 (97%)	2 (1%)	0 (0%)	12	217
Rwanda	152 (84%)	10 (5%)	3 (2%)	17 (9%)	39	221
Total	156	209	5	17	51	**438**

B.	Rwandan Males	Rwandan Females	Zambian Males	Zambian Females	Total
HIV +	111 (RIM)	110 (RIF)	109 (ZIM)	108 (ZIF)	438
HIV -	102 (RUM)	110 (RUF)	97 (ZUM)	89 (ZUF)	398
Total	213	220	206	197	**836**

Table 1: Description of cohorts A. Subtypes of HIV-1 infected individuals by country B. Number of study participants by country, sex, and HIV status. (ZUM=Zambian Uninfected Males, ZUF=Zambian Uninfected Females, ZIM=Zambian Infected Males, ZIF=Zambian Infected Females, RUM=Rwandan Uninfected Males, RUF=Rwandan Uninfected Females, RIM=Rwandan Infected Males, RIF=Rwandan Infected Females).

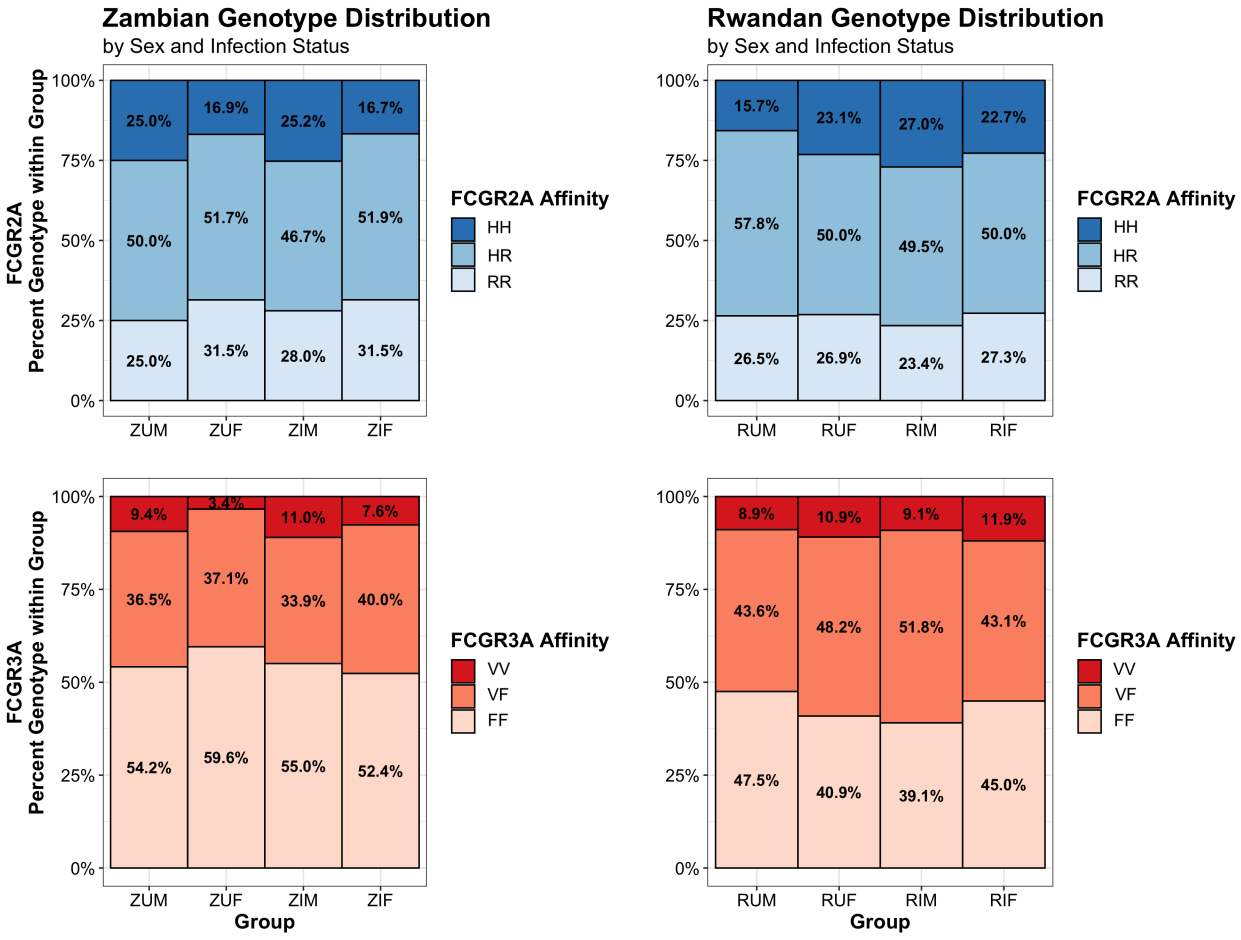


Figure 1: Distribution of FcγRIIA and FcγRIIIA genotypes across country, sex, and HIV status FcγR genotype distribution in Rwanda and Zambia does not differ by sex and infection status. Chi-square tests comparing genotypes by infection status and sex, within and across countries, were non-significant at alpha=0.05. Receptor affinities from high to low are from single nucleotide polymorphisms (SNPs) in the alleles FCGRIIA – 131 HH, HR, RR and FCGRIIIA – 158 VV, VF, FF, respectively. N = 836. (ZUM=Zambian Uninfected Males, ZUF=Zambian Uninfected Females, ZIM=Zambian Infected Males, ZIF=Zambian Infected Females, RUM=Rwandan Uninfected Males, RUF=Rwandan Uninfected Females, RIM=Rwandan Infected Males, RIF=Rwandan Infected Females).

A. Genotype (Zambia)	Exposure Level	Cases	Controls	Total	Odds of Exposure	Odds Ratio (95% CI)
RR & FF	0	41	36	77	1.14	0.97 (0.55, 1.70)
	1	74	63	137	1.17	1.00 (ref)
	2	60	57	117	1.05	0.90 (0.55, 1.47)
	3	30	26	56	1.15	0.98 (0.53, 1.83)
HH & VV	4	7	2	9	3.5	2.98 (0.60, 14.86)
	Total	212	184	396		

B. Genotype (Rwanda)	Exposure Level	Cases	Controls	Total	Odds of Exposure	Odds Ratio (95% CI)
RR & FF	0	32	27	59	1.19	1.46 (0.80, 2.69)
	1	64	79	143	0.81	1.00 (ref)
	2	75	61	136	1.23	1.52 (0.95, 2.43)
	3	38	36	74	1.06	1.30 (0.74, 2.29)
HH & VV	4	10	6	16	1.67	2.06 (0.71, 5.96)
	Total	219	209	428		

Table 2: Odds of exposure to high affinity FcγRs among HIV+ cases and HIV- controls

Exposure level was calculated based on combined FcγRIIA and FcγRIIIA genotypes representing total number of high affinity FcγRs. Homozygous low affinity (RR, FF) genotypes are worth 0, heterozygous (HR, VF) worth 1, and homozygous high affinity (HH, VV) worth 2. The sum of their score at both loci yields the exposure level. Only individuals where both FcγR

genotypes were identified were included; this excluded 12 subjects. A. Extended Mantel-Haenszel chi square for linear trend = 0.13, p-value = 0.72; B. Extended Mantel-Haenszel chi square for linear trend = 0.67, p-value = 0.41.

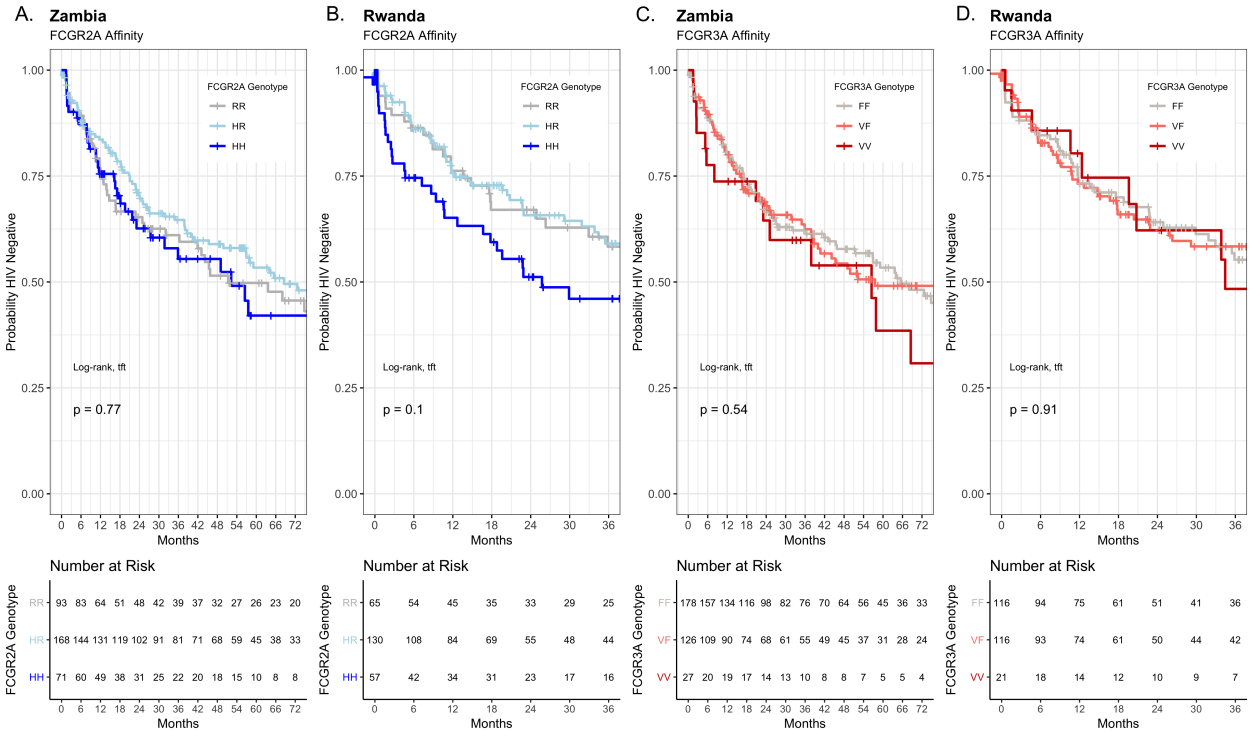


Figure 2: Kaplan-Meier survival analysis for time-to-infection (TTI) in Rwanda and Zambia by FcγR locus TTI does not appear to vary by FcγR genotype in either Zambia (A, C) or Rwanda (B, D). For Rwanda, individuals with TTI greater than 36 months were right-censored due to small sample size. (tft = test for trend).

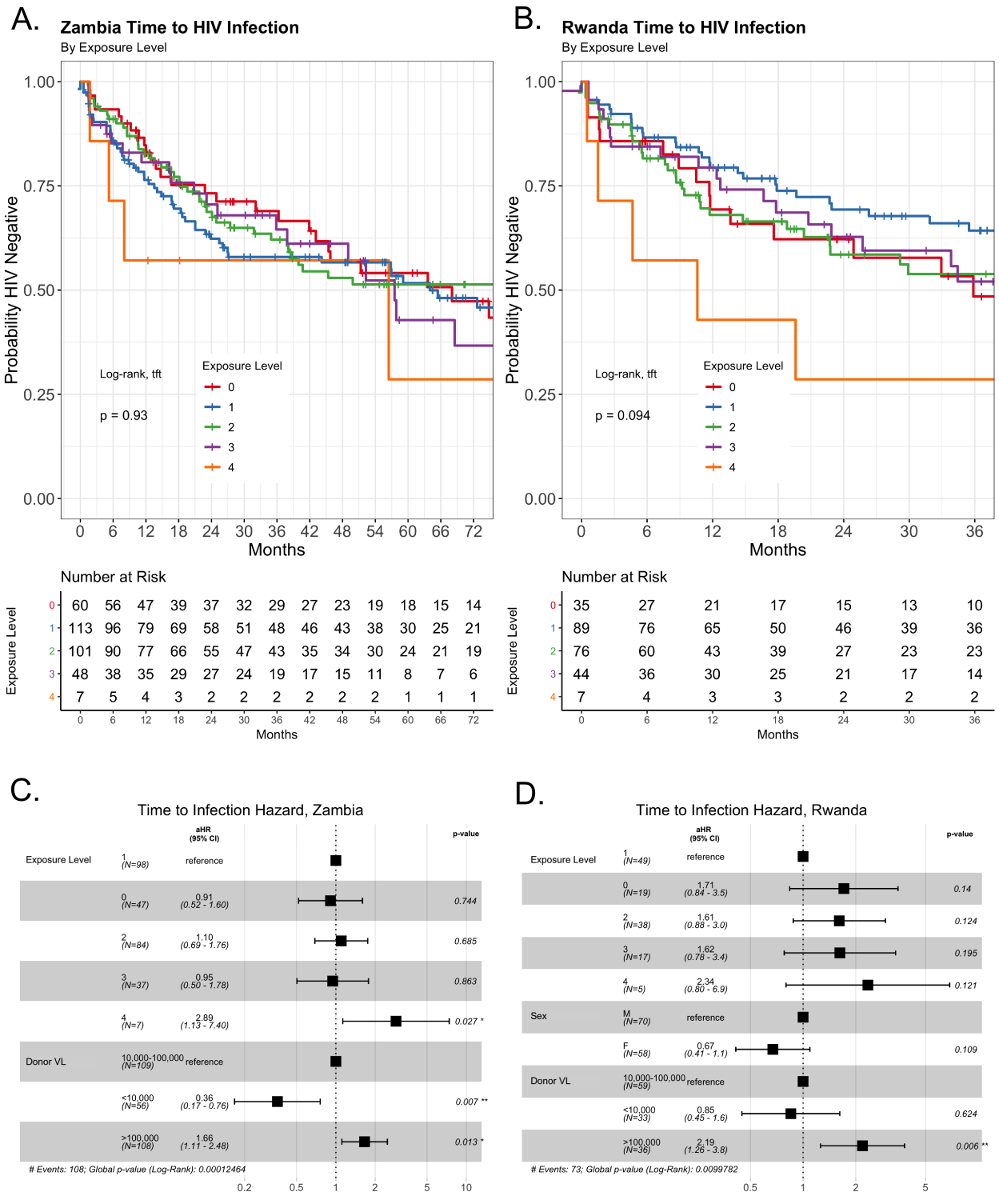


Figure 3: Kaplan-Meier survival analysis and adjusted Cox proportional hazard regression models for time-to-infection (TTI) based on exposure to high affinity FcγRs A. TTI does not

vary by exposure level in Zambia. B. There is no dose-dependent trend demonstrating decreased TTI is associated with increased number of high affinity FcγRs in Rwanda ($p = 0.09$). Rwandan individuals with TTI greater than 36 months were right-censored. C. Controlling for donor viral load, Zambian individuals with exposure level 4 became infected more quickly than those with exposure level 1 ($p = 0.03$). D. Controlling for biological sex and donor viral load in Rwanda, exposure level to high affinity FcγRIIA and FcγRIIIA genotypes do not significantly affect TTI compared to those with exposure level 1. (aHR = adjusted hazard ratio, VL = viral load (copies/mL), * = <0.05, ** = <0.01).

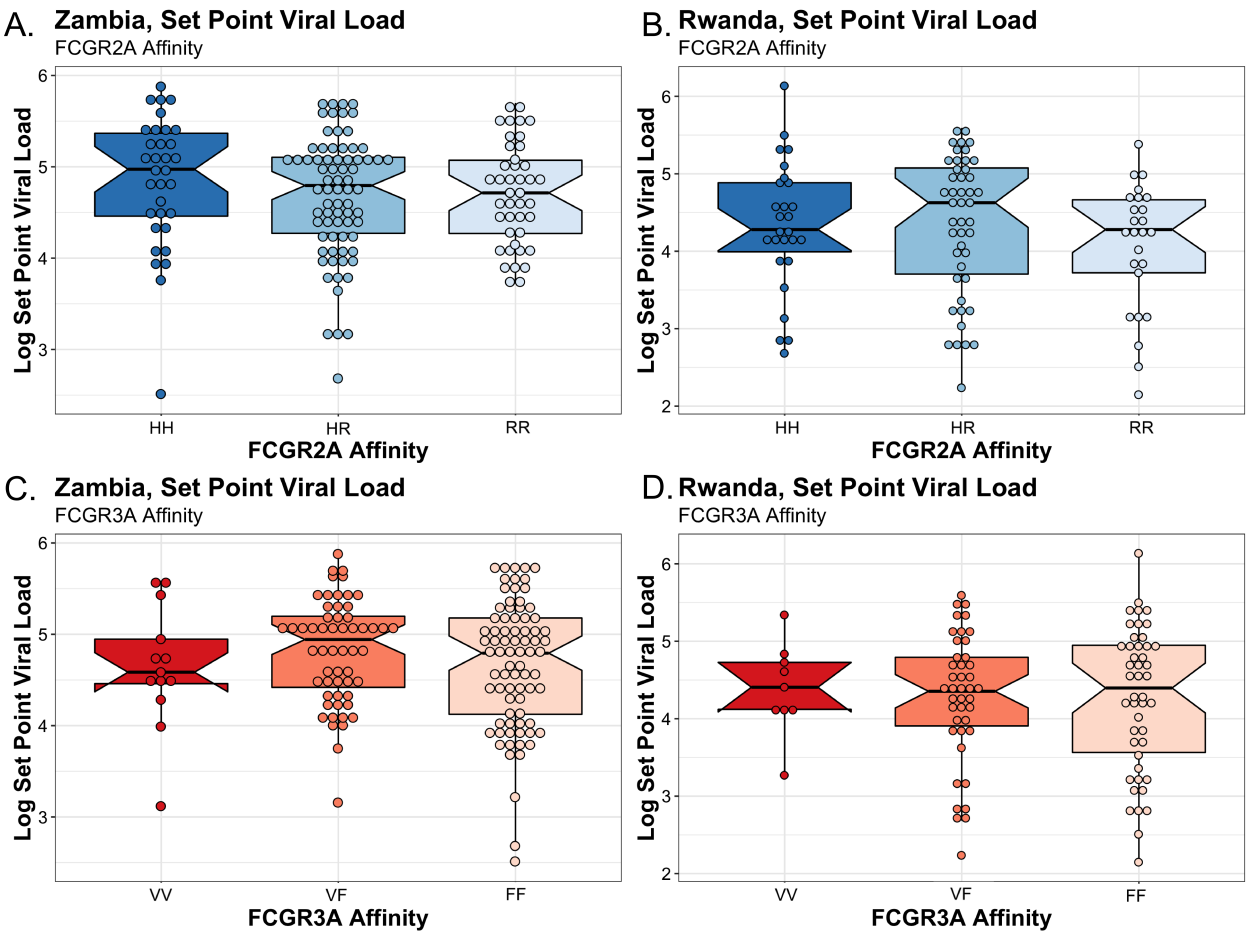


Figure 4: Comparing set point viral load and FcγR genotype by country (A-D) FcγR genotype does not appear to be associated with set point viral load in Zambia (Kruskal-Wallis FcγRIIA p = 0.33, FcγRIIIA p = 0.41) or Rwanda (Kruskal-Wallis FcγRIIA p = 0.30, FcγRIIIA p = 0.99). Y-axis is $\log_{10}$(set point viral load copies/mL). Notches in the box plot represent the 95% confidence interval.

A. Zambia

Parameter	β Estimate	Std. Error	t Value	p Value
Exposure Level 0	0.07	0.17	0.435	0.664
Exposure Level 2	0.15	0.15	1.054	0.294
Exposure Level 3	0.19	0.19	1.031	0.305
Exposure Level 4	0.04	0.33	0.136	0.892
Sex – Female	-0.30	0.12	-2.475	0.015*
B.42	-0.22	0.17	-1.313	0.192
B.57	-0.65	0.20	-3.306	0.001**
RC - Low	-0.18	0.13	-1.363	0.176

B. Rwanda

Parameter	β Estimate	Std. Error	t Value	p Value
Exposure Level 0	-0.63	0.25	-2.487	0.015*
Exposure Level 2	-0.05	0.22	-0.215	0.830
Exposure Level 3	-0.36	0.28	-1.312	0.193
Exposure Level 4	-0.03	0.39	-0.069	0.945
Sex – Female	-0.32	0.18	-1.818	0.073
B.42	0.49	0.26	1.861	0.066
B.57	-0.79	0.36	-2.169	0.033*

Table 3: Adjusted linear regression model of set point viral load based on exposure to high affinity FcγRs A. Exposure level, compared to those with exposure level 1, is not significantly

associated with set point viral load in Zambia after controlling for biological sex, human leukocyte antigen (HLA) alleles B.42 and B.57, and *in vitro* viral replicative capacity (RC). B. In Rwanda, individuals with exposure level 0 experience a lower set point viral load compared to those with exposure level 1 after controlling for biological sex and HLA alleles B.42 and B.57. (* = <0.05, ** = <0.01)

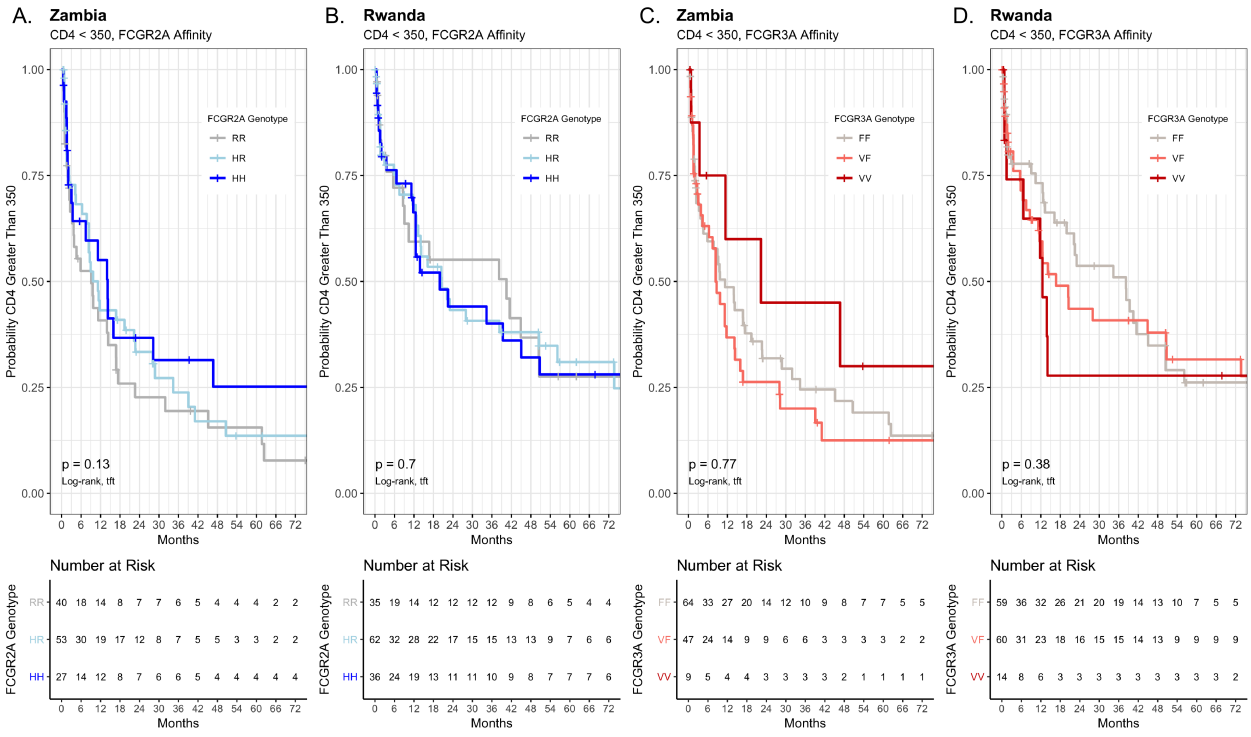


Figure 5: Kaplan-Meier survival analysis for CD4 decline from time of infection to < 350 cells/µl by country and FcγR genotype (A-D) FcγR genotype is not associated with CD4 decline to <350 cells/µl in Rwanda or Zambia. Only decline to <350 cells/µl is shown here, but decline to other thresholds was also not significant.

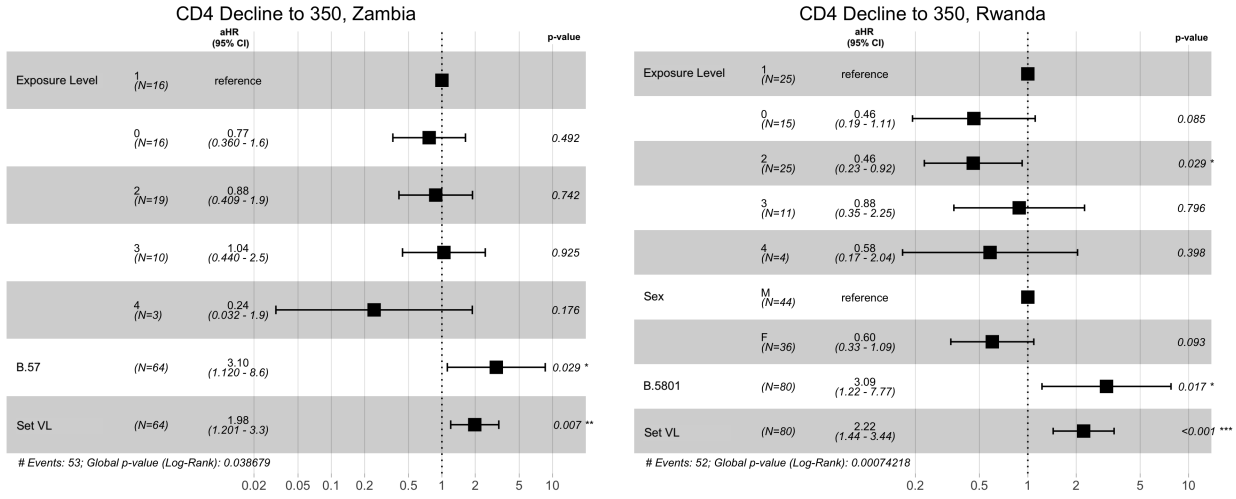
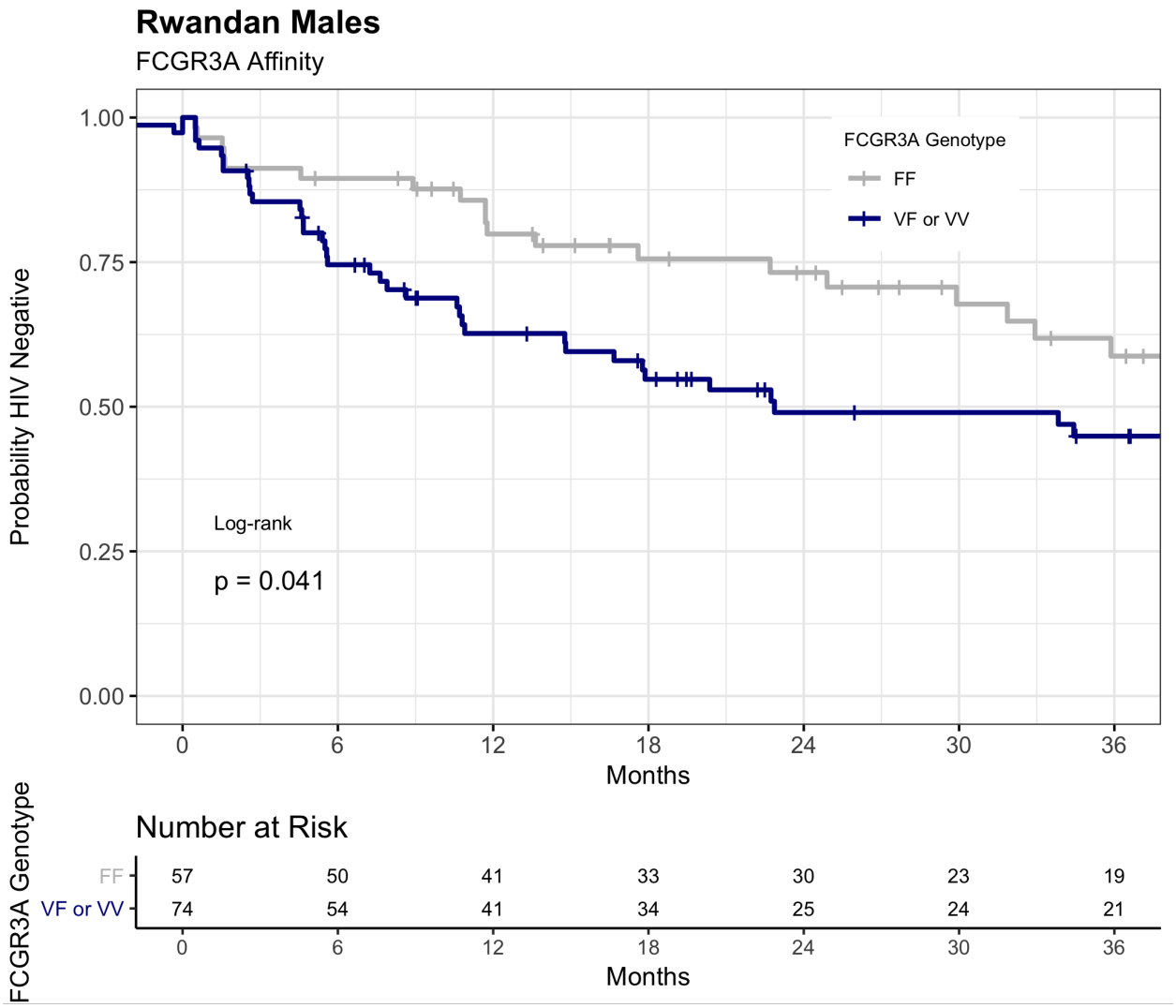
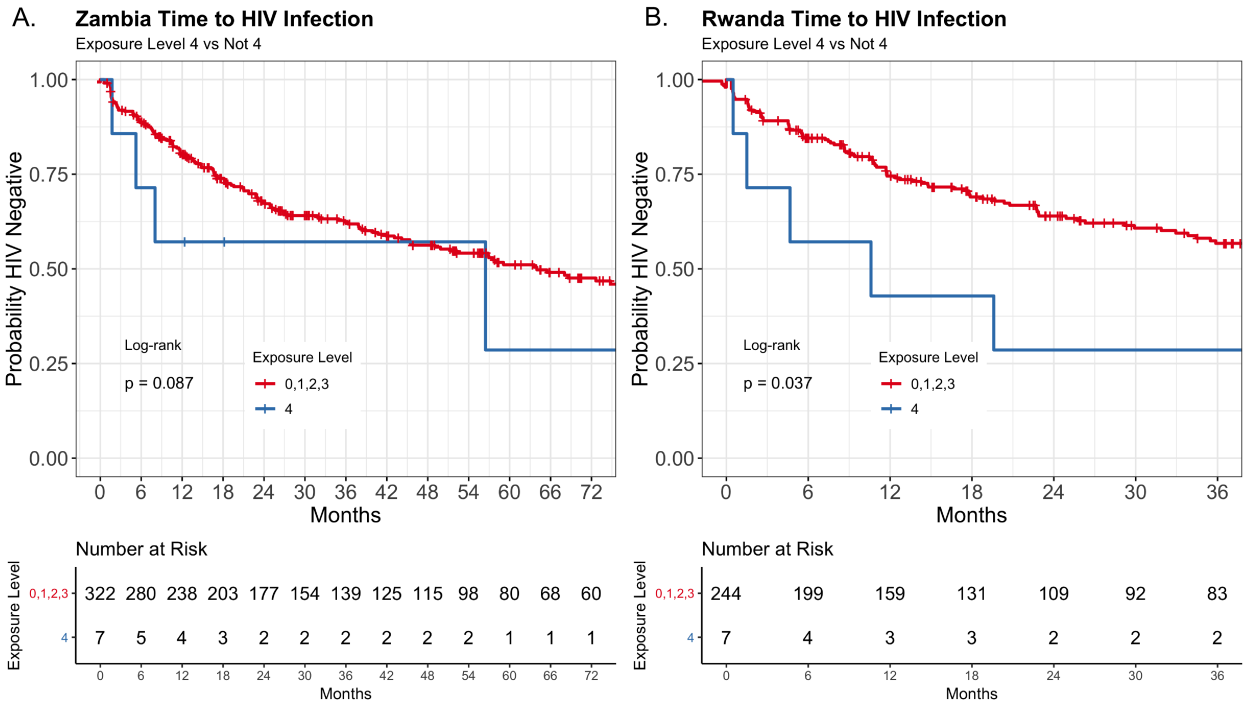


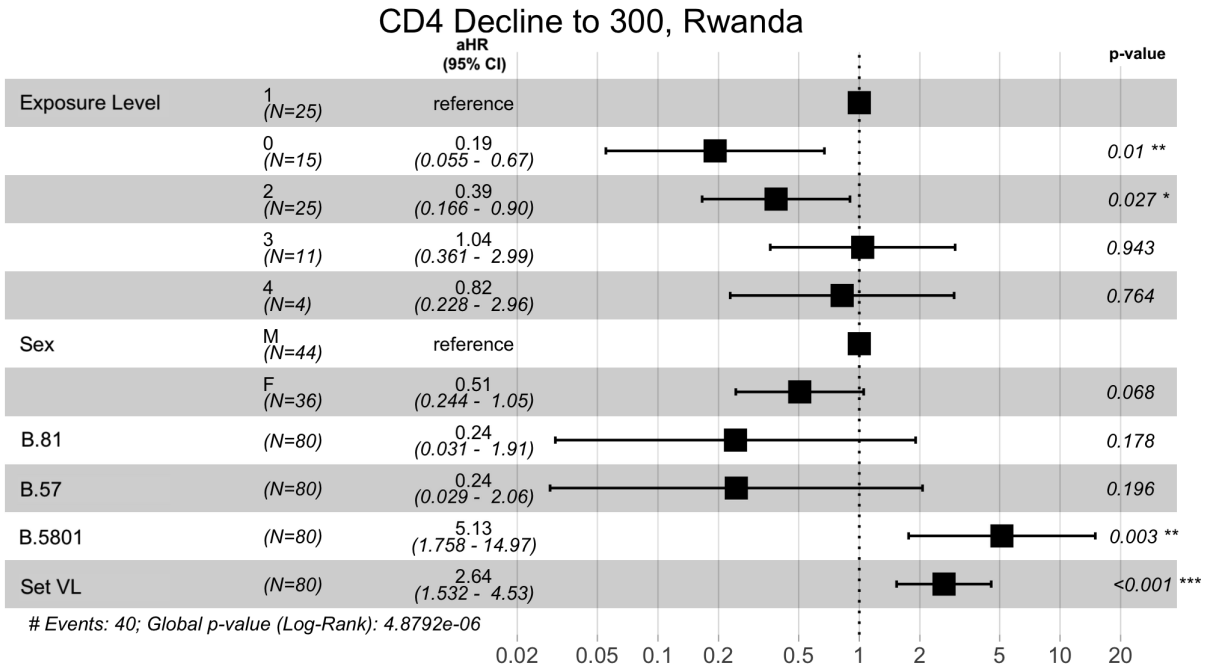
Figure 6: Adjusted Cox proportional hazard regression models for CD4 decline to < 350 cells/µl by exposure level to high affinity FcγRs alleles A. Cox proportional hazard ratios do not suggest that exposure to high affinity FcγR alleles is associated with CD4 decline in Zambia when controlling for human leukocyte antigen (HLA) B.57 and set point viral load. B. In Rwanda, individuals with exposure level 2 experienced a statistically significantly slower decline to CD4 <350 cells/µl versus individuals with exposure level 1 after controlling for biological sex, HLA B.5801, and set point viral load. (aHR for set point viral load indicates hazard per unit (cells/µl) increase, aHR = adjusted hazard ratio, VL = viral load (copies/mL), * = <0.05, ** = <0.01, *** = <0.001)



Supplemental Figure 1: Rwandan men with at least one high affinity FcγRIIIA allele become HIV infected more quickly than men with the homozygous low affinity genotype



Supplemental Figure 2: Rwandan individuals with exposure level 4 become infected more quickly than those with other exposure levels



Supplemental Figure 3: Adjusted Cox proportional hazard regression models for CD4 decline to <300 cells/μl in Rwanda

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Chapter III: HLA-associated preadaptation in HIV Vif is associated with higher set point viral load and faster CD4 decline in Zambian transmission pairs

Abstract

Objective(s): We examined the relationship between HLA-associated preadaptation and HIV-1 disease progression for the entire subtype C HIV-1 proteome in a cohort of heterosexual linked transmission pairs in Zambia.

Design: An adaptation model was used to calculate an adaptation score for each virus-HLA combination in order to quantify the degree of preadaptation of the transmitted virus to the linked recipient's HLA alleles. These scores were then assessed for their relationship to viral load and longitudinal CD4 decline in the recipient.

Methods: Viral RNA was extracted from the plasma of the donor partner and the linked recipient near the time of transmission, as well as longitudinally from the linked recipient. Adaptation scores were calculated for each individual and each protein in the subtype C HIV-1 proteome.

Results: The majority of HLA-associated sites were located in Gag, Pol, and Nef, however proportional to protein length the accessory and regulatory proteins contained a relatively high proportion of HLA-associated sites. Over the course of infection, HLA-adaptation increased for all proteins except Vpu and gp120. Preadaptation was positively associated with higher early set point viral load and faster CD4 decline. When examined by protein, preadaptation in Pol and Vif were statistically significantly associated with these markers of disease progression.

Conclusions: Despite containing a large proportion of HLA-associated sites, preadaptation in HIV-1 regulatory and accessory proteins, with the exception of Vif, did not correlate with disease progression.

Introduction

Viruses coexist with their host in a delicate balance of survival and escape. HIV-1 is particularly adept at striking this balance through its error-prone reverse transcriptase(1), hypervariable(2) and heavily glycosylated envelope proteins(3), and viral proteins with key immune evasion functions(4). Most of these escape mechanisms occur within functional constraints, with some mutations incurring a fitness cost which forces the virus to sacrifice replicative capacity in order to dodge the immune response(5, 6). In cases where an escape mutation carries a large fitness detriment, the mutation often reverts to the original sequence after transmission to a new host with a different immune environment because the benefit no longer outweighs the cost(7). However, if the mutation does not incur a fitness cost, it may be retained in the viral sequence through future transmission events, and become part of the circulating consensus sequence(8).

In defense, human evolution has built diverse immune responses between individuals with a wide variety of antibodies and human leukocyte antigen (HLA) genes which encode the major histocompatibility complex (MHC) proteins that present antigens to T cells. In spite of this, through decades of coevolution, the HIV genome has accumulated low-fitness-cost immune escape mutations, particularly to HLA alleles, which has led to instances where the transmitted virus is preadapted and carries host-specific HLA mutations from the moment of transmission. A study of transmission pairs from Zambia found that 77% of polymorphisms in the HIV-1 *gag* gene were attributable to evasion of HLA class I alleles in the Zambian population, and that on average 19% of polymorphisms that were transmitted were already suited to escape the new host's HLA alleles(9).

This preadaptation has negative outcomes in terms of disease progression. In the same study of Zambian transmission pairs, the ratio of polymorphisms preadapted to the recipient's HLA alleles versus polymorphisms that were not preadapted, was the strongest predictor of higher early set point viral load and CD4 decline to below 350 cells/μl(9). Similar findings were reported in a study of subtype C Gag, Pol, and Nef where greater preadaptation was associated with higher early set point viral load in the recipient and more rapid CD4 decline(10).

The majority of research in this area has focused on the HIV-1 proteins Gag, Pol, and Nef. However, one study which examined the entire subtype C HIV-1 proteome observed that several accessory and regulatory proteins, including Nef, Rev, Tat, and Vif, had a higher proportion of HLA-associated amino acid sites, relative to length, than Gag and Pol(11). Their findings also suggested that mutations in Vpr, Gag, and Rev may be associated with a higher fitness cost, due to a higher ratio of susceptible to resistant amino acid residues(11). Thus, the goal of our study was to examine viral adaptation to HLA class I alleles across the entire subtype C HIV-1 proteome in a cohort of epidemiologically-linked transmission pairs from Zambia in order to determine the impact of HLA-associated adaptations in all proteins on disease progression.

Methods

Study population: Samples were obtained from a historical cohort study conducted by the Zambia-Emory HIV Research Project (ZEHRP) on heterosexual HIV-1 serodiscordant couples. Both partners were enrolled for longitudinal follow-up starting in 1994 in Zambia. Individuals were provided with free healthcare, couples' voluntary HIV counseling and testing(12, 13), and risk-reduction counseling. All samples used in this study were from ART-naïve participants.

Ethics statement: Written informed consent was obtained from all participating couples. This study was approved by the Institutional Review Board of Emory University and the University of Zambia.

Sequencing: Viral RNA was extracted from 140µl of plasma from both the donor and linked recipient partners near the time of transmission, as well as at 3, 6, 9, 12, 18, and 24 months post-infection from the linked recipient using the Viral RNA Extraction Kit (Qiagen) and eluted in 60µl of elution buffer. Viral RNA (5µl) was then amplified in two half genome fragments in a 50µl reaction with SuperScript III One-Step RT-PCR System (ThermoFisher Scientific) and 1ul of 20µM primers. First round RT-PCR primers included: 5' segment, Gag Outer For (forward) 5′-ATTTGACTAGCGGAGGCTAGAA-3′ and VIF OR (reverse) 5′-TTCTACGGAGACTCCATGACCC-3'; 3' segment, Vif1 (forward) 5′-GGGTTTATTACAGGGACAGCAGAG-3′ and OFM19 (reverse) 5′-GCACTCAAGGCAAGCTTTATTGAGGCTTA-3′. A second round of PCR was performed with Expand High Fidelity enzyme (Roche), 1µl of the first round PCR product, and 0.5µl of 20µM primers in a 25µl reaction. Second round primers included: 5' segment, Gag Inner For

(forward) 5′-TTTGACTAGCGGAGGCTAGAAGGA-3′ and VIF IR (reverse) 5′-TCCTCTAATGGGATGTGTACTTCTGAAC-3′; 3’ segment, VIF2 (forward) 5′-GCAAAACTACTCTGGAAAGGTGAAGGG-3′ and OFM19 (reverse). Amplicons from three positive PCR reactions for each individual were pooled and sequenced by 454 Sequencing separately by gene using gene-specific primers. Sequences for Gag, Pol, and Nef were published previously(14).

Modeling adaptation score: Adaptation scores were calculated for each virus/HLA pair relative to an HLA-null model of amino acid residue probabilities, as described previously(10). Briefly, adaptation scores represent the average probability of observing a given viral amino acid sequence in the context of a particular set of HLA alleles. An adaptation score is computed for every sequence-allele pair, yielding a total of six adaptation scores for each individual (a score for each of their HLA-A, HLA-B, and HLA-C alleles), which are averaged to produce an overall adaptation score. Adaptation scores for HLA-A, B, or C separately can be calculated by averaging the two corresponding adaptation scores. Each adaptation score is a product of ratios, one for every amino acid position in the sequence, dividing the probability of observing each amino acid in the presence of the particular HLA allele by the probability of observing each amino acid in an HLA null model: $\Pr(S = s \mid H = h) / \Pr(S = s \mid H = \varnothing)$, where S is the amino acid present in the sequence and H is the HLA allele. An important assumption of this model is the independence of amino acid positions.

The model was trained on 430 full-length chronic subtype C HIV-1 sequences from Zambia and South Africa for which HLA type was also available, in order to identify HLA-

associated amino acid residues. Phylogenetic-correction was performed with phylogenetic trees constructed in RaxML and the adaptation model was run in Matlab.

Autologous adaptation scores were calculated by matching the viral sequence from a given individual with their HLA-alleles. Heterologous adaptation scores were calculated by pairing random viral sequences in the study population to randomly selected host's HLA alleles from the study population. This randomization was completed for 10,000 random virus-HLA combinations.

The number of HLA-associated sites was counted and included all matches below a false-discovery threshold of 0.2. Each associated site was only counted once, regardless of the number of associations detected for that position. The proportion of associated sites was determined relative to the viral sequence length in nucleotides of the model input files, divided by three and rounded to the nearest whole number. The calculated length in amino acids by protein was: Gag 660, gp120 145, gp41 380, Nef 244, Pol 1088, Rev 129, Tat 110, Vif 202, Vpr 101, Vpu 110.

Disease progression: Set point viral load was calculated for 133 individuals as the geometric mean of all viral load measurements between 30-365 days post-infection. Longitudinal CD4 count measurements were available for 66 individuals with follow-up time ranging from 3.5 months to over 6.5 years, with the average CD4 count follow-up time being 3.9 years.

Statistical analysis: R was used for all analyses and statistical tests, aside from the adaptation model. The association between set point viral load and adaptation score was tested by Spearman correlation, separately for each protein as well as overall. "Overall" represents the average adaptation score of all HIV-1 proteins in a given individual. CD4 decline was plotted on Kaplan-

Meyer survival curves with adaptation score dichotomized at the median for visualization. This association was quantified for each protein separately in a univariate Cox proportional hazard model with adaptation score as a continuous predictor. For these analyses, adaptation score was scaled by a factor of 10 such that the hazard ratios are representative of the increase in hazard for a 0.1-unit increase in adaptation score.

Results

Accessory proteins contain a high relative proportion of HLA-linked sites

We counted the number of amino acid positions in each HIV-1 protein associated with any HLA allele in a population of Zambians chronically infected with HIV-1. This included sites for which a particular amino acid was more likely to be present in the presence of a particular HLA allele as well as sites for which a particular amino acid was less likely to be present in the presence of a particular HLA allele. Across all proteins, we identified a total of 362 HLA-associated amino acid sites. Of these, 105 had an association with more than one class of HLA allele, for example the site was associated with at least one HLA-A and HLA-B allele. Overall, there were 149 (32%) sites associated with HLA-A, 203 (43%) sites associated with HLA-B, and 115 (25%) sites associated with HLA-C.

Gag, Pol, and Nef contained the majority of the HLA-associated sites in the HIV-1 genome (Figure 1a). However, considering that Gag and Pol are the largest viral proteins, and Nef the most immunogenic, HLA-associated sites were also quantified in proportion to protein length (Figure 1b). After normalizing by number of amino acids, it was observed that the accessory (Nef 24%, Vif 15%, Vpr 19%, and Vpu 12%) and regulatory proteins (Tat 23% and Rev 19%) contained a similar proportion of HLA-associated sites, and surpassed the proportion of sites identified in Gag (9%) and Pol (11%). HLA-associated sites were less common in the Env glycoproteins, gp120 (3%) and gp41 (3%).

Viral proteins are adapted to host-matched HLA alleles

Autologous adaptation is the degree to which a viral sequence is adapted to its host-matched HLA alleles. Heterologous adaptation, on the other hand, is the degree to which a viral

sequence is adapted to a randomly selected host's HLA alleles, from the same study population. Comparing these types of adaptation with a numeric adaptation score contextualizes and quantifies viral evolution in response to host HLA pressure. Over all HLA alleles and viral proteins, autologous adaptation scores were statistically significantly higher than heterologous adaptation scores ($t=41.2$, $p=<0.0001$), indicating that on average, a virus is more adapted to host-matched HLA alleles than to a random set of HLA alleles (Figure 2a). Similarly, autologous adaptation in Gag, Pol, and Nef to all HLA alleles was statistically significantly higher than heterologous adaptation ($t=41.0$, $p=<0.0001$), as has been shown previously (Figure 2b)(10).

The majority of adaptation, autologous and heterologous, was observed in Gag, Pol, and Nef (Figure 2c). A modest amount of adaptation was observed in the regulatory and accessory proteins, with overall HLA adaptation in Vif being the most pronounced. Heterologous adaptation was greatest towards HLA-B alleles. Autologous adaptation was observed overall, as well as to HLA-A and HLA-B alleles. Autologous adaptation to HLA-C was only detected in Gag, Pol, and Nef, and was lower compared HLA-A and HLA-B.

Host-specific adaptation increases over time

Longitudinal viral sequences from epidemiologically-linked transmission pairs allowed us to examine the evolution of HLA-adaptation over the first two years of infection. Overall HLA adaptation score increased from the time of transmission to 24 months in all proteins, except for gp120 and Vpu which decreased (Figure 3a).

Over the same time period, viral sequences compared to the previous host's (donor partner) HLA alleles maintained a positive adaptation score, decreasing slightly from time of

transmission to 24 months after infection of the linked recipient partner (Figure 3b). The greatest decrease in overall adaptation score is observed in Gag, Pol, and Nef.

Higher transmitted adaptation is associated with accelerated disease progression

Higher set point viral load is a clinical indicator of more severe HIV-1 disease progression. Among linked recipients, those infected with a more preadapted virus overall experienced a higher set point viral load in the first year of infection (Spearman ρ=0.25, p=0.004) (Figure 4). This positive association remains statistically significant for Pol (Spearman ρ=0.26, p=0.004) and Vif (Spearman ρ=0.25, p=0.005) when analyzed by protein.

Another clinical indicator of disease progression is the decline of $CD4^+$ T cells. In a univariate Cox proportional hazard model using the scaled adaptation score as a continuous predictor, individuals infected with a virus having a higher transmitted adaptation score reached the CD4 endpoint of less than 250 cells/μl at a faster rate than individuals with a less preadapted virus (HR=3.03, p=0.04) (Figure 5a). When examined by protein, higher preadaptation in Pol (HR=1.37, p=0.05) and Vif (HR=1.71, p=0.03) was statistically significantly associated with this marker of HIV-1 disease progression (Figures 5b, 5c).

Discussion

We observed that the subtype C HIV-1 accessory and regulatory proteins contained a relatively high proportion of HLA-associated sites, concurrent with previous findings(11). Similarly, most of these associations were with HLA-B, followed by HLA-A(11, 15-17). However, despite the high proportion of HLA-associated sites in these accessory and regulatory proteins, little correlation was observed with disease progression, aside from Vif.

Interestingly, in macaques, those vaccinated against Gag and Vif $CD8^+$ T-cell epitopes controlled SIV infection upon challenge, whereas those vaccinated against Nef epitopes did not(18). Several other SIV studies have also suggested that Vif-specific responses are important for viral control(19, 20). These findings complement those of our study because if a Vif-specific $CD8^+$ T cell response is an important element of control and an individual is infected by a virus with a higher degree of Vif preadaptation, it argues that this evasion in Vif would correlate with faster markers of disease progression, as we observed.

In chronically infected individuals, as demonstrated by us and others(10), viruses are on average more adapted to their host-matched HLA alleles than to a randomly-matched host's HLA alleles from the same population. Nevertheless, the distributions of heterologous and autologous adaptations overlap indicating there remains a population of viruses that are more adapted to a random set of HLA alleles than to the current host, which highlights the possibility of transmission of a preadapted virus.

The median heterologous adaptation to HLA-B was especially low for Gag, Pol and Nef, likely due to the wider diversity of HLA-B alleles in a population(21). If a virus adapts to evade a specific HLA-B allele, the probability of the virus being transmitted to another individual carrying the same HLA-B allele is small due to the larger number of HLA-B variants,

exemplifying the evolutionary benefit of HLA diversity. Conversely, preadaptation would be more likely for HLA-A, which represents approximately one third of all HLA-associated sites in the HIV-1 genome and fewer allele variants in the population. We found this to be true in our study for Nef, Rev, and Vif, where the median HLA-A heterologous adaptation score was positive.

Proteins adapt from the time of transmission to reach this chronic level of host-specific adaptation. Gag, Pol, and Nef showed the greatest increase in adaptation to the new host immune environment, as well as the greatest decrease in adaptation to the previous host's HLA alleles, consistent with previous findings(10). In all proteins however, except Vpu, we found that after 24 months post-transmission, the virus was still more adapted to the donor's HLA alleles than to the recipient. This suggests that many HLA-associated adaptations do not carry heavy fitness burdens that revert upon transmission to a new host, and are being retained in the viral sequence which could then be propagated in future transmission events.

Interestingly, and despite the large proportion of HLA-associated sites, longitudinal adaptation in the accessory and regulatory proteins was minimal, both with respect to the donor and recipient HLA alleles, and no significant association with disease progression was detected. Two hypotheses might explain these observations. The first is that these proteins have lower structural and functional constraints than Gag and Pol(22). Supporting this hypothesis is the observation that HLA-associated adaptation in Pol is relatively limited for the first 9 months of infection, after which there is a rapid increase. It is possible that compensatory mutations are required before HLA-associated mutations can be supported. Building on this, in the absence of such stringent functional constraints, the accessory and regulatory proteins would have more positions within HLA-restricted epitopes that could tolerate mutations. Because our adaptation

model assumes all sites to be independent, and we did not group mutations by epitope, it is possible we did not have sufficient power to detect associations at these sites with disease progression.

Second, HLA-associated mutations in the accessory and regulatory proteins may truly have less impact on disease progression because these proteins are expressed, and therefore presented as peptides, in lower levels compared to Gag and Pol and at different times in the viral life cycle(23). In particular, we believe HLA-associated adaptations to Env glycoproteins were especially limited because Env is primarily targeted by antibodies, not T cells. Furthermore, the amount of glycosylation present on gp120 and gp41 prohibits efficient presentation of Env antigens to T cells(24).

One limitation of our study is the resolution with which we identified HLA-associated sites. Previous studies extended their HLA-associated sites to include amino acid polymorphisms located in known CTL epitopes(9), as well as sites flanking known HLA-restricted epitopes(15). In both of these studies, a statistically significant association was observed between HLA-adaptation in Gag and viral load. However, when Goepfert et al. examined viral load in relationship to all HLA-associated mutations in Gag, not limited to those flanking CTL epitopes, the association was no longer significant(15). Perhaps our inability to detect a statistically significant association between preadaptation in Gag and set point viral load could be due to our inclusion of all HLA-associated sites identified in Gag, without narrowing our focus to known CTL epitopes. The lack of association between disease progression and HLA-associated adaptation in Nef is consistent with previous findings(5, 10, 15).

Overall, we have demonstrated that the levels of HLA-associated preadaptation in the HIV-1 regulatory and accessory proteins were not associated with HIV-1 disease progression, with the

exception of Vif. Vif plays a crucial role in viral infectivity and reverse transcription(25) and its ability to evade the early CTL immune response could grant the virus a significant advantage. Similarly, due to the length of time required for HLA-associated adaptations in Pol to develop, it makes sense that preadaptation in Pol would have notable consequences in terms of disease progression. There could be several reasons why preadaptation in the other accessory and regulatory proteins were not associated with disease progression, ranging from functional constraints to lower levels of protein expression. Future studies focusing on HLA-associated adaptation in HIV-1 accessory and regulatory protein epitopes are warranted to increase power and lead to a better understanding of the role these adaptations may play in disease progression. Furthermore, exploring the specific epitopes responsible for preadaptation in Vif could be relevant to designing T-cell targets for a preventive HIV-1 vaccine.

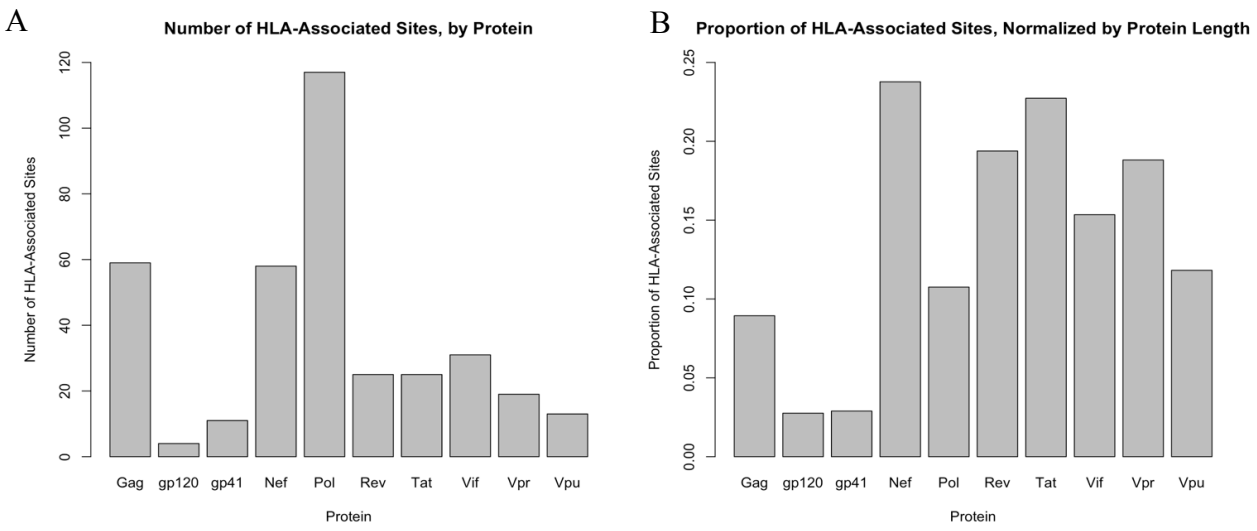

Figure 1: HLA-associated sites by protein (A) Number and (B) proportion of amino acid positions associated with HLA allele(s). Pol contains the greatest number of HLA-associated sites (117), followed by Gag (59) and Nef (58). Nef contains the highest proportion of HLA-associated sites (24%), followed by the regulatory proteins Tat (23%) and Rev (19%).

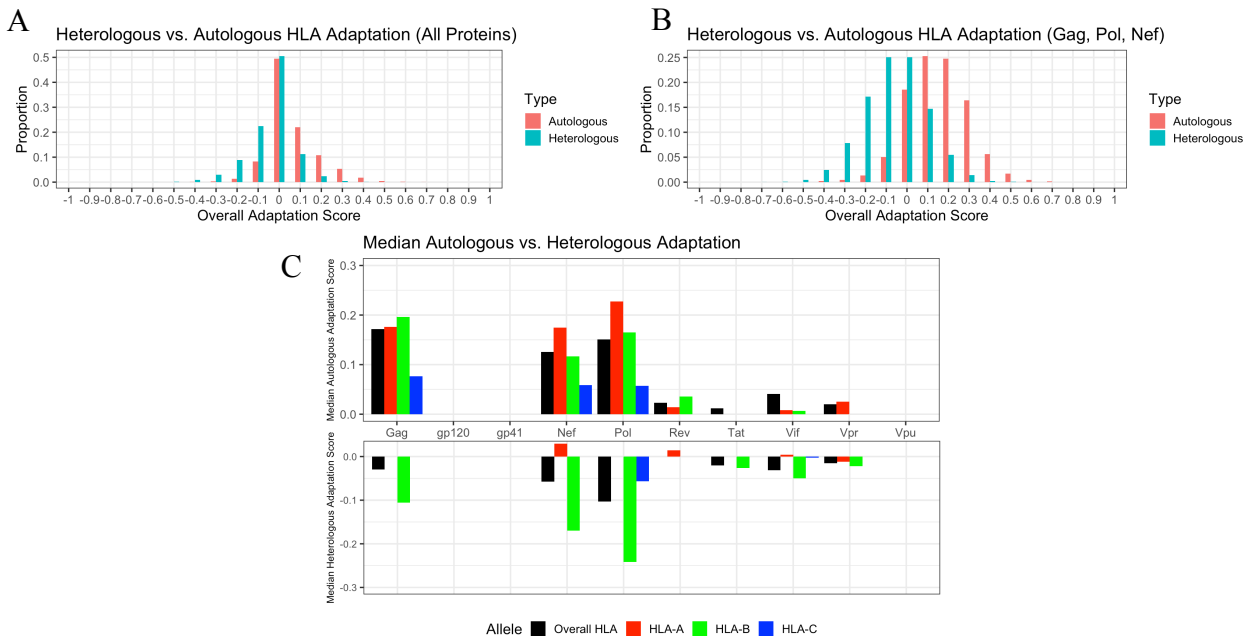

Figure 2: Heterologous vs. autologous adaptation (A) Autologous adaptation scores are statistically significantly higher than heterologous adaptation scores for all proteins, across all HLA alleles (t = 41.2, p = <0.0001). Mean overall autologous adaptation score is 0.06, mean overall heterologous adaptation score is -0.03. (B) Autologous adaptation scores are statistically significantly higher than heterologous adaptation scores for Gag, Pol, and Nef combined, across all HLA alleles (t = 41.0, p = <0.0001). Mean overall autologous adaptation score is 0.15, mean overall heterologous adaptation score is -0.06. (C) Median autologous and heterologous adaptation scores by protein and HLA allele.

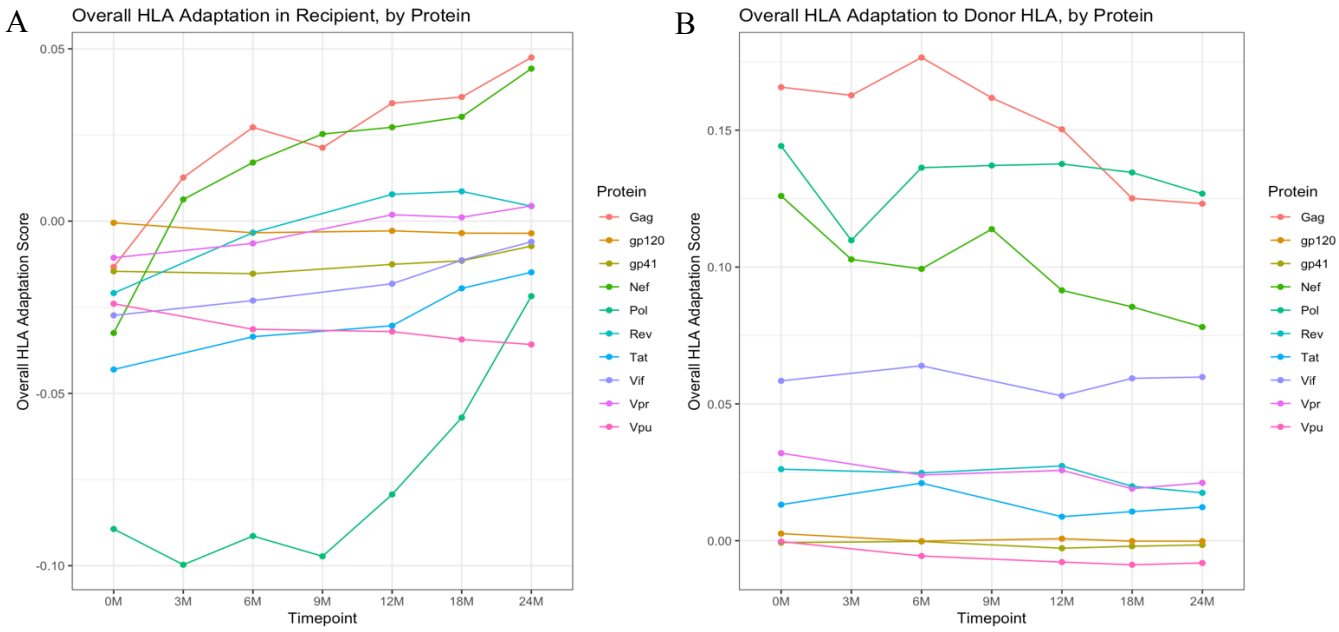

Figure 3: Longitudinal adaptation by protein (A) Overall HLA adaptation to the linked recipient's HLA alleles increases from the time of transmission to 24 months. (B) Overall HLA adaptation to the donor partner's HLA alleles decreases from the time of transmission.

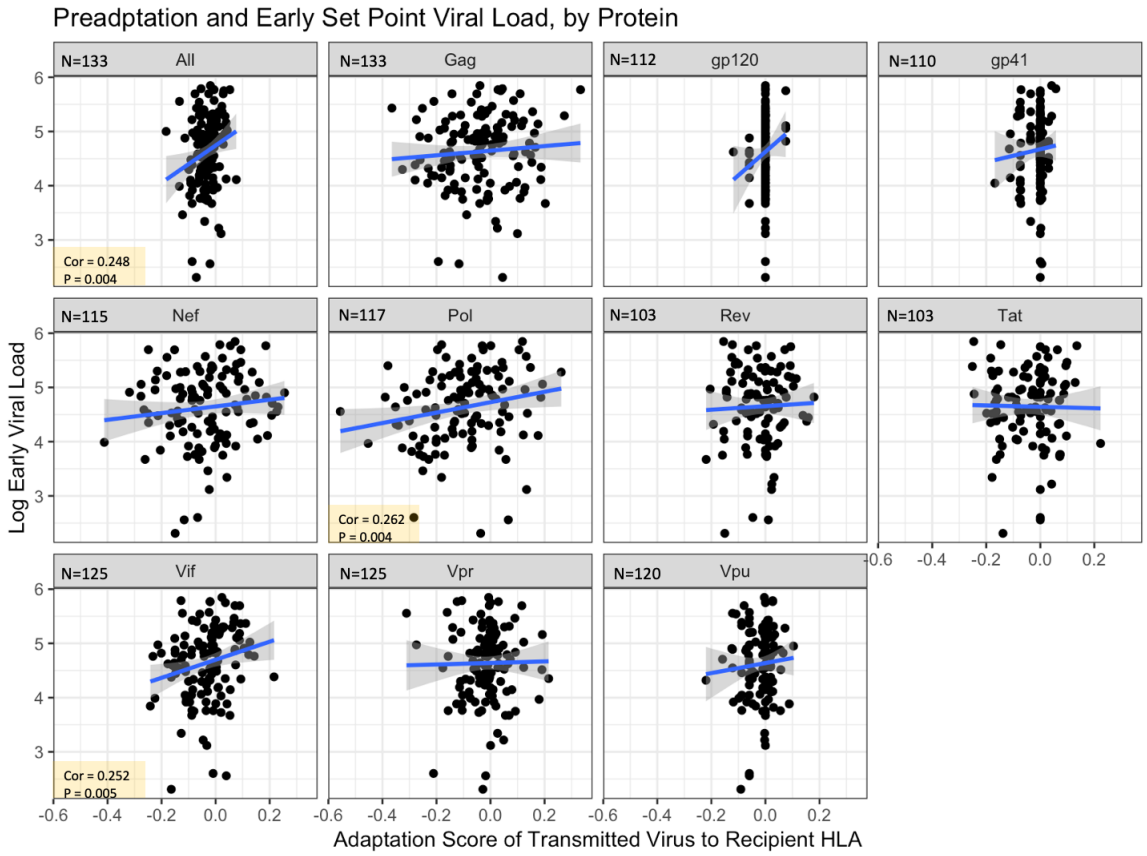

Figure 4: Preadaptation vs. viral load by protein Increased overall adaptation of the transmitted virus to the recipient's HLA alleles is correlated with higher early set point viral loads. Separately, higher preadaptation in Pol and Vif are correlated with higher viral load.

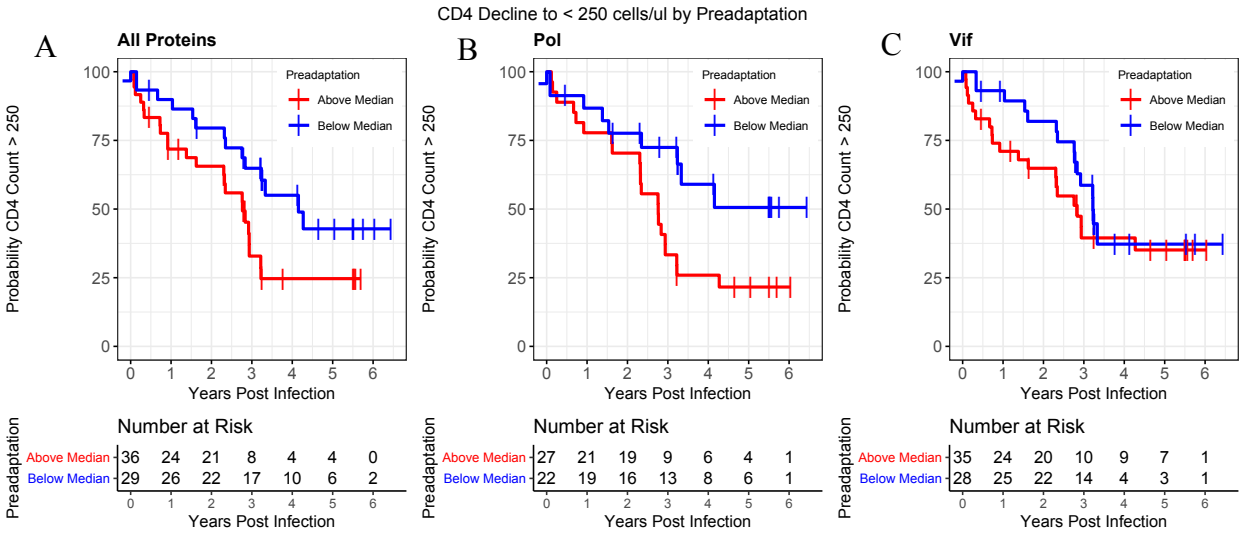

Figure 5: Preadaptation and CD4 decline to < 250 cells/µl Disease progression measured by CD4 decline to less than 250 cells/µl, is more rapid for individuals with higher preadaptation in (A) all proteins (HR=3.03, p=0.04), (B) Pol alone (HR=1.37, p=0.05), and (C) Vif alone (HR=1.71, p=0.03). For visualization, adaptation score was dichotomized above and below the median. Scaled adaptation score as a continuous predictor was used in the univariate Cox proportional hazard model.

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Chapter IV: Sociodemographic factors and STIs associated with *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infection in Zambian female sex workers and single mothers

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Abstract

Sexually transmitted infections (STIs) in women caused by *Chlamydia trachomatis* (CT) and/or *Neisseria gonorrhoeae* (NG) are epidemiologically distinct. In this study, associations with sociodemographic and clinical risk factors are explored separately for CT and NG. Multivariate logistic regression (MLR) models quantify associations between potential CT and/or NG risk factors within a cross-sectional study of high-risk women in two Zambian cities, Lusaka and Ndola. CT was associated with living in Lusaka, younger age, and literacy. Long-acting reversible contraception (LARC) was predictive of CT in Ndola, but protective in Lusaka. In Lusaka only, CT was associated with lower education and reported unprotected sex. NG was associated with younger age, lower education, concurrent *Trichomonas vaginalis*, bacterial vaginosis, and incident syphilis infection. Signs and symptoms were rare and not associated with either infection. CT was more prevalent, nearly 11%, compared to NG, 6.8%. The higher prevalence of CT could explain the lack of association with other STIs. The associations observed with NG could be the result of high-risk sexual networks or lack of protective immunity. Risk factors for CT and NG are distinct and may differ geographically, which should be considered when developing diagnostic tools or guiding presumptive treatment in specific populations.

Introduction

Chlamydia trachomatis (CT) and *Neisseria gonorrhoeae* (NG) are bacteria that result in sexually transmitted infections (STIs) worldwide. In women, these infections are frequently asymptomatic and can cause pelvic inflammatory disease, pregnancy complications, and infertility, as well as increase the risk for HIV acquisition(1-3). Both infections are curable with antibiotics, however the rise of antibiotic resistant NG strains are an increasing threat to treatment(4).

Longitudinal and cross-sectional studies have suggested partial protective immunity to CT. In a study of female sex workers (FSW) in Nairobi, younger age and fewer years working as a FSW were associated with increased incidence of CT, suggesting that despite continued exposure, protective immunity prevented recurrent infections in older and more experienced FSWs(5). Similarly, among FSWs in Benin, longer duration of sex work was associated with protection against CT/NG infection(6). Animal studies have shown that the immunity to CT is mediated by $CD4^+$ T cells(7, 8) and the cytokines INF-y and IL-13 have been associated with CT/NG protection in humans(5). No protective immunity has been described in NG, but rather an array of host-evasion strategies precludes the establishment of an effective immune response(9).

Although the mode of transmission for CT and NG is the same, each pathogen has unique characteristics. Due to similar symptomology and limited resources, symptomatic women may be treated for both infections under syndromic STI management(10). However, with potential protective immunity to CT and the mounting risk of antibiotic resistance by NG(4), it is important to investigate how the risk profiles for infection by each of these unique bacteria differ within the same study population.

Methods

Study Population: An established prospective cohort of HIV-uninfected high-risk women (HRW) in Lusaka and Ndola, Zambia was examined(11-13). The women included were either FSW or single mothers with children under the age of five (SM) referred to the Zambia-Emory HIV Research Project (ZEHRP) from local post-natal clinics or invited from community outreach at sex work hot spots.

Data from a 2016 cross-sectional sub-study on intra-vaginal practices (IVP) in this cohort was the source of STI test results and clinical observations(14). The IVP study included a survey of sociodemographic factors, IVP, symptoms, and sexual behaviors. A clinical exam was performed where venous blood and endocervical swabs were collected by a nurse. All participants were STI tested, regardless of reported symptoms. Samples were tested for HIV by rapid test (Determine HIV-1/2 Ag/Ab Combo, Uni-Gold confirmatory) and GeneXpert; syphilis by rapid plasma reagin (RPR); *Trichomonas vaginalis* (TV), candida, and sperm by wet mount microscopy; and bacterial vaginosis (BV) by KOH whiff test and clue cell identification. Women with bacterial STIs received free treatment onsite and partners were invited for treatment. Women testing HIV positive were referred to government clinics for antiretroviral therapy. A total of 825 unique clinic visits where CT/NG GeneXpert testing was performed were available for analysis. There were several instances where the same individual was tested at two separate clinic visits, however these entries were not excluded from the analysis because GeneXpert testing and survey responses were available for both time points.

Variables: All samples and data were gathered between September 2016 and January 2019. The two outcome variables for this study were CT (including CT/NG co-infected), and NG (including

CT/NG co-infected). The reference group for both outcomes was the CT and NG uninfected population. In the models where CT was the outcome of interest, those infected with NG only were omitted from the analysis; the reverse is true for the models where NG was the outcome of interest.

Clinical signs were any of the following on either the external or internal genitalia during pelvic examination: inguinal adenopathy, inflammation, ulceration, condyloma or warts, cervicitis, cervical discharge or pus, vaginal discharge, erosion or friability of the cervix or vagina, non-menstrual bleeding, or adnexal tenderness or mass. Symptoms were recorded if the participant spontaneously reported the symptom or if they reported it after being specifically prompted, and whether the symptom was currently present or previously treated elsewhere and was now resolved. The following symptoms were evaluated: cystitis, dysuria, vaginal itching, vaginal discharge, dyspareunia, lower abdominal pain, or acute/chronic/recurrent genital ulcer.

Laboratory tests assessed the presence of TV, HIV, sperm on a vaginal swab, candida, BV, and syphilis. BV was considered present if a woman had both a positive KOH whiff test and clue cells observed on microscopy. The presence of an incident syphilis infection was determined by a proxy, receiving treatment for syphilis, due to the high prevalence of serofast RPR results in Lusaka(15, 16).

Age groups (18-24 and 25+) were tabulated based on birth year and age in 2016. Literacy was determined based on whether the participant could read English or the predominant local dialect (Nyanja, the lingua franca in Lusaka or Bemba, the predominant language of communication in Ndola) without difficulty. Education was based on the level of schooling completed and grouped by either none/primary or secondary/college.

Long-acting reversible contraception (LARC) was considered to be either the hormonal implant or the intrauterine device (IUD); injectable contraception was not categorized as LARC. Other variables considered were city of residence, reported sex without a condom at least once in the past one-three months, reported history of transactional sex, and self-reported pregnancy.

Logistic Regression Modeling: Overall frequencies of each covariate, as well as frequencies stratified by city, were first compared via chi-square or Fisher's exact test for each outcome to the uninfected group. Instances where the stratified chi-square or Fisher's exact p-value was less than 0.05 in one city but not the other, or significant in both cities but not overall, were included as an interaction term by city in the logistic regression model.

The probability of a positive outcome follows a binomial distribution. Each covariate and the interaction terms were initially considered in bivariate logistic regression models. Variables that resulted in a significant association at the bivariate level were included in the full multivariate logistic regression model (MLR). For each of the full models, a data subset was created that excluded missing values for all predictors in the model. The full model then underwent bi-directional stepwise model selection using the R function stepAIC to arrive at the final MLR model.

Collinearity of the full model was assessed by examining the correlations between each of the variables in the model and the variance inflation factors (VIF). All of the correlation coefficients were below 0.5 and all of the VIFs were below 10, so no variables were eliminated due to collinearity.

Ethics: These investigations were approved by the Institutional Review Board of Emory University and the Research Ethics Committee of the University of Zambia. Participants completed written informed consent before survey administration or study specimen collection.

Results

Clinical signs and symptoms are not significantly associated with CT/NG status

There were a total of 68 CT-only cases (26 Lusaka, 42 Ndola), 34 NG-only cases (6 Lusaka, 28 Ndola), and 22 CT & NG co-infected cases (4 Lusaka, 18 Ndola). The remaining 701 individuals were both CT and NG uninfected (169 Lusaka, 532 Ndola). The prevalence of CT was higher in Lusaka compared with Ndola (15% vs. 10%, p=0.053), while the prevalence of NG was not different (5% vs. 7%, respectively). Twenty-four women seroconverted to HIV during cohort follow-up and were HIV+ at the time of sample collection for CT/NG testing. HIV was not associated with either CT and/or NG (4/24 HIV+ vs. 119/794 HIV-, p=0.77).

Pelvic exams were performed on 530 participants at the time of CT/NG testing. The majority of women, across all infection categories, had vaginal discharge not associated with CT or NG (Table 1). The number of women with other abnormalities noted on gynecologic exam was small: external ulceration in 2 women and internal ulcers in 1; cervicitis in 2; and pus in the cervix in 5. None of these signs were associated with CT and/or NG (not shown). The only clinical sign that was statistically significantly related with CT or NG was internal genitalia adnexal tenderness (Fisher's exact p=0.03, n=2); both women who presented with this sign were infected with CT, whereas there were zero women with this sign in any other infection status group. All other signs and reported symptoms were present at low frequencies and were not statistically associated with either CT or NG.

Symptoms reported by 559 women were also uncommon, with cystitis/dysuria reported by 3, vaginal itching by 6, dyspareunia by 2, lower abdominal pain by 4, and acute genital ulcer by 3. No symptoms were associated with CT and/or NG (not shown).

STIs and vaginal dysbiosis are associated with prevalent CT/NG infection

Due to the limited number of positive STI observed, many covariates contained only a small sample size, however several statistically significant associations were detected. TV infection was statistically significantly associated with NG overall and within each city (Table 2), as well as with CT overall and within Lusaka. Receiving treatment for an incident syphilis infection and the BV composite variable of positive KOH whiff test and presence of clue cells were statistically significantly associated with NG overall and within Ndola, however were not associated with CT. Visualization of sperm on microscopy was statistically significantly associated with CT overall. Visualization of candida and testing newly positive for HIV by rapid test were not associated with either of the outcomes regardless of stratification.

Various sociodemographic factors are associated with prevalent CT and/or NG infection

Sociodemographic and risk behaviors varied in their associations to each outcome when stratified by city (Table 3). Age group and education level, which also appeared to interact with city, were associated with both outcomes. Literacy, unprotected sex in the last 1-3 months, and LARC usage were statistically associated with at least one outcome in at least one city. Transactional sex and pregnancy status were not associated with any outcome in either city.

Of the IVP that were included in this study, only washing the external genitalia with detergent was found to be statistically significantly associated with NG (Fisher's exact $p=0.05$) (Table 1).

CT infection is associated with younger age, higher literacy, lower education, unprotected sex, and LARC usage in a multivariate logistic regression model

Bivariate associations with CT infection were seen with city, age group, literacy, unprotected sex, LARC, and TV (Table 4). Interaction terms with city for age group, literacy, education, unprotected sex, LARC usage, and TV were included based on statistical significance in Tables 2 and 3. The full MLR model contained all interaction terms and their lower order terms. Stepwise model selection eliminated the interaction terms with TV, literacy, and age group, as well as the main effect for TV. The data subset used for the MLR, which removed missing observations, included 68 cases of CT and 461 CT/NG uninfected individuals.

Women in the 18-24 age group had 2.05 (p=0.02) times higher odds of being infected with CT compared to women 25 years or older, controlling for literacy, education by city, unprotected sex by city, and LARC by city (Table 4). Interestingly, women that could not easily read English, Bemba, or Nyanja had less than half the odds (Adj. OR 0.48, p=0.02) of being infected with CT compared to women that could easily read English, Bemba, or Nyanja, controlling for all other factors in the model.

Significant associations were observed in Lusaka, but not in Ndola, for education and unprotected sex interaction terms. Women who did not attend secondary school in Lusaka had 4.46 (p=0.01) times the odds of being infected with CT than women living in Lusaka who attended secondary school, controlling for all other factors. In Ndola, the odds of not attending secondary school and CT infection was only 1.25 (p=0.53) times higher. Reporting at least one unprotected sex act within the last 1-3 months, compared to none, increased the odds of CT infection by 4.38 (p=0.01) in Lusaka and 1.37 (p=0.38) in Ndola, when controlling for all other factors. Using the implant or IUD had an inverse relationship between cities; controlling for all

other covariates in the model, in Lusaka women who used a LARC method had 0.14 (p=0.02) times the odds of being infected with CT, compared to women who were not using a LARC method. The opposite was true in Ndola, where women using a LARC method had 2.22 (p=0.01) times the odds of being infected with CT compared to women not using LARC.

NG infection is associated with younger age, lower education, TV, BV, and incident syphilis infection in a multivariate logistic regression model

In the initial bivariate analyses of NG infection, age group, education, TV, BV, incident syphilis infection, and washing the external genitalia with detergent were statistically significantly associated (Table 5). Interaction terms by city were included for education, BV, and incident syphilis infection as these variables suggested different effects on NG infection by city, based on chi-square and Fisher's exact test results (Tables 2 & 3). The full MLR model contained all interaction terms, their main effects, and predictors for age group, TV, and external detergent IVP. Stepwise model selection reduced the model to ultimately contain age group, education, TV, BV, and incident syphilis infection. City was no longer represented in the model in interaction terms or as a main effect. The data subset on which the model was tested contained 34 cases of NG and 389 CT/NG uninfected individuals.

Controlling for all other factors in the model, increased odds of NG were observed for age 18-24 (Adj. OR 3.43, p=0.01), not completing secondary school (Adj. OR 3.53, p=0.01), testing positive for TV (Adj. OR 6.84, p<0.01), BV (Adj. OR 2.31, p=0.03), or being treated for syphilis (Adj. OR 9.12, p<0.01) (Table 5).

Discussion

In this study, no clinical signs or symptoms were associated with CT or NG, which implies limited sensitivity of syndromic management. Opposing associations between CT and LARC usage were observed between cities, with LARC significantly increasing the odds of CT infection in Ndola, and significantly decreasing the odds of CT infection in Lusaka. Interestingly, the prevalence of LARC in both cities is nearly identical, 33.3% in Lusaka and 34.6% in Ndola. We have previously shown that, when sensitive measures of condomless sexual exposure in HIV discordant couples are included, hormonal contraception overall, and LARC in particular, are not associated with HIV transmission(17, 18). We also found that LARC use was associated with less condomless sex in discordant couples(19). In the HRW studied here, the measure of condomless sex was imprecise, therefore the interpretation of the LARC associations with CT must be interpreted with caution.

Similar to other studies, we detected associations between NG and BV(20), incident syphilis, and TV. The association between NG and BV may be related to the association observed between IVP and NG in this study due to an underlying relationship between IVP and BV(21), although this connection appears inconsistent(14). The association between NG and other STIs may indicate that women infected with NG engage in risky behavior within a sexual network with a high prevalence of STIs, or hint at potential immunomodulatory aspects of NG infection which create an immunosuppressive environment advantageous for NG and other vaginal pathogens(9).

The lack of association between CT and other STIs may be an artifact of the higher prevalence of CT in this population overall, nearly 11%, compared to NG at 6.8%. This can be demonstrated by the prevalence of CT/NG co-infections in each group, where only 24% of those

infected with CT were also infected with NG, whereas among those infected with NG, 39% were co-infected with CT. Our observations are consistent with CT/NG co-infection rates in comparable populations(22). In particular, within a similar cohort of HIV-uninfected high-risk South African women, 100% (n=43) of the women infected with NG were also co-infected with another STI, whereas only 84% (n=130) of those infected with CT had an STI co-infection(23).

Our data do not strongly support partial immunity to CT. Compared to women over 25 years, young women had twice the odds of being infected with CT controlling for other variables in the model, whereas they had more than 3 times the odds of being infected with NG, a pathogen which does not establish protective immunity. If protective immunity to CT developed, we would expect the adjusted odds ratio for young age in the CT model to be higher than that in the NG model, rather than less. Furthermore, apart from lacking protective immunity, young women may have additional unmeasured risk factors such as increased risk-taking behavior or physiological risk factors such as an immature cervix or cervical ectopy, which has been shown to be associated with CT infections(24). Together, our findings support that young women are more likely to be infected with CT or NG than older women, but do not indicate that this may be the result of protective immunity.

Other limitations include the fact that this was a cross-sectional design examining women who were part of an existing prospective cohort and who did not come to the clinic due to CT/NG symptoms, which likely dampened any associations we might have detected with clinical indicators. However, on the other hand, this highlights the prevalence of asymptomatic CT and NG infections. Finally, the data regarding risk behaviors and sexual practices were obtained via self-report, which could be subject to recall and social desirability bias.

Overall, the results presented here describe various demographic, social, and clinical factors that are associated with CT/NG infections among HRW in Zambia. This information is useful for parsing out unique risk factors for CT versus NG and understanding how the epidemiology of these diseases vary within the same population. These data could also be useful for developing risk assessment tools to improve detection strategies in low-resource settings, especially among asymptomatic women. However, detection and treatment should be accompanied by social intervention, such as risk-reduction counseling, voluntary couples testing(25), and partner services(26). Without such services reinfection will occur, potentially at an increased rate as it is hypothesized that early treatment disrupts the development of protective immunity to CT(27, 28). Together with a comprehensive intervention program or effective vaccine, and epidemiologic guidelines, case detection and clinical outcomes can improve.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Table 1: Prevalence of signs and IVP by CT and/or NG status and city, Zambian HRW, 2016

	Overall								Lusaka				Ndola	
	CT Only (n=68)		NG Only (n=34)		CT & NG Co-infected (n=22)		CT/NG Uninfected (n=701)		Fisher's p-value CT vs Uninfected	Fisher's p-value NG vs Uninfected	Fisher's p-value CT vs Uninfected	Fisher's p-value NG vs Uninfected	Fisher's p-value CT vs Uninfected	Fisher's p-value NG vs Uninfected
	N	(%)	N	(%)	N	(%)	N	(%)						
Signs (n=530)[a]														
Internal genitalia discharge vagina	29	(57)	16	(67)	12	(80)	304	(69)	0.33[b]	0.71	1.00	1.00	0.71	0.41
Internal genitalia adnexal tenderness	2	(4)	0	(0)	0	(0)	0	(0)	0.03	1.00	0.17	1.00	0.10	1.00
No clinical signs observed	20	(39)	7	(29)	3	(20)	130	(30)	*ref*	*ref*	*ref*	*ref*	*ref*	*ref*
Pelvic exam not performed (Missing)	17	(25)	10	(29)	7	(32)	261	(37)						
Washes External Genitalia with Water									1.00	1.00	1.00	1.00	1.00	1.00
Yes	52	(100)	23	(100)	12	(100)	438	(100)						
No	0	(0)	0	(0)	0	(0)	1	(0)						
Washes Internal Genitalia with Water									1.00	1.00	1.00	1.00	1.00	1.00
Yes	45	(100)	22	(100)	11	(100)	385	(100)						
No	0	(0	0	(0)	0	(0)	1	(0)						
Washes External Genitalia with Soap									0.21[b]	0.14[b]	0.80[b]	0.14	0.17[b]	0.34[b]
Yes	19	(37)	7	(30)	4	(33)	194	(44)						
No	33	(63)	16	(70)	8	(67)	244	(56)						
Washes Internal Genitalia with Soap									0.06[b]	0.60	0.34	1.00	0.16[b]	0.60
Yes	2	(4)	2	(9)	1	(9)	56	(15)						
No	43	(96)	20	(91)	10	(91)	330	(85)						
Washes External Genitalia with Detergent									0.34[b]	0.05[b]	1.00	1.00	0.07[b]	0.21[b]
Yes	19	(37)	10	(43)	9	(75)	165	(38)						
No	33	(63)	13	(57)	3	(25)	274	(62)						
Washes Internal Genitalia with Detergent									0.59	0.17	1.00	1.00	0.39	0.18
Yes	2	(4)	2	(9)	3	(27)	28	(7)						
No	43	(96)	20	(91)	8	(73)	357	(93)						

CT, *Chlamydia trachomatis*; HRW, high-risk women; IVP, intra-vaginal practices; NG, *Neisseria gonorrhoeae*; ref, reference group.

[a]The following signs were also investigated during physical examination, but none where clinically observed: External genitalia inguinal adenopathy >1cm unilateral, External genitalia inguinal adenopathy >1cm bilateral, External genitalia inflammation, External genitalia condyloma/warts, Internal genitalia inflammation of vagina, Internal genitalia ulcer vagina, Internal genitalia erosion or friability cervix, Internal genitalia erosion or friability vagina, Internal genitalia non-menstrual bleeding cervix, Internal genitalia non-menstrual bleeding vagina, Internal genitalia condyloma/warts cervix, Internal genitalia condyloma/warts vagina, Internal genitalia adnexal mass.

[b]$\chi 2$ p-value.

Table 2: Prevalence of lab results by CT and/or NG status and city, Zambian HRW, 2016

	Overall										Lusaka		Ndola	
	CT Only (n=68)		NG Only (n=34)		CT & NG Co-infected (n=22)		CT/NG Uninfected (n=701)		Fisher's p-value CT vs Uninfected	Fisher's p-value NG vs Uninfected	Fisher's p-value CT vs Uninfected	Fisher's p-value NG vs Uninfected	Fisher's p-value CT vs Uninfected	Fisher's p-value NG vs Uninfected
	N	(%)	N	(%)	N	(%)	N	(%)						
Trichomonas vaginalis (microscopy)									0.01	<0.01[a]	0.03	0.01	0.08	0.03
Positive	5	(7)	4	(13)	4	(19)	23	(3)						
Negative	62	(93)	27	(87)	17	(81)	651	(97)						
Incident syphilis infection (based on RPR)									0.26	<0.01	1.00	0.26	0.15	<0.01
Yes	2	(4)	4	(16)	2	(13)	13	(3)						
No	54	(96)	21	(84)	13	(87)	454	(97)						
HIV (rapid test)									0.50	0.24	1.00	1.00	0.61	0.08
Positive of Discrepant	1	(1)	3	(9)	0	(0)	20	(3)						
Negative	66	(99)	31	(91)	22	(100)	675	(97)						
BV (Both KOH+ & Clue Cells)									0.23[a]	<0.01[a]	1.00	1.00	0.08[a]	<0.01[a]
Positive	14	(27)	14	(50)	9	(45)	147	(26)						
Negative	37	(73)	14	(50)	11	(55)	425	(74)						
Sperm (microscopy)									0.05[a]	0.56	1.00	1.00	0.07	0.77
Positive	1	(2)	4	(14)	0	(0)	43	(7)						
Negative	64	(98)	25	(86)	20	(100)	609	(93)						
Candida (microscopy)									0.76	0.25	1.00	1.00	1.00	0.24
Positive	2	(4)	0	(0)	0	(0)	24	(4)						
Negative	53	(96)	27	(100)	20	(100)	555	(96)						

BV, bacterial vaginosis; CT, *Chlamydia trachomatis*; HRW, high-risk women; KOH, potassium hydroxide; NG, *Neisseria gonorrhoeae*; RPR rapid plasma reagin.

[a]$\chi 2$ p-value.

Table 3: Prevalence of demographic factors and risk behaviors by CT and/or NG status and city, Zambian HRW, 2016

	Overall													
	CT Only (n=68)		NG Only (n=34)		CT & NG Co-infected (n=22)		CT/NG Uninfected (n=701)		χ^2 p-value CT vs Uninfected	χ^2 p-value NG vs Uninfected	χ^2 p-value CT vs Uninfected	χ^2 p-value NG vs Uninfected	χ^2 p-value CT vs Uninfected	χ^2 p-value NG vs Uninfected
	N	(%)	N	(%)	N	(%)	N	(%)						
City									0.06	0.29				
Lusaka	26	(38)	6	(9)	4	(18)	169	(24)						
Ndola	42	(62)	28	(91)	18	(82)	532	(76)						
HRW Group									0.33	0.09	0.25	0.68[a]	0.66	0.11
Single Mothers	38	(68)	11	(46)	4	(29)	250	(54)						
Female Sex Workers	18	(32)	13	(54)	10	(71)	215	(46)						
Age Group									0.01	<0.01	0.17	0.05[a]	0.02	<0.01
Age 18-24	44	(67)	26	(79)	21	(95)	412	(59)						
Age 25+	22	(33)	7	(21)	1	(5)	284	(41)						
Literacy									0.05	0.96	0.78	0.09[a]	0.02	0.35
Reads English, Bemba, or Nyanja	45	(66)	17	(52)	12	(55)	361	(52)						
Illiterate	23	(34)	16	(48)	10	(45)	328	(48)						
Education									0.81	0.02	0.04	0.01[a]	0.73	0.27
No School or Primary School Only	42	(62)	28	(85)	15	(68)	426	(62)						
Secondary School or Higher	26	(38)	5	(15)	7	(32)	261	(38)						
Sex in Exchange for Money									0.73	0.12	0.95	0.50[a]	0.71	0.19
Never	38	(56)	13	(39)	8	(36)	339	(49)						
Ever	30	(44)	20	(61)	14	(64)	350	(51)						
Unprotected Sex (Last 1-3 months)									0.03	0.22	0.01	1.00[a]	0.23	0.34
None	16	(31)	9	(41)	4	(22)	201	(42)						
At least once	35	(69)	13	(59)	14	(78)	272	(58)						
Pregnancy									0.43[a]	0.29[a]	0.52[a]	1.00[a]	0.37[a]	0.25[a]
Not Pregnant	54	(96)	23	(96)	13	(93)	453	(97)						
Pregnant	2	(4)	1	(4)	1	(7)	12	(3)						
Uses LARC Method									0.12	0.11	0.05	0.72[a]	<0.01	0.13
No LARC	43	(63)	20	(65)	9	(43)	449	(67)						
Uses Implant or IUD	25	(37)	11	(35)	12	(57)	224	(33)						

CT, *Chlamydia trachomatis*; HRW, high-risk women; LARC, long-acting reversible contraception; IUD, intrauterine device; NG, *Neisseria gonorrhoeae.*
[a]Fisher's exact p-value.

Table 4: Logistic regression models of factors associated with CT, Zambian HRW, 2016

Predictor of CT[a]	Bivariate Association				Multivariate Final Model[b]			
	Odds Ratio	Lower 95% CI	Upper 95% CI	p-Value	Adj. Odds Ratio	Lower 95% CI	Upper 95% CI	Adj. p-Value
Age Group								
Age 18-24	1.76	1.08	2.85	0.02	2.05	1.13	3.72	0.02
Age 25+	*ref*	-	-	-	*ref*	-	-	-
Literacy								
Reads English, Bemba, or Nyanja	*ref*	-	-	-	*ref*	-	-	-
Illiterate	0.64	0.40	1.00	0.05	0.48	0.26	0.89	0.02
Education x City								
No School or Primary School Only vs Secondary School or Higher in Lusaka	2.26	1.03	4.99	0.04	4.46	1.42	13.98	0.01
No School or Primary School Only vs Secondary School or Higher in Ndola	0.90	0.51	1.61	0.73	1.25	0.62	2.54	0.53
Unprotected Sex (Last 1-3 months) x City								
At least once vs None in Lusaka	3.72	1.39	9.96	0.01	4.38	1.44	13.30	0.01
At least once vs None in Ndola	1.51	0.77	2.96	0.23	1.37	0.68	2.76	0.38
Uses LARC Method x City								
Uses Implant or IUD vs No LARC in Lusaka	0.37	0.13	1.02	0.06	0.14	0.03	0.69	0.02
Uses Implant or IUD vs No LARC in Ndola	2.43	1.41	4.2	<0.01	2.22	1.17	4.20	0.01

Adj, adjusted; CI, confidence interval; CT, *Chlamydia trachomatis*; HRW, high-risk women; LARC, long-acting reversible contraception; IUD, intrauterine device; ref, reference group.

[a]The following variables were not significant in bivariate analyses and are not tabled: cohort, sex in exchange for money, pregnancy, HIV rapid test result, sperm (microscopy), candida (microscopy), BV, and new syphilis infection.

[b]Stepwise model selection done in both directions using the stepAIC function in R. The following variables were not significant in initial adjusted analyses and were removed from the final multivariate model via bi-directional stepwise selection and are not tabled: age group x city, literacy x city, TV, and TV x city. The final model contained 529 observations, including 68 with the outcome of interest.

Table 5: Logistic regression models of factors associated with NG, Zambian HRW, 2016

Predictor of NG[a]	Bivariate Association				Multivariate Final Model[b]			
	Odds Ratio	Lower 95% CI	Upper 95% CI	p-Value	Adj. Odds Ratio	Lower 95% CI	Upper 95% CI	Adj. p-Value
Age Group								
Age 18-24	3.57	1.72	7.40	<0.01	3.43	1.38	8.53	0.01
Age 25+	*ref*	-	-	-	*ref*	-	-	-
Education								
No School or Primary School Only	2.20	1.14	4.24	0.02	3.53	1.35	9.21	0.01
Secondary School or Higher	*ref*	-	-	-	*ref*	-	-	-
Trichomonas vaginalis (microscopy)								
Positive	4.63	1.99	10.77	<0.01	6.84	2.20	21.28	<0.01
Negative	*ref*	-	-	-	*ref*	-	-	-
BV (Both KOH+ & Clue Cells)								
Positive	2.64	1.46	4.79	<0.01	2.31	1.10	4.87	0.03
Negative	*ref*	-	-	-	*ref*	-	-	-
Incident syphilis (based on RPR)								
Yes	5.98	2.18	16.42	<0.01	9.12	2.71	30.66	<0.01
No	*ref*	-	-	-	*ref*	-	-	-

Adj, adjusted; BV, bacterial vaginosis; CI, confidence interval; HRW, high-risk women; KOH, potassium hydroxide; NG, *Neisseria gonorrhoeae*; ref, reference group; RPR rapid plasma reagin.

[a]The following variables were not significant in bivariate analyses and are not tabled: cohort, literacy, sex in exchange for money, unprotected sex, pregnancy, LARC usage, HIV rapid test result, sperm (microscopy), and candida (microscopy).

[b]Stepwise model selection done in both directions using the stepAIC function in R. The following variables were not significant in initial adjusted analyses and were removed from the final multivariate model via bi-directional stepwise selection and are not tabled: city, education x city, BV x city, new syphilis infection x city, and washes external genitalia with detergent. The final model contained 423 observations, including 34 with the outcome of interest.

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Chapter V: Discussion

HIV is a Game of Cat and Mouse

The human immune system has been under construction for millennia, with facets of our innate immune system tracing all the way back the evolutionary lineage to fruit flies, and even amoebas(163). The adaptive immune system traces back approximately 500 million years to specific antigen receptors found in jawed fish(164). Perhaps surprisingly, viruses have been around even longer(165) and retroviruses have shaped much of the human genome in the form of endogenous retroviral elements(166). Viruses are more than just pathogens, in fact some would argue they are building blocks of our very selves(167). This astonishingly intertwined legacy of coevolution has created a system of mutual survival and coexistence on a population level.

The identity of each arm, virus and host response, are inextricably linked and have molded the path of evolution for each other. In an ironic sense, these two seemingly opposing forces would not exist in their current forms if not for the influence of the other. Characteristics of the virus enable it to deftly counteract the host immune response, and even manipulate host signs and symptoms, in order to ensure its successful transmission to the next vulnerable individual. The goal of any individual human immune system, on the other hand, is to eliminate the infection before succumbing to the disease and to guard against future infections by the same pathogen. On a population level, the immune system's goal is to be diverse enough such that the entire species would not be decimated by any particular pathogen that has evolved to escape particular aspects of the immune response.

Examples of the incredible diversity of the human immune system are found at all levels, from innate to adaptive, from CNV in the genes that encode innate defensins(112) and adaptive

FcγRs(65), to the thousands of HLA haplotypes(168), and billions of T-cell receptor and B-cell receptor clonotypes possible(169, 170). Furthermore, species have entered into symbiotic relationships with microbes which provide additional protection against pathogens by competing for physical space, nutrient resources, and secreting defensive molecules which benefit both the commensal microbe as well as the host(171).

HIV has evolved an arsenal of techniques to subvert every aspect of the host response. An error-prone reverse transcriptase and the ability to recombine with genomes from other virions leads to a high rate of mutations, a diverse viral quasi-species, and number of different viral subtypes. The ability to integrate into the host genome and enter latency creates a stable reservoir of viruses that persists for the life of host. A myriad of glycosylation patterns blocks attachment of antibodies to the viral envelope and creates an ever-changing target. The collection of viral accessory proteins specifically antagonizes host defense mechanisms. And finally, the strategy of attacking the very cells central to mounting an effective immune response topples the entire defensive effort. HIV has covered all the bases in terms escaping the immune response and remains an extremely evasive target.

The impressive strategies employed by HIV to escape the immune system are actually a testament to the potency of our immune system itself, which is equally impressive. Our immune system has fought back against the virus on all fronts, forcing the virus to adapt, and in some cases sacrifice its own replicative ability, simply in order to survive in such a hostile environment. With every transmission event, the virus encounters a novel landscape of HLA alleles, antibodies, and T cell receptors which exert new selective pressures on the viral population.

However, in the true spirit of coevolution, the virus adapts overtime by maintaining the low-fitness-cost escape mutations in the circulating consensus sequence(129). Characteristics such as this could be one reason why consensus amino acids are preferentially transmitted(150). This archiving of escape mutations over time leads to instances of preadaptation to host HLA alleles(126, 150) and gives the virus a fitness advantage in certain immune environments with little associated fitness cost. The virus employs the same strategy against other selective pressures as well. Tools that humans have developed to fight HIV, such as ART, have sparked drug-resistance mutations which can lead to treatment failure within an individual. Furthermore, the transmission of drug-resistance mutations has presented a challenge for treatment(172) and has necessitated the use of combination therapies to target the virus at multiple stages of its lifecycle(173).

Other tactics of conquering the virus have proved similarly challenging. Developing an HIV-1 cure has centered on a strategy called "shock-and-kill"(174). The premise of this approach lies in identifying all corners of the latent viral reservoir, awakening the virus within those cells, and leveraging the power of the host's killer T cells to eliminate all virally infected cells, all while ART prevents the infection of naïve cells. However, the viral reservoir remains elusive and the shock-and-kill approach has yet to prove successful.

To date, there are only two documented cases of HIV-1 cure. One in the "Berlin patient"(175), and the other in the "London patient"(176). In both of these cases, the individuals were diagnosed with life-threatening cancer which necessitated treatment by stem cell transplant. The physicians in both cases selected a bone marrow donor carrying a deleterious mutation in CCR5 which eliminated this HIV-1 co-receptor. Furthermore, part of the treatment required complete ablation of the patient's existing immune system. This combination of essentially

erasing the individual's entire immune system, and therefore viral reservoir, followed by transplantation with immune cells resistant to HIV-1 infection, resulted in the cure of these two patients. Studies attempting to broadly replicate this cure strategy have proven ineffective(177). Moreover, this highly invasive and risky medical intervention is hardly scalable and not justifiable in the presence of highly effective ART, which allows infected individuals to live a full life in the absence of a cure(178).

Another major challenge is HIV-1 vaccine development. Efforts are underway to design both a therapeutic vaccine and a preventive vaccine. A therapeutic vaccine would suppress viral loads for a prolonged period of time without daily ART, render them noninfectious, and effectively "cure" the individual without clearing the virus. A preventive vaccine would be administered to uninfected individuals, stimulate the production of a protective immune response either in the form of neutralizing antibodies, a targeted cellular response, or some combination of the two. Aspects of both the virus and the host complicate these efforts. Unlike most other vaccine preventable diseases, there is no template for an effective anti-HIV-1 immune response. No human has effectively cleared themselves of the virus, and infected individuals continue to be susceptible to infection by additional HIV-1 viruses even once they are infected, leading to cases of superinfection(179). In the absence of a working prototype which we could design a vaccine to emulate, it begs the question of whether a protective, preventive immune response against HIV-1 is even possible.

Furthermore, even if there was a reliable model of protective immunity against one type of HIV-1, the virus is remarkably variable with at least nine distinct genetic subtypes, in addition to subsubtypes and recombinant forms(15-17). At this level of variation, combined with its high mutation rate, the immune response would have to be incredibly broad, or targeted specifically to

highly conserved common epitopes, in order to protect against all the possible HIV-1 variants. Host factors must also be considered in the vaccine development process. Efforts to guide affinity maturation of broadly neutralizing antibodies(134), depend on the population of germline B cell receptors available, which varies by individual. Once antibodies are generated, their effector functions should be considered, particularly through binding to different genotypes of FcγRs, which has proved to be an unanticipated risk factor in some vaccine trials(79, 80). The design of a safe and effective HIV-1 vaccine will depend on an awareness of both host and viral factors alike.

Lessons on FcγRs, Preadaptation, and STIs

We can explore both sides of the host-pathogen relationship in search of weaknesses to exploit and strengths to bolster. In these studies, we have shown that first, binding affinity differences between FcγR genotypes does not play a role in natural transmission of HIV-1. Second, almost all subtype C HIV-1 proteins contain sites that are influenced by HLA pressure, however within the limitations of the current study only preadaptation in the proteins Pol and Vif were related to more severe disease progression. And third, that risk factors for CT and NG in HRW are distinct, which could better inform screening and treatment to reduce the prevalence of this important HIV-1 risk factor.

We found that individuals with high affinity and low affinity FcγR genotypes were equally at risk for HIV-1 infection and progressed similarly in terms of viral control and CD4 decline. This contrasts what was observed in vaccine studies where individuals with high affinity genotypes were infected at a more rapid rate(79, 80), and studies of disease progression where individuals with low affinity genotypes progressed more quickly to AIDS(81). One possible

explanation of the differences in our findings is that the previous studies were conducted in men. It is widely known that the immune reactivities of men and women are different(180), and that they respond differently to many viral pathogens, including HIV-1(181), as well as to vaccination(182). These sex differences in HIV-1 infection are an active area of research in our laboratory, however even when we stratified our FcγR data by biological sex, we did not observe these published differences in our subset of men. Another potential explanation is that the individuals in the published acquisition study were part of an HIV-1 vaccine trial, which would generate an HIV-1 specific antibody response. The newly infected individuals in our study were unvaccinated and therefore would not have HIV-1 specific antibodies at the time of transmission, which could modulate this risk of infection.

Another host factor we examined, this time from the viral perspective, was HLA-associated adaptation. We analyzed each viral protein individually to identify sites of HLA-associated adaptation and then quantified this adaptation in a numeric score. In transmission events where the virus already carried HLA-associated mutations that allowed it to immediately evade the new host's T cell response, we found these individuals had increased viral loads and faster CD4 decline, as has also been reported previously(126, 132). Specifically by protein, this association with disease progression was apparent for transmitted viruses with preadaptation in Pol and Vif. We speculate that due to stringent structural constraints in Pol and the amount of time required for HLA-associated adaptations to emerge following transmission, preadaptation in Pol is particularly advantageous to the virus. Vif plays a critical role in viral infectivity and also is involved in reverse transcription, alongside Pol proteins. Thus HLA-associated adaptations in Vif warrant further investigation as they could point to potential new vaccine targets in the HIV-1 accessory proteins.

If we frame our perspective of the host as a "superorganism", or a shared host to many other species of microbes(183), STIs represent another important host factor outside of the direct immune response that modulates risk of HIV-1 infection. The collection of microorganisms colonizing the human body at any particular moment shapes the immune milieu and, in some instances, predisposes a person to infection by other pathogens. This is especially true for individuals infected with HIV-1, who become increasingly susceptible to opportunistic infections as the disease progresses. Not to mention, it was this salient characteristic of coinfection-induced susceptibility that led to the discovery of HIV/AIDS and is the namesake of the virus.

Taking a step back and examining risk factors for risk factors allows us to add another level of primary prevention to reducing risk of HIV-1 acquisition. In our study of Zambian women at high-risk for HIV infection, we found that CT was associated mainly with sociodemographic and behavioral risk factors such as self-reported unprotected sex, younger age, and lower education. Interestingly, CT was associated with higher literacy and the relationship between CT and LARC usage differed by city; increasing the risk of CT infection in Ndola, Zambia but lowering the risk in Lusaka, Zambia. NG was also associated with younger age and lower education; however, the other statistically significant risk factors were other STIs or vaginal dysbioses, including TV, syphilis, and BV. Importantly, neither of these infections were associated with signs or symptoms, which underscores the disadvantage of using syndromic surveillance as the primary means of case detection and diagnosis. CT and NG are often discussed and tested for together, but it is important to remember that these infections are distinct and even within the same study population have unique risk factors. This knowledge can be used to build more effective screening tools to diagnose and treat the specific infections, preventing overtreatment and the unnecessary use of antibiotics, which contributes to antibiotic resistance.

Building on this work, the field can continue to advance in several areas. First, while we have demonstrated that FcγR genotype is not a host factor that contributes significantly to natural HIV-1 infection, it may, as shown previously, still be a risk factor in vaccine trials. We feel that host factors such as these, especially where there is defined genetic variability between individuals, should be included in the data analysis component of vaccine trials in order to capture the true effect of the vaccine on an immunologically diverse population. Second, our findings on the unique risk factors for CT and NG infection could provide foundation for improved screening and treatment methods for STIs, particularly in resource-limited settings. Finally, sites of viral adaptation to host HLA alleles are critical to study further. Through these sites, the virus is revealing precise immune system targets. We can leverage this information to build more effective immune responses to these areas. More crucial though is the knowledge of where the immune system is not targeting; we can investigate further to determine the reasons for this, whether it be that these sites are not immunogenic epitopes, or perhaps that the immune system is targeting these sites but the virus cannot escape due to insurmountable fitness costs. It is towards these immutable regions that we should focus our vaccination efforts.

Furthermore, if the goal of vaccine development is to produce a single vaccine that would effectively prevent HIV-1 infection from multiple subtypes, researchers should compare these sites of immune escape across different viral subtypes. In Uganda, subtypes A1 and D cocirculate and researchers found that 34% of the HLA-associated sites were different between the subtypes, despite circulating in the same population(184). One way that our group could investigate these differences is by comparing HLA-associated adaptations in our two distinct heterosexual transmission cohorts. The Zambian cohort is representative of subtype C HIV-1, whereas the Rwandan cohort is representative of subtype A HIV-1 infection. By performing

similar analyses to those described here, we could generate a list of HLA-associated sites in subtype A HIV-1, examine their associations with disease progression, and compare the findings with those from the Zambian subtype C cohort. Sites of the virus that are immune targets in both subtypes, or sites that are heavily conserved between both subtypes, would be vital areas to explore further in the field of vaccinology.

Outside the realm of vaccines, the HIV-1 subtypes are intriguing because the host response to them is different. Individuals infected by subtype A HIV-1 typically have less severe HIV-1 disease progression than individuals infected by subtype C or subtype D HIV-1(185-187). It is still unclear whether this difference is due to dampened fitness of subtype A viruses, or perhaps the explanation is that the subtype A viruses are evolutionarily younger(1) and therefore less adapted to their host population, which would result in fewer low-fitness-cost mutations fixed in the consensus sequence and ultimately less preadaptation of the transmitted viruses.

Is Ending the Epidemic Possible?

While scientists continue working to discover a cure and develop and test a vaccine, work can still be done now to slow and even stop the epidemic. HIV-1 meets all of the requirements for a theoretically eradicable disease(188), including having an effective intervention that interrupts the cycle of transmission, practical diagnostic tools, and no zoonotic or environmental reservoir. Furthermore, basic public health practices are incredibly successful in reducing transmission. One noteworthy example is CVCT, which reduced the rate of HIV-1 transmission between serodiscordant couples by over 60% through testing, joint counseling, and condom provision; when CVCT was combined with ART in the HIV-1 infected partner, the reduction in transmission rate approached 80%(189). This is a remarkable achievement and a higher success

rate than any HIV-1 vaccine trail to date. Increasing access to testing so that everyone knows their HIV-1 status is a huge part of the 90-90-90 goals set by UNAIDS(190). Other educational messages are also important, such as proper use of condoms and risks associated with breastfeeding for mothers with HIV-1. Finally, identifying other risk factors, such as STIs, and reducing them could be pivotal points of intervention.

Next, we should capitalize on the highly effective tools that we already possess, such as ART. The first step is identifying infected individuals and linking them to care, then improving treatment retention. We can embrace the use of ART not only to treat infected individuals and prolong their healthful lives, but also to reduce their risk of spreading the virus to others through TasP(31, 41). We can also use ART in uninfected individuals at high-risk of infection in the form of PrEP(30). A key population to continue successful intervention is testing pregnant women and their husbands - prescribing ART to those infected with HIV-1 in order to prevent acute infections during pregnancy and mother-to-child transmissions.

Humans have, in the past, conquered viral scourges in the past, such as smallpox and rinderpest(191), which have been eradicated from the face of the planet thanks to dedicated human intervention. Similarly, much progress has been made in the fight against polio, with clear eradication goals in sight(192). In each of these examples an effective vaccine was available, which helped to prevent new infections and build lasting immunity. However, there are success stories where no vaccine or cure was available and natural immunity did not exist, such as the incredible progress towards eradication of guinea worm disease. Through committed efforts of basic public health such as community education, outreach, and surveillance, cases of guinea worm disease dropped from 3.5 million in 1986 to only 28 in 2018(193). This remarkable success story gives hope for the eradication of other diseases, like HIV-1, for which no natural

immunity, effective vaccine, or cure currently exist, but for which tools are available and community infrastructure is in place to make a dramatic difference in the future of the epidemic.

Summary

This work has explored a variety of host factors and their relationship to HIV-1 acquisition and disease progression. Starting with an intrinsic aspect of the human immune system, the FcγR, to ways that the virus responds to the selective pressure of HLA alleles, all the way to other organisms that colonize our bodies and impact our risk of HIV-1 infection. When we frame our research from the perspective of this host-pathogen intersection, we can learn about both sides. Throughout the extremely long process of coevolution, viruses and the host immune response have sculpted each other. Evidence of this principle is present in the many viral characteristics that enable it to be genetically flexible, and the accessory proteins it has designed to antagonize the host response; it is also evident on the host side by the incredible diversity of our antibodies, HLA alleles, and T cell receptors.

At the end of the day, humans are the ones with the advantage. We have numerous external tools at our disposal and effective public health strategies that can halt chains of transmission and stop the replication of the virus in the hosts it infects. Research efforts are currently underway to develop a cure for HIV-1 and produce a safe and effective preventive vaccine. Humans have demonstrated that victory over viruses is possible and even with the tools that already exist, the eradication of HIV-1 is attainable.

References: Introduction and Discussion

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