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Effects of systemic biomarkers on HIV-1 acquisition and subtype A and C infections

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Abstract

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Despite the wealth of knowledge gathered since its discovery in the 1980s, HIV-1 remains one of the greatest health crisis's facing the world today. In particular, sub-Saharan African bears almost two-thirds of the global disease burden. Within this region, the most common subtypes of HIV-1 seen are subtype A and C. While the geographical distribution and clinical disease progression of different subtypes in Africa is known, there is less known about the immunological baseline state of the individuals living in this high-risk region. One way to study this is by examining the levels of biomarkers, cytokines and chemokines, in these individuals. Levels of biomarkers can help identify different stages of HIV infection, the presence of other infections/diseases, and help us further understand the effect that HIV-1 has on the innate immune response.

In the first study presented in this dissertation, we studied biomarker levels in two groups of individuals in serodiscordant couples from sub-Saharan Africa: uninfected individuals that remained HIV-negative and uninfected individuals that would finally seroconvert and become HIV-positive. Through our analysis, we found that individuals that eventually became HIV-positive had significantly higher levels of multiple biomarkers compared to individuals that remained HIV-negative. In addition, we were able to identify several biomarkers that could predict individuals who would become HIV-positive; the ones identified were non-classical inflammatory biomarkers and suggests that other infections or chronic diseases may increase an individuals' risk for HIV acquisition.

In the second study presented in this dissertation, we investigated the basis for the higher viral loads and faster CD4 loss seen in subtype C vs subtype A infected individuals. We showed that the replicative capacity of the transmitted founder viruses from HIV-1 subtype A and C infections were similar and therefore was unlikely to differentially impact disease progression. While levels of the biomarkers during acute infection were similar between the two subtypes, we noticed that there was a higher number of biomarkers whose levels significantly increased from their pre-infection levels with subtype C compared to subtype A infections. This shows that the clinical differences observed during infection by these two subtypes is not reflected in biomarker levels during acute infection and therefore islikely is linked to the adaptive immune response. Clearly this is a complicated question that is still unanswered.
The use of biomarkers helped show the increased risk of individuals with elevated biomarkers for HIV acquisition and showed subtle differences in the biomarker response between HIV-1 subtype A and C acute infections. Biomarker studies can help reveal additional information about all phases of HIV-1 infections.

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Chapter I: Introduction

HIV-1 is a global epidemic that has infected individuals on six out of the seven continents and deeply impacted millions of lives. While the epidemiology, pathology, and etiology of HIV-1 infection and disease are widely studied, no effective vaccine or curative treatment has been developed. Current antiretroviral treatment has saved millions of lives and extended the lifespan of those infected with HIV-1, these individuals carry the burden of taking medication every day for the rest of their lives. So, while a vast amount of research has been performed, there is still a need to improve what we know about this virus. This chapter will focus on the genetic diversity of HIV-1 and the differences observed between HIV-1 subtypes in clinical disease presentation and disease progression. This chapter will also cover some ways that HIV-1 interacts with the innate immune system, a field that has largely been under-researched. This chapter will frame the issue of subtype and innate immunity in the context of the HIV-1 transmitted-founder virus and how these factors affect the selection of the transmitted-founder virus and the overall disease progression of new HIV-1 infections.

Discovery of HIV-1 and beginning of global epidemic

HIV-1 first appeared in North America in the early 1980s when a strange illness was sweeping through the homosexual population in San Francisco, California. This mystery illness was characterized by a reduction of total number of T cells in patients, as well as other unexplained lymphadenopathies (1). This unknown disease was eventually named acquired immune deficiency syndrome, or AIDS (2). Early epidemiological analysis found that this disease was spread by multiple different routes

of transmission, such as sexual, intravenous drug use, and blood transfusions (2). With growing numbers of infections, identification of the causative agent became dire. Using samples taken from AIDS patients, multiple groups tried to determine the agent responsible for the growing epidemic. Early findings suggested that the causative agent was a form of the human T-cell leukemia viruses (HTLV). However, antibodies made against HTLV-1 were not able to precipitate proteins from the isolates taken from AIDS patients (2). This revealed that the cause of this epidemic was a novel retrovirus.

In 1983, Francoise Barre-Sinoussi and Luc Montagnier from the Pasteur Institute in Paris were the first individuals to isolate and describe the causative agent of AIDS from the lymph node of a homosexual patient with multiple lymphadenopathies (2). Initially thought to be a member of the human T-cell leukemia family of viruses, Barre-Sinoussi and Montagnier showed that this novel retrovirus was able to propagate in T lymphocyte cultures (2). Three years later, J. Steven McDougal determined that this virus targeted $CD4^+$ T cells (3) These important findings jumpstarted the research field that would eventually identify the causative agent of AIDS, the human immunodeficiency virus (HIV).

Current state of the HIV-1 epidemic

Since its discovery in the early 1980s, HIV has become a global problem. The virus has spread globally and has caused millions of AIDS-related deaths worldwide. In 2019, there was a total of 37.9 million people infected with HIV globally, with 1.7 million new infections and 770,000 AIDS-related deaths (4).

Globally, there is different distribution of HIV-1 burden. Within the western world (Western and Central Europe and North America), there are 2.2 million individuals infected with HIV (4). However, the greatest HIV-1 disease burden is located in sub Saharan Africa where 20.6 million individuals are infected. That means that 54% of HIV-1 infected individuals are located within one region (4). While the number of HIV-1 infected individuals currently on antiretroviral treatment (ART) in sub-Saharan Africa had increased to over 50% in 2016, AIDS-related illnesses still remain the leading cause of death in this region (4). This highlights the need for continued research in this field in order to decrease the number of new infections each year, in addition to the number of AIDS-related deaths.

HIV-1 Infection

HIV-1 is an enveloped virus with a single-stranded RNA genome (ssRNA). However, HIV-1's genome, like that of all retroviruses, is unusual in the fact that it is pseudo-diploid, meaning that it contains two copies of its genome in each viral particle from which a single pro viral genome is generated. HIV-1 binds to the CD4 and CCR5/CXCR4 molecules on $CD4^+$ T cells to facilitate its fusion and entry into susceptible host cells. Once in the cytoplasm of a host cell, the single strand RNA genome is converted, or reverse transcribed, into double-stranded DNA (5). This double-stranded DNA is then integrated into the host cell's genome. This integration into the host cell's genome leads to the formation of the viral reservoir. The viral reservoir is composed of latently infected $CD4^+$ T cells that escape immune detection (5).

When these latently infected $CD4^+$ T cells become activated, this leads to the integrated viral genome being transcribed by cellular RNA polymerases (5). This results in the production of new HIV-1 genomes which will be packaged at and eventually bud from the host cell's plasma membrane (5). The end result is the formation of new virions which can spread and infect more host cells.

HIV-1 Genetic Diversity

As an RNA virus, HIV has a large degree of genetic diversity. In its replication cycle, HIV-1 uses reverse transcriptase to convert its ssRNA genome to double stranded DNA that can be integrated into the host cell's genome (5). Reverse transcriptase has a high error rate, which results in about one error per genome per replication cycle (6). In addition, reverse transcriptase does not have proof-reading abilities, meaning that any mistake that is made by the enzyme is not corrected (6). Additional genetic diversity can occur during the replication process in the form of recombination events. In a recombination event, the viral reverse transcriptase enzyme switches templates during replication (6). This can occur between the two genomes within the viral particle when two different HIV-1 viruses have previously infected the same cell; this is how recombinant subtype viruses are formed (which will be covered later in the chapter). Genetic diversity also arises due to pressure from the the innate immune system. One example is the mutations caused by APOBEC3G, a restriction factor that will be discussed more later in the chapter.

This great degree of genetic diversity has necessitated the classification of HIV-1 into different groups and subtypes based on their phylogeny. The major phylogenetic

groups are: group M, group N, and group O; the greatest disease burden is due to group M (6). Group M is further divided into nine subtypes/clades. The differences between these subtypes will be discussed at great length below.

Differences in HIV-1 subtypes

As previously described, HIV-1 has great genetic diversity. Due to this diversity, HIV-1 group M is divided in at least 9 individual subtypes/clades that are determined based on nucleotide sequencing of gag, pol, and nef or based on the sequence of the entire HIV-1 genome (6). The 9 subtypes are as follows: A, B, C, D, F, G, H, J, and K (4). Recombinant or multiple subtype viruses also exist. These recombinant forms of the virus are generated when two viruses of different subtypes infect the same cell in an individual, allowing virions with two genetically different RNAs to be produced. During the reverse transcription process, the viral enzyme, reverse transcriptase, uses the RNA genomes from both viruses as a template for replication. The resulting single DNA genome is a patchwork of different genomic regions from two different subtypes. There are numerous different recombinants that are currently distributed around the globe and are known as circulating recombinant forms (CRFs).

Each of the different subtypes have a different geographical distribution. The predominant subtype in North America and Western Europe is subtype B, South America is burdened by subtypes B and F (and recombinants between them), and Russia is inflicted with subtypes A and B (4). Within the African continent, a majority of the different subtypes and numerous HIV-1 recombinant forms can be found. Overall, a

majority of African individuals are infected with either subtype C (the most abundant), A, D, or a recombinant form of the virus (4).

Within the HIV-1 field, there has been a bias to focus research on subtype B. This is because this is the subtype that is affecting the regions where the majority of HIV researchers reside (North America and Western Europe). However, out of the 37.9 million people infected with HIV-1 in 2016, 54% of infected individuals are located in sub-Saharan Africa (4). Within this region, subtype B infections are rarely if ever found. As a result, there is a lack and an urgent need to research the subtypes that affect the highest burdened population within this pandemic.

As research in the HIV-1 field advanced, researchers noticed that the infections with different subtypes resulted in distinct clinical presentations. In a limited study in 2009, researchers looked at the occurrence of dementia in 33 individuals infected with subtypes A, C, D, or A/D recombinant with advanced HIV-1 infection. Dementia is considered to the most severe form of HIV-associated neurocognitive disorders (HAND) (7). This study found that individuals that were infected with subtype D were most likely to develop dementia in advanced stage HIV-1 infection compared to individuals who were infected with subtype A (7).

In a more extensive study in 1999, researchers followed a group of HIV-positive female sex workers for 12 years and monitored the disease progression of these women. This cohort contained women who were infected with subtypes A, G, D, and C. Overall, this study found that women infected with a non-subtype A were 8 times more likely to develop AIDS compared to women infected with subtype A viruses (8). This study showed that subtype A has a slower disease progression compared to other HIV-

1 subtypes. Experimentally, George Shaw's group released a paper in 2012 that illuminated a possible reason why subtype A has a slower disease progression. When subtype A, D, and A/D recombinant transmitted-founder viruses were tested for replicative ability in *in vitro* assays, it was found that subtype D viruses replicated significantly faster than subtype A viruses (9). This reduced replication rate of subtype A viruses may explain why individuals infected with subtype A have a slower disease progression compared to individuals infected with other subtypes.

Additionally, researchers have noticed that there are subtype differences in clinical presentation in early HIV infections. In a 2013 study, 218 women who were classified as being in the early stages of HIV-1 infection were evaluated based on the symptoms they were experiencing. Women included in the study were infected with either subtype A, C, or D. This study found that women infected with subtype C were more likely to experience unexplained fever, fatigue, and abnormal vaginal discharge compared to women infected with subtypes A or D (10). Despite the increased number of symptoms associated with subtype C infections, women infected subtype D had the highest prevalence of symptoms; this is in agreement with the belief that subtype D is more pathogenic than other subtypes such as A and C (10).

While the current observation is that subtype C is more pathogenic than A, the exact cause of differences between subtypes is unclear. In contrast to these previous reports, a study published in 2016 found that subtype C viruses replicated slower than subtype A and D viruses *in vitro*, and in their cohort individuals infected with subtype C had slower $CD4^+$ T cell decline, and lower viral loads compared to individuals infected

with A or D viruses (11). These results also contradict the findings from the cohorts of HIV subtype A and C infected individuals in our studies.

The cohorts that we use for our studies were established in 1986 in Rwanda and 1994 in Zambia by Dr. Susan Allen, which offered Couples Voluntary Counseling and HIV testing (CVCT) as a prevention intervention. At the site in Lusaka, Zambia, heterosexual Zambian HIV-serodiscordant couples were enrolled into a longitudinal follow-up study; at the establishment of cohort, there were 3049 couples enrolled and these couples had been together for a median of 7 years. The CVCT services that were offered at these sites included group counseling, rapid HIV testing, and mutual disclosure of the results to couples. Enrolled couples came in every 3 months for additional testing. In addition, once antiretroviral treatment (ART) was available in Zambia government clinics in 2007, HIV-positive partners were referred to them for treatment (12). An additional study site in Kigali, Rwanda implemented similar CVCT procedures (13).

Participants in these cohorts also provided a number of demographic, behavioral, and clinical data at enrollment; these included, but were not limited to, age, duration of cohabitation, number of prior pregnancies, pregnancy status, history of sexually-transmitted infections (STIs), STI diagnosed clinically, clinical stage and viral load of HIV+ partner, self-reported sex with or without condoms, self-reported sex with outside partners, etc (12). Overall, the CVCT services provided at these sites resulted in a reduction of self-reported unprotected sex and a two-thirds reduction in HIV transmission independent of who was the HIV+ partner (12). The existence of this cohort not only helped educate the participants of the cohort on how to prevent HIV

transmission, but it also provided valuable samples that allowed researchers to study early HIV transmission and infection, such as the ones presented in this dissertation.

In unfortunate incidents where transmission occurred, plasma samples were available from very early points in infection since the seronegative individuals involved in the study were tested on a regular basis. Not only did it allow our laboratory to isolate the transmitted-founder virus from newly infected individuals (discussed later in this chapter), it also allowed us to follow these patients and track their disease progression longitudinally as part of a multi-site protocol named Protocol C.

There are numerous different groups who work with the same cohort of patients that have examined differences in clinical presentation of individuals infected with different subtypes. In a study published in 2014 by one of our collaborators at the University of Alabama in Birmingham (UAB) found that individuals infected with subtype C had significantly higher viral loads between 9-24 months post infection compared to individuals infected with subtype A (14).However in a study published in 2017, researchers in Kenya investigated differences in symptoms with individuals infected with either subtype A, C, or D. This study found that individuals infected with subtype A had higher viral loads and overall had more symptoms compared to individuals infected with subtype C or D (15).

Recently, our laboratory became interested in the differences between subtype A and C infections. Our interest in this subject was piqued due to several reports published using our cohort. The first was a study published in 2013. This paper showed that individuals infected with subtype C had a 60% faster disease progression compared to individuals infected with subtype A. Disease progression was defined by

three characteristics: $CD4^+$ T cell decline, viral load, and time to AIDS. This study found that individuals infected with subtype C had faster $CD4^+$ T cell decline, higher viral loads, and shorter time to development of AIDS than individuals infected with subtype A (16). This can be seen in Figure 1. These findings corroborated the findings from our collaborators at UAB.

An additional study published in 2015 examined HIV incidence rates in different countries in Africa. Within Africa, Rwanda is predominantly afflicted with subtype A while Zambia has mostly subtype C infections. This study determined the HIV incidence rates in both countries before and after the counseling that I previously described. In both pre- and post-counseling, Zambia was found to have higher HIV incidences rates compared to Rwanda (17). This study suggests that there is something about the viruses present within those two countries that are fundamentally different in order to see a difference in transmission rates, since the counseling protocols in both countries are nearly identical. The final finding that lead us to address this question was a paper from a collaborator published in 2017 showed that there is higher proportion of non-progressors (individuals who can control their viral load without the help of antiretroviral drugs) infected with subtype A compared to those infected with subtype C (18).

Overall, these findings signal to us that subtype A viruses are less fit or less pathogenic than subtype C viruses and that these differences may exist within the transmitted founder virus.

Characterization of the transmitted-founder virus

Work in the Hunter laboratory has centered on the HIV-1 transmitted-founder virus with the goal of determining the characteristics of this virus and the factors that are key for selecting which viral variant will establish an infection. A study from the Hunter laboratory published in 2009 showed that there is a genetic bottleneck that exists in HIV-1 subtype A and C infections. The result of this bottleneck was that up to 90% of heterosexual transmissions examined within the study resulted due to a single viral variant despite the fact that a chronically infected donor would theoretically contains a diverse viral quasispecies (19). This means that despite the great viral diversity that exists within donors, the majority of new heterosexual infections are established due to a single viral variant, which is visualized in Figure 2.

This study also examined the phenomena of multi-variant transmission, in which multiple viral variants from the donor establish a new infection in a recipient. While this is a less common occurrence in heterosexual transmission, 5 out of 42 newly infected individuals were shown to have been infected by multiple viral variants (19). Further analysis showed that these 5 individuals had evidence of genital inflammation or genital ulcers. This shows that one of the factors that allows for the transmission of multiple variants is the presence of inflammation or ulceration in the genital tract (19).

The next breakthrough made in the lab was the discovery that there was selection bias for more consensus-like variants (the sequence of amino acids that is the consensus of all viral sequences in the cohort under study) during heterosexual transmission. This study of 137 virologically linked transmission pairs showed that donor amino acids that matched the cohort consensus were transmitted 99.65% of the time compared to donor amino acids that did not match the consensus, which were only

transmitted 92.61% of the time, as shown in Figure 3A (20). In addition, this study found that presence of genital ulcers or inflammation decreased the selection bias, in addition to allowing multiple variants to be transmitted (20).

This study also found that there was a higher selection bias in female-to-male transmission vs. in male-to-female transmission. This was determined based on the observation that females tended to be infected with viruses that contained more non-consensus polymorphisms vs. the viruses that infected males (20). This means that females are infected with less fit viruses compared to males. Moreover, when they examined these transmitted polymorphisms longitudinally, the transmitted polymorphisms reverted to consensus faster in females vs. male recipients (20).

With these new findings, a major focus in the laboratory became determining the characteristics of the HIV-1 transmitted-founder virus. One of the characteristics that our laboratory uses to phenotype these viruses is replicative capacity, which is defined as the ability of a virus to replicate and spread to other susceptible host cells. This is a characteristic that we were able to test for and determine using *in vitro* experiments.

In a study published in 2012, our laboratory examined whether the replicative capacity of a virus had any associations to other aspects of viral infection. Since the products of the *gag* gene were found to be critical for replication, the *gag* genes from 149 subtype C infected individuals were isolated and used to construct *gag* chimeric viruses in the MJ4 backbone to determine replicative capacity. This study found a significant correlation with the replicative capacity of the transmitted-founder virus and the setpoint viral load in the donor; this means that if a donor had a higher viral load, the transmitted founder virus would have a higher replicative capacity compared to a donor

who had a lower setpoint viral load (21). In addition, this study found an association between $CD4^+$ T cell count and the replicative capacity of the transmitted-founder virus, suchthat being infected with viruses that have a lower replicative capacity confers a survival benefit to the infected individual, meaning these individuals tended to have higher $CD4^+$ T cell counts compared to individuals infected with a transmitted-founder virus with a high replicative capacity (21). This difference could be observed within the first 3 years after infection (21). Similar findings were also found when examining replicative capacity using reverse-transcriptase-integrase chimeric viruses (22).

In a second study published in 2015, the replicative capacity of a transmitted-founder virus was shown to be predictor of $CD4^+$ T cell decline in newly infected individuals. This study found that individuals who were infected with viruses that had a high replicative capacity progressed to $CD4^+$ T cell counts lower than 200 faster than individuals infected with viruses with a low replicative capacity; in short, individuals infected with transmitted founder viruses with high replicative capacity progressed to the development of AIDS faster than individuals infected with low replicative capacity viruses (23). This study also found that high replicative capacity viruses lead to a significantly higher amount of proviral DNA residing in central memory $CD4^+$ T cell than low replicative capacity viruses (23). It was important because it clearly showed that the phenotype of the transmitted-founder virus is directly associated with the disease progression of the infected individual. Around this time, our laboratory developed a protocol in order to generate infectious molecular clones (IMCs) for full-length HIV-1 genomes, which is essential for all the *in vitro* assays we perform (24).

In 2015, our laboratory also published a study of 6 subtype C transmission pairs from whom full-length genomes were PCR amplified, sequenced and used to derive infectious molecular clones; this allowed us to directly examine factors that may play a role in the transmission bottleneck. With these 6 transmission pairs, the study found that the transmitted-founder viruses were closer to a subtype C consensus in both nucleotide and amino acid sequence compared to non-transmitted donor viruses, as shown in Figure 3B (25). The transmitted-founder viruses were also found to more sensitive to plasma derived from the donor and were able to infect dendritic cells with 50% efficiency. Additional findings from this study showed that the transmitted-founder virus did not have higher particle infectivity nor higher replicative capacity compared to non-transmitted donor viruses. The transmitted-founder viruses were also found not to be more resistant to *in vitro* interferon-alpha treatment, a finding that remains debated within the field (discussed later) (25).

Overall, the Hunter laboratory has been influential in the characterization of the transmitted-founder virus and trying to determine the selecting factors involved in the transmission bottleneck.

Physiological predisposition to HIV-1 infection

It is well-known that sex workers, men who have sex with men, intravenous drug users, and transgender individuals can result in an increased risk for acquiring HIV-1 (26). However, there is a number of innate biological properties that can increase an individuals' risk for acquiring HIV-1. When looking at heterosexual transmission between men and women, it has been found that about 90% of heterosexual

transmissions are caused by a single viral variant that comes from the diverse viral quasispecies of the transmitting partner (19). This severe genetic bottleneck can be mitigated by the presence of inflammatory genital infections in the at-risk partner, which was shown by vaginal/urethral discharge, ulceration, or presence of lower abdominal pain (19).

In men, a risk factor for HIV acquisition is being uncircumcised. Uncircumcised men who acquired HIV were more likely to have higher levels of IL-8 and MIG (monokine induced by gamma interferon) and innate anti-microbial proteins, such as neutrophil derived α-defensins in their sub-preputial space than those who remained uninfected (27). The presence of the α-defensins may be promoting epithelial remodeling and inflammation, which can attract HIV susceptible cells to the site and increase the risk of HIV infection (27).

In the female genital tract, women have an increased surface area of mucosal tissues (which contains HIV susceptible cells) compared to men; this can result in increased mucosal HIV exposure (28). Young women have a pro-inflammatory immune environment in their genital tract and single-cell columnar genital epithelium at the cervix, increasing the chance for HIV to interact with a susceptible cell (28). In addition, women have an increased mucosal expression of the HIV co-receptor CCR5 and a greater probability of virus exposure on the rectal mucosa compared to men (28).

Additional studies have found that the risk of HIV acquisition was significantly higher in women with evidence of genital inflammation. One study found that women who acquired HIV had higher genital concentrations of MIP1α (macrophage inflammatory protein 1α), IL-8, and IP-10 (interferon gamma-induced protein 10) prior to

infection compared to women who remained HIV-negative (29). These elevated biomarkers persisted for multiple weeks after HIV infection. As a result, risk of HIV infection was significantly higher in women who had elevated genital inflammatory biomarkers (29).

Another study of the female genital tract observed a greater than 4-fold increase in HIV acquisition in women with high-diversity, low *Lactobacillus* abundance genital flora communities compared to women with *Lactobacillus cripastus* dominant genital flora (30). These women had a 17-fold increase in HIV target cells in the female genital tract compared to women with *L. crispatus* dominant floras. In addition, women with low *Lactobacillus* abundance in their genital tracts had elevated levels of MIP-1α and MIP-1β (which attracts CCR5 expressing cells), but also had elevated levels of IL-1α, TNF-α, IL-8, IL-12p70, IFN-γ, and IL-10 (30). Upon examination of the different floras in the genital microbiota, *Prevontella melaninogenica, Veillorella montpellierensis, Mycoplasma, Prevontella bivia, Sreathia sanguinegens* were all associated with increased genital inflammation and were all positively associated with HIV acquisition (30).

These studies show that there are intrinsic properties in individuals that can predispose an individual to being at a higher risk for HIV acquisition.

Innate Immune Responses to HIV-1 Infection

HIV-1 infection is commonly associated with the adaptive immune response since its main target cell are $CD4^+$ T cells and the subsequent decline of those cells is linked directly to disease progression. However, the virus still has to deal with the first

line of defense in susceptible hosts: the innate immune system. The innate immune system recognizes invading pathogens in a non-specific and rapid manner and plays a key role in the development of the adaptive immune response. In recent years, the interaction between HIV-1 and the innate immune system has gained more attention and researchers have been uncovering the numerous ways that HIV-1 either is targeted or evades the innate immune response.

One of the major innate immune responses to viral infections, including HIV-1, is the interferon response. Virally-infected cells secrete type I interferons (IFN-α and IFN-β) which can induce the expression of interferon-stimulated genes (ISGs) and create an antiviral or non-permissive state in uninfected cells (31). While interferon has been shown to be effective in early stages of HIV-1, the continued production of type I interferons and expression of ISGs can have detrimental effects in chronic infection. The issue of interferons in HIV infection will be addressed more fully later in the chapter.

One feature of the innate immune system is the use of pathogen pattern receptors. Pattern recognition receptors (PRRs) are receptors that recognize molecules that are associated with pathogens, such as LPS from gram-negative bacteria or viral envelope proteins. PPRs can located on the surface of host cells, in endosomes, or the cytoplasm of the host cell. One classic PRR is is a class of molecules known as Toll-like receptors or TLRs. Two of these, TLR-7 and TLR-8 are located in endosomes and recognize foreign single-stranded DNA (ssDNA) and single-stranded RNA (ssRNA). As a result, these two receptors can recognize intermediate products of reverse transcription. These two receptors can then activate an interferon response within the infected cell (32). A paper from 2010 showed that HIV-1 can activate the NF-kB

pathway in infected dendritic cells (DCs) via TLR8, but that HIV-1 has evolved a way to use the activation of this pathway to its advantage. When TLR8 recognizes HIV-1 ssRNA and activates the NF-kB pathway, its activation results in recruitment of a subunit of NF-kB to the HIV-1 LTR which facilitates initiation of transcription, leading to replication of the HIV-1 genome and a productive infection (32).

In the past decade, a new innate sensor of HIV-1 has been discovered in the cytoplasm of cells. Cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS) recognizes HIV-1 infection and produces cyclic guanosine-adenosine monophosphate (cGAMP) which subsequently binds to the adaptor STING. STING, an ER-associated protein, activates IRF3 and results in the production of type I interferons and other cytokines (33). It was discovered that when reverse transcription was blocked, the production of cGAMP was blocked (33). This shows that the recognition of HIV-1 by cGAS is dependent on reverse transcription and the conversion of the viral RNA into DNA. In addition, a paper from 2015 found that PQBP1 (polyglutamine binding protein 1) serves as a co-receptor for cGAS and it binds directly to reverse-transcribed DNA and leads to the expression of IRF3 and the subsequent antiviral genes (34). The activation of these different sensors and subsequent pathways leads to the expression of a number of different ISGs and biomarkers.

An additional sensor that is capable of detecting HIV-1 is interferon-inducible protein 16 (IFI16). In a landmark paper published by the Greene laboratory in 2014, these researchers showed that IFI16 was required for up to 60-70% of bystander $CD4^+$ T cell death in HIV-1 infections (35). This finding reversed the thinking in the field that $CD4^+$ T cell death was due to HIV-1 infected cells dying. Instead, the majority of $CD4^+$ T

cell death is from uninfected bystander $CD4^+$ T cells. This paper showed that when IFI16 was knocked out, bystander cell death was significantly decreased (33). Upon further research, this article was able to determine that IFI16-induced cell death was actually due to abortive infection; in this process, reverse transcription products accumulate in the $CD4^+$ T cell cytoplasm and are unable to integrate into the host cell's genome (35). The accumulation of HIV-1 genomic products is sensed by IFI16 which then activates caspase-1 leading to cell death via pyroptosis (35). This was one of the first links that HIV-1 infection would be linked to the inflammasome.

In a study published in 2017, it was found that IFI16 and cGAS cooperate with each other in order to sense foreign DNA within keratinocytes. The research presented shows that IFI16 was required for STING phosphorylation, which is a required step in the activation of the STING pathway (36). The researchers showed that IFI16 was able to interact with STING through its pyrin domain and lead to its activation (36). This study also showed that cGAS and IFI16 are physically brought together when they bind to the same strand of DNA within the cell (36).

One group of ISGs capable of inhibiting HIV-1 is the interferon-induced transmembrane proteins (IFITMs). This family of proteins are 125-135 amino acid proteins that span the membrane of the host cell (35). Research done to date has shown that IFITM proteins incorporate themselves into the viral membrane and can prevent the entry of the virus into an uninfected cell; they are also able to inhibit the processing of the envelope glycoprotein (37). While the exact antiviral mechanism of these proteins against HIV-1 remains unclear, other studies have shown that HIV-1 has specific adaptations that it has developed in order to evade IFITMs. In a study published

in 2016, HIV-1 transmitted-founder viruses were shown to be more resistant to IFITMs vs. paired viruses from 6-months post infection (37). Another study published in 2013 showed that HIV-1 viruses that started out susceptible to IFITM1 restriction gained resistance to this protein through multiple *in vitro* passages in cell lines (38). This shows that while the mechanism of this protein is still unknown, HIV-1 can select for mutations in order to evade these proteins.

While humans have evolved multiple different defenses against HIV-1, the virus has developed methods to overcome any restriction these defenses may have been able to provide. A prime example of this is the use of accessory proteins to overcome the protection offered by restriction factors. Restriction factors are constitutively expressed by different cell types and have different antiviral responses. One family of restriction factors is APOBEC proteins. These are cytidine deaminases that extensively mutate the HIV-1 genome to prevent the virus from productively infecting the host cell (39). Other examples of restriction factors are TRIM5a (which induces premature uncoating of the virus in the cytoplasm allowing for recognition of the infection) and tetherin (which prevents the release of new virions from the infected cell membrane) (39).

These proteins would be extremely effective at preventing a successful HIV-1 infection if it wasn't for the existence of HIV-1 accessory proteins, which have evolved to target these restriction factors and counter their antiviral properties. The viral protein Vif is able to target APOBEC3G for degradation by the proteasome so it is unable to be incorporated into virions and mutate the HIV-1 genome during reverse transcription (39). Vpu from HIV-1 also targets tetherin for degradation via the proteasome (39).

These two examples show how HIV-1 is able to utilize its accessory proteins to counteract defenses within the host in order to be able to infect potentially restrictive cells. Another example of HIV-1's ability to evade detection from the innate system is its use of CPSF6 and cyclophilins to hide from PRRs. Cleavage and polyadenylation specificity factor subunit 6 (CPSF6) and cyclophilins (Nup358 and CypA) are cofactors that the host cell uses for normal cell functions such as DNA replication and inflammation, respectively. A study published in 2013 showed that HIV-1 uses these three proteins to hide from innate immune receptors and prevent activation of antiviral innate responses in macrophages (40). This paper hypothesized the CPSF6/cyclophilins prevent premature viral DNA synthesis, which could lead to recognition of the virus by the many different cytoplasmic sensors in host cells.

Biomarker studies in HIV research

Biomarker studies, which examine levels of cytokines or chemokines, are commonly seen in HIV-1 research and are often used to try to distinguish between different phases of HIV-1 infection. One such study compared biomarker levels between individuals who either had acute HIV infection or were non-HIV-infected individuals who had reported fevers. This study found that individuals with acute HIV infections had a higher fold change of many biomarkers, including IP-10, IL-10, CRP, MCP-1, FasL, BAFF, MIG, TNFR2, FABR2, GSCF, CXCL16, IgG3, IgG1, MIP1a, sCD23, and EGF (41). This study also found a strong association between IP-10 and viral load in the acute HIV infection group (41). This same group of researchers also found that individuals in the early stages of HIV infection (Fiebig I-IV) and late HIV infection (Fiebig

V-VI) had significant differences in the expression levels of plasma B-cell activating factor, MCP-1, sCD163, and MIG (42). This finding allowed these researchers to distinguish between early and late HIV infection in HIV+ individuals (42).

Another group used biomarkers to be able to distinguish between rapid progressors, progressors, and slow progressors. They found that rapid progressors showed a higher number of increased plasma proteins than progressors or slow progressors (43). Overall, their research found that plasma IP-10 levels during primary infection were predictive of rapid disease progression and could identify individuals who would be rapid progressors (43). Along similar lines, a separate group of researchers used biomarker levels to be able to develop profiles of women who were elite controllers (EC), women who were on ART, non-controllers (NC), and HIV-negative women. This study found that elite controllers had elevated levels of CCL14, CCL27, CCL21, XCL1 compared to ART-treated or non-controller women; elite controllers also had 43% higher levels of stromal cell-derived factor-1 (SDF-1) compared to non-controllers (44). This combination of five biomarkers associated with elite controllers induced the expression of anti-HIV host restriction factors (IFITM1 and IFITM2) and suppressed the expression of RNaseL and SAMHD1 (44). This combination of chemokines also suppressed CCR5 and CXCR4 virus replication in resting $CD4^+$ T cells (44).

Biomarker studies have also been performed in cohorts of men who have sex with men. One such study followed men who had initially enrolled in the cohort as HIV-negative individuals and eventually seroconverted. When the biomarkers of the men enrolled in this study were examined, the researchers found that the levels of 18 chemokines/cytokines were changed significantly during primary HIV infection relative

to the pre-HIV infection levels (14 biomarkers were upregulated and four were downregulated) (45). In the HIV-positive men, CXCL9, CXCL10, and CXCL11 showed the most significant increase and the levels of these chemokines were much higher in the subset of HIV-positive men who had a high viral setpoint compared to HIV-positive men with a low viral setpoint (45).

In the Hunter laboratory, previous work has also examined biomarker levels associated with *in vitro* replicative capacity, a viral phenotype that positively correlates with disease progression. This study found that viral replicative capacity also correlated with IL-6 and IL-1b, cytokines that have been implicated in driving aberrant $CD4^+$ T cell turnover and impairing homeostatic proliferation (23). In addition, viruses that had high replicative capacities exhibited with a distinct inflammatory cytokine profile characterized by a heightened type I and II IFN response and elevated levels of IL-6 and IL-1b (23).

As shown, biomarker studies have helped to characterize HIV-1 infection, in terms of both disease progression and different stages of HIV-1 infection.

The Interferon dilemma: Important in early HIV-1 infection

The importance of interferon in HIV-1 infection has long been studied and understood. Many studies have found that depending on when interferon is administered, it is either beneficial or detrimental to HIV pathogenesis. When type I interferon receptors were blocked in macaques acutely infected with SIV, it resulted in delayed and reduced antiviral gene expression, higher VLs, and more rapid disease progression (46). Conversely, administration of type I interferon (IFN-α2a) prior to and

during viral challenge of rhesus macaques resulted in initial increases of antiviral genes that decreased with subsequent doses. Prior administration of IFN-α2a was shown to delay SIV acquisition and systemic infection in rhesus macaques, but, in part due to subsequent decreased PRR expression, disease progression, as assessed by proviral DNA loads and CD4/CD8, ratio was exacerbated compared to placebo controls (46).

In contrast, comparison studies between African green monkeys and rhesus macaques confounds the above results. While both African green monkeys and rhesus macaques can be infected with SIV, $CD4^+$ T cell depletion is only observed in rhesus macaques, which is why it serves as the animal model for pathogenic HIV infection (47). This study showed that while both species of monkeys induced IFN-α production and ISG expression during the acute stage of infection, these responses are inhibited over time in African green monkeys but not in rhesus macaques (47). This study also revealed that in rhesus macaques increased interferon responses and ISG expression, which can lead to T cell activation, during chronic infection can lead to a situation where innate antiviral responses benefit the virus (45).

These findings have raised the question of the importance of IFN in the selection of the transmitted-founder virus, a question that the Hunter laboratory has investigated. A previous study from 2015 from our laboratory used 6 subtype C transmission pairs to address the question of whether transmitted-founder viruses were more sensitive or resistant to *in vitro* IFN-α treatment. If these viruses were more resistant, then that would serve as a possible selection factor in the transmission bottleneck. However, when the sensitivity of transmitted-founder viruses was compared against viruses

isolated from epidemiologically linked donors, the transmitted-founder virus was not found to be more resistant to IFN-α treatment compared to the donor viruses (25).

In contrast, a study published in 2017 by Beatrice Hahn's group showed the opposite results. When they tested transmitted-founder viruses and donor viruses isolated from patient plasma, they found that the transmitted-founder virus was more resistant *in vitro* IFN-α and IFN-α treatment (48). Whether IFNs play a significant role in the earliest stages of infection was brought into question by studies of Barouch et al., who analyzed gene expression in the first cells infected following SIV challenge and found that genes in the inflammasome pathway, some of which block the IFN response, were elevated (ref). Thus, it is still unclear what the importance of IFN is in the initiation of HIV infection *in vivo*, and whether it is a selecting factor in the transmission bottleneck, these opposing findings highlight the need for further research into the relationship between the IFN response and HIV-1.

Conclusion

This chapter has covered HIV-1 subtypes, how infections differ depending on subtypes, and predisposition to HIV-1 acquisition. It has also covered some basics on how HIV-1 interacts with the innate immune system and the possible role that innate immunity plays in the establishment and development of a new HIV-1 infection. Continued research into different aspects of HIV-1 infection are necessary in order to fully understand how a new HIV-1 infection is established and to bring the scientific community closer to finding a cure.

Figures

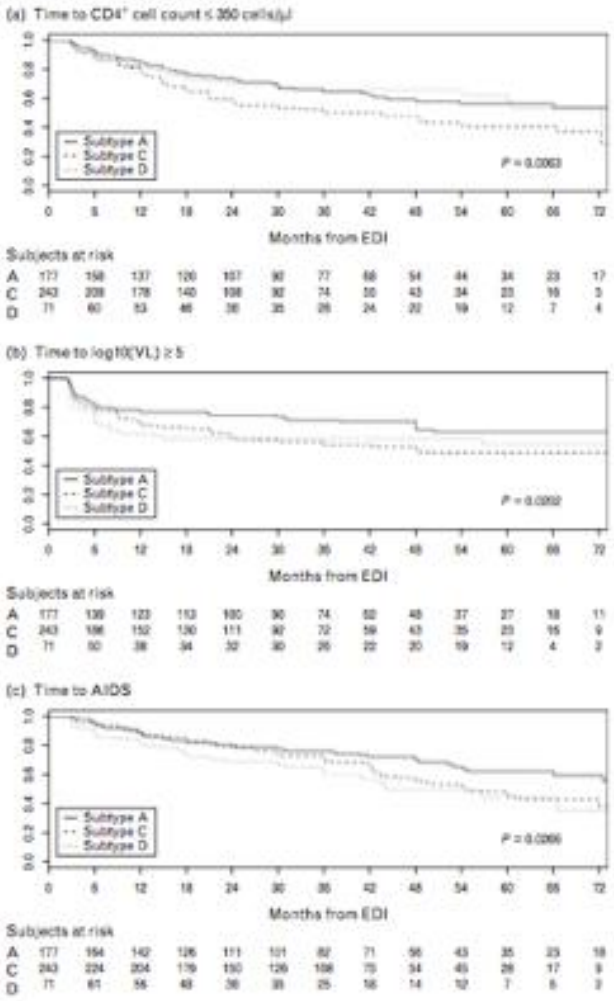

Figure 1. Differences in clinical disease progression in HIV-1 subtype A, C, and D infections. 615 individuals infected with HIV-1 subtype A, C, and D were followed from seroconversion for 5 years and came into the clinical every 3-6 months for follow-up. At these follow-up visits, blood was taken to determine viral load and $CD4^+$ T cell counts. It was found that subtype C and D had significantly faster disease progression compared to subtype A based on $CD4^+$ T cell count, viral load levels, and overall time to development of AIDS. Figure taken from Amornkul 2013 (16).

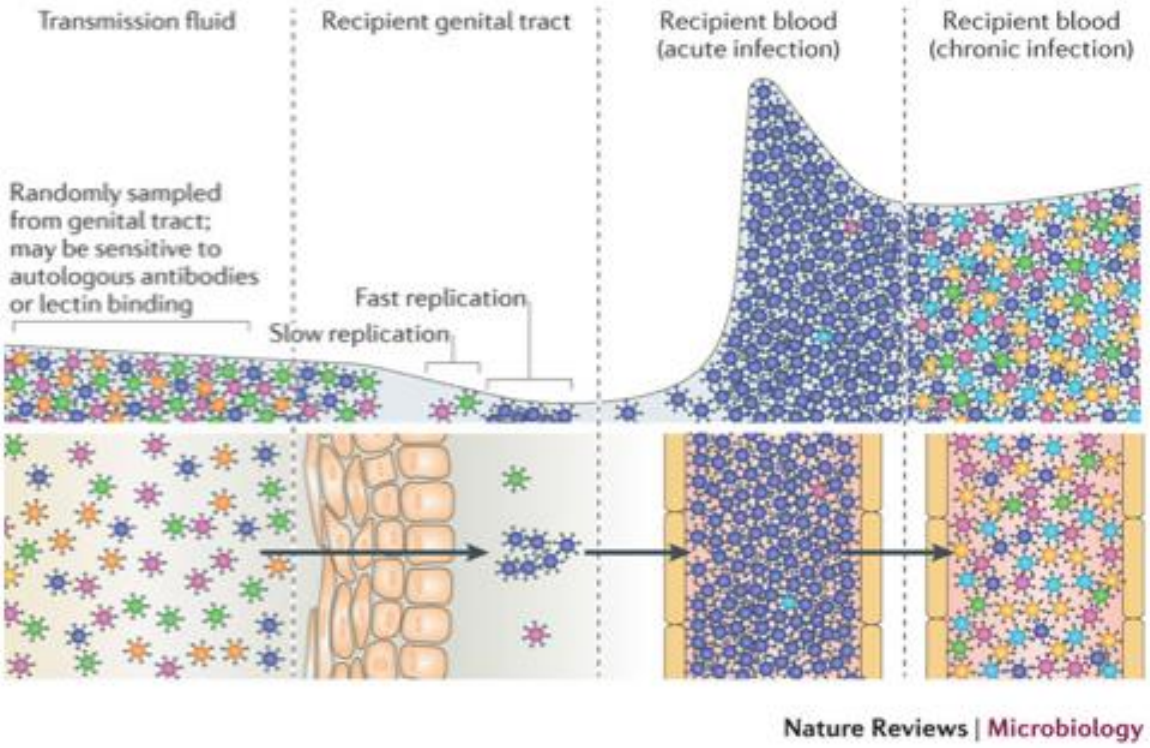

Figure 2. Selection Bottleneck in HIV-1 genital transmission. In a chronically infected HIV-1 individual, there is a genetically diverse quasispecies of viral variants that develop over years of mutations in the infected host. In a transmission event, the new recipient is exposed to this diverse quasispecies in the transmission fluid. However, in 80-90% of heterosexual transmission, the HIV-1 infection in the newly infected recipient is established by a single genetic viral variant, known as the transmitted-founder virus. It remains unclear how the transmitted-founder virus is selected out of the diverse donor quasipecies. As the the newly infected individual progresses to chronic stages, the viral quasispecies diversifies from a single viral variant to a diverse viral quasispecies. Figure taken from Joseph 2015 (49).

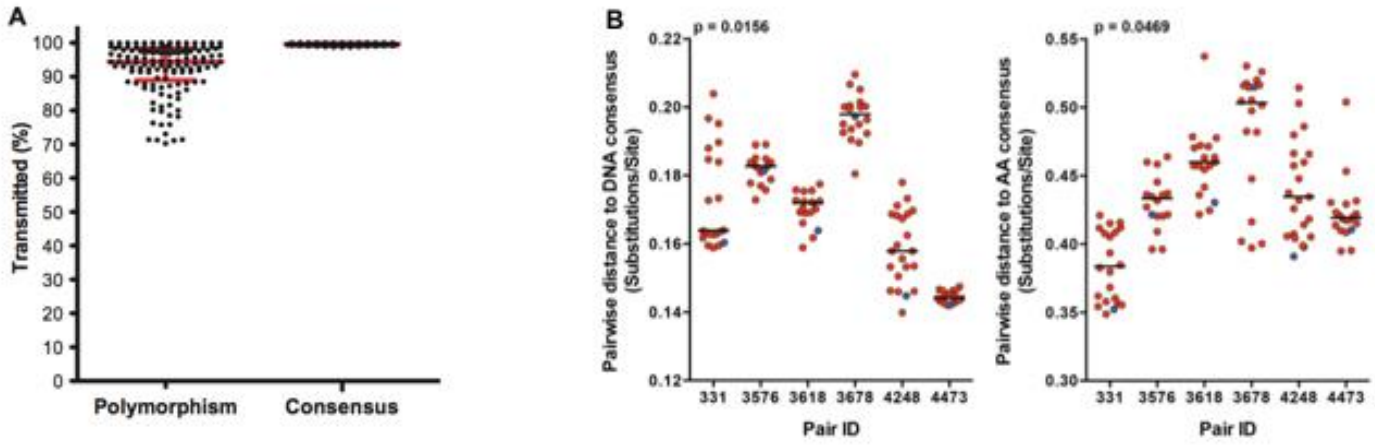

Figure 3. Consensus bias exists for HIV-1 subtype C transmission. (A) Analysis of amino acid sequences gag, pol, and nef examined 137 subtype C transmission pairs from Zambia determined that donor variants that matched the cohort consensus were transmitted 99.65% of the time to the recipient compared to donor variants that did not match the consensus, which were only transmitted 92.61% of the time. Figure take from Carlson 2014. (B) A follow-up analysis looking at six subtype C transmission pairs from Zambia examined the full-length genome nucleotide and amino acid sequences. When compared to a LANL subtype C consensus sequence, this study found that the transmitted-founder viral variant had shorter pairwise distance to the subtype C consensus compared to the non-transmitted donor variants, in both the nucleotide and amino acid analyses. Figure taken from Deymier, Ende 2015 (25).

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Chapter II: Elevated levels of inflammatory plasma biomarkers are associated with risk of HIV infection

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Abstract

Background: To determine if individuals, from HIV-1 serodiscordant couple cohorts from Rwanda and Zambia, who become HIV-positive have a distinct inflammatory biomarker profile compared to individuals who remain HIV-negative, we compared levels of biomarkers in plasma of HIV-negative individuals who either seroconverted (pre-infection) and became HIV-positive or remained HIV-negative (uninfected).

Results: We observed that individuals in the combined cohort, as well as those in the individual country cohorts, who later became HIV-1 infected had significantly higher baseline levels of multiple inflammatory cytokines/chemokines compared to individuals who remained HIV-negative. Genital inflammation/ulceration or schistosome infections were not associated with this elevated profile. Defined levels of ITAC and IL-7 were significant predictors of later HIV acquisition in ROC predictive analyses, whereas the classical Th1 and Th2 inflammatory cytokines such as IL-12 and interferon-γ or IL-4, IL-5 and Il-13 were not.

Conclusions: Overall, the data show a significant association between increased plasma biomarkers linked to inflammation and immune activation and HIV acquisition and suggests that pre-existing conditions that increase systemic biomarkers represent a factor for increased risk of HIV infection.

Keywords: Cytokines, chemokines, HIV pathogenesis, HIV acquisition, HIV discordant couples

Background

HIV-1 remains a major health crisis facing the world today. At the end of 2019, 37.9 million people were infected with HIV-1 globally (1). It is well known that sex workers, men who have sex with men, intravenous drug users, and transgender individuals are at increased risk for HIV acquisition (1), as are the seronegative partners in HIV-1 discordant cohabiting couples (2). Genital inflammation and ulceration clearly contributes to increased risk of HIV-1 acquisition. Studies in discordant couples in Rwanda and Zambia, as well as studies in other cohorts, have shown that individuals with genital ulceration/inflammation have a 5-10-fold higher risk for HIV infection (3-10). Genital ulceration/inflammation has also been shown to affect the transmission bottleneck in heterosexual HIV-1 infection. The presence of genital inflammation has been shown to increase the likelihood of transmission of two or more transmitted-founder viruses, or multivariate transmission (3, 11). Sexually transmitted infections (STI) are frequently associated with increased genital inflammation. *Schistosoma haematobium*, which colonizes the venous plexus in the bladder, has also been shown to induce a similar increase in the risk of HIV acquisition (12-17). In a study of the prevalence of *Schistosomiasis* antibodies in sera from a heterosexual HIV-discordant couple cohort in Lusaka, Zambia, it was found that Schistosome infections were linked to increased HIV-1 transmission in both sexes, increased acquisition of HIV-1 in women, and increased progression to death in HIV-positive women (18).

In a large study of at-risk HIV-negative women in South Africa it was observed that higher levels of inflammatory cytokines in the female genital tract of individuals prior to infection was associated with increased HIV susceptibility (6), allowing a more defined

approach to quantitating this risk factor for acquisition. It is clear that in women the composition of the genital microbiome can also greatly influence the genital tract inflammatory state. Consistent with this notion, a study of the FRESH cohort in Durban, South Africa, showed that women who presented with *Lactobacillus*-deficient microbiota in their reproductive tract produced higher levels of inflammatory cytokines (19). A follow-up study showed that these women had a greater risk of HIV acquisition compared to women with *Lactobacillus crispatus-rich* genital microbiota (20). However, the genital cytokine levels observed were not correlated with those in the plasma (20). In this study, we examined systemic plasma biomarker levels in individuals who would eventually seroconvert (pre-infection) and those who remained HIV-negative (uninfected) in a cohort of serodiscordant couples from both Zambia and Rwanda. We compared these two groups to determine if there was any association between systemic plasma biomarker levels and increased acquisition of HIV in HIV-negative individuals, and whether those biomarkers were associated with any pre-existing urogenital infections.

Results

Elevated systemic plasma cytokine and chemokine levels characterize individuals prior to infection.

A major goal of this study was to determine whether prior to infection seroconverting partners in the two discordant couple cohorts under study exhibited a different inflammatory cytokine or chemokine profile compared to those who remained seronegative (uninfected). Plasma from a total of 38 Zambian participants (19 uninfected, 19 pre-infection) and 30 Rwandan participants (17 uninfected, 13 pre-infection) were analyzed in a Luminex multiplex assay. All of the individuals included in this study were negative partners in an HIV-1 serodiscordant couple, and were analyzed a median of >1,000 days following enrolment (Zambia) and >450 days (Rwanda) (See Additional File 1 - Supplemental Table 1). Initial analyzes showed that the levels of the biomarkers were similar in the two countries, allowing an initial analysis of the combined Zambia and Rwanda cohorts (See Additional File 2 - Supplemental Figure 1). We observed that 18 of the 21 biomarkers measured were significantly increased in the pre-infection group compared to the uninfected group (Fractalkine, GMCSF, ITAC, IL-1ß, IL-2, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-17a, IL-21, IL-23, MIP-1α, MIP-1ß, MIP-3α, and TNFα) (Figure 1 and Additional File 3 - Supplemental Table 2). All p-values have undergone FDR-correction to account for the multiplicity of the assay. A majority of these upregulated cytokines and chemokines are involved in the inflammatory response. Because many of these cytokines are upregulated in coordinated pathways, we analyzed the data using a random forest model to establish those exerting most influence on the phenotype. Five biomarkers (ITAC, IL-8, IL-7, TNFα, and Fractalkine)

were identified as the most significant contributors to the signature associated with future HIV-1 infection (Figure 2A). We also analyzed the data using a Partial Least Squares (PLS) analysis. The PLS showed that the levels of GMCSF, Fractalkine, IFN-γ, ITAC, IL-1ß, IL-2, IL-5, IL-7, IL-8, IL-10, IL-12, IL-17α, IL-21, IL-23, MIP-1ß, and TNFα had higher impact on the separation between the uninfected and the pre-infection cohort (Figure 2B and Additional File 4 - Supplemental Table 3A). This result confirms our initial analysis that multiple inflammatory biomarkers are significantly elevated in individuals who later become infected. To further interrogate these findings, we analyzed the data from the Zambia and Rwanda combined cohorts to try and determine if genital inflammation contributed to the elevated profile observed in the pre-infection cohort.

Presence of genital inflammation or ulceration in the combined cohort does not explain elevated biomarker levels in pre-infection group.

Past studies of the Zambian and Rwandan cohorts, as well as other studies, have shown that the presence of genital inflammation/ulceration increases an individual's susceptibility to infection by HIV (3, 4, 6-9, 19, 20). To test the possible role of genital inflammation or ulceration in the elevated biomarker profile, we compared the cytokine levels in individuals from the pre-infection group for whom any form of genital inflammation or ulceration had been reported in the 6 months prior to sample collection with those for whom no genital inflammation or ulceration was reported. There was no significant difference in the levels of any of the 21 analytes analyzed. Similarly, genital inflammation/ulceration did not significantly impact systemic biomarkers in the

uninfected group. Moreover, after removing individuals with genital inflammation from the analysis, 11 of the biomarkers remained significantly higher in the pre-infection group compared to the uninfected group (Fractalkine, GMCSF, ITAC, IL-1ß, IL-6, IL-7, IL-8, IL-21, MIP-1α, MIP-3α, and TNFα) (Figure 3). We next analyzed the Rwandan and Zambian cohorts separately to determine whether any differences existed between the two cohorts when analyzed in a similar manner.

Elevated systemic plasma cytokine and chemokine levels associated with pre-infection individuals in both Zambian and Rwandan cohorts.

Despite the smaller numbers analyzed when the data was separated by country, we still observed that several inflammatory biomarkers were significantly higher in the pre-infection group than the uninfected group. In Rwanda, Fractalkine, GMCSF, ITAC, IL-1ß, IL-6, IL-7, IL-8, MIP-1α, and TNFα concentrations were significantly increased in the pre-infection group compared to the uninfected group (Figure 4A and Additional File 5 - Supplemental Table 4A). The median, interquartile range, and p-values for this analysis is shown in Additional File 5 - Supplemental Table 4A. When analyzed in a PLS model, Fractalkine, GMCSF, IFN-γ, ITAC, IL-1ß, IL-2, IL-5, IL-6, IL-7, IL-8, IL-10, IL-17α, IL-21, MIP-1α, MIP-1ß, and TNFα were found to be the major contributors to the separation of the groups (Figure 4B and Additional File 4 – Supplemental Table 3B). This supports our initial univariate analysis of the Rwanda cohort since there were a number of additional biomarkers that were trending towards significance (See Additional File 6 - Supplemental Figure 2).

In Zambia, Fractalkine, ITAC, IL-7, IL-8, IL-23, and TNFα concentrations were significantly increased in the pre-infection group compared to the uninfected group in univariate comparisons (Figure 5A and Additional File 7 – Supplemental Figure 3). The median, interquartile range, and p-values for this analysis are shown in Additional File 5 - Supplemental Table 4B. While elevated levels of Fractalkine, ITAC, IL-7, IL-8, and TNFα are observed for both countries in their respective pre-infection groups, they differ in that they exhibit additional biomarkers unique to each country. The PLS analysis showed that levels of ITAC, IL-5, IL-7, IL-8, and MIP-1α were associated with pre-infection (Figure 5B and Additional File 4 - Supplemental Table 3C).

Taken together, we observed that the pre-infection group had elevated biomarker levels in both Rwanda and Zambia even when the cohorts are analyzed separately.

Presence of *Schistosomiasis* antibody titers does not appear to be a major contributor to the elevated biomarker profile in pre-infected individuals.

We have recently reported that infection with *Schistosoma haematobium* is common even in urban settings in Zambia and that infection was associated with increased susceptibility to HIV-1 infection (18). Because this parasite colonizes the venous plexus in the bladder, we investigated prior infection as evidenced by antibodies resulted in elevated cytokines. However, screening of the plasma from both the uninfected and pre-infection groups for antibodies to the schistosome showed that a similar proportion of individuals in both had detectable antibody titers (9/19 uninfected; 11/19 pre-infection). The Zambian cohort was therefore divided into four groups: uninfected individuals with negative *Schistosomiasis* antibody titers, uninfected individuals with positive antibody

titers, pre-infection individuals with negative titers, and pre-infection individuals with positive titers (See Additional File 8 - Supplemental Figure 4). We did not observe any significant differences in biomarker levels between seropositive and seronegative individuals in either the uninfected group or the pre-infection group. With the proviso that we are analyzing very small groups of individuals, this suggests that schistosome infection is not associated with the elevated biomarker levels observed in the pre-infection group.

Predictive analyses identify elevated levels of several biomarkers as markers of pre-infection

Based on preliminary results from partial least squares analysis (figures 2B, 4B, and 5B), we wanted to determine which biomarkers included in the study were associated with future HIV-1 acquisition. To achieve this, we analyzed the combined Zambian and Rwandan cohort using Receiver Operating Characteristics (ROC) curves and established a cutoff for the area under the curve (AUC) of 0.8. Using a cutoff of 0.8 means that any biomarker identified in the analysis has over an 80% chance of distinguishing pre-infection individuals from those that remain uninfected. ITAC, Fractalkine, IL-7, IL-8, and TNFα were identified as markers predictive for pre-infection (Figure 6). ROC curves for individual Zambia and Rwanda cohorts are shown in Additional Files 9 and 10 - Supplemental Figures 5 and 6. This finding is supported by our random forest analysis (Figure 2). Using a kfold validation model, we found that concentrations of ITAC above 30.98 pg/ml was highly predictive of a seronegative partner who would become infected (probability of 0.91; sensitivity of 0.75; specificity

0.94). If an IL-7 value above 6.99 pg/ml was added to the model, the probability of identifying pre-infection individuals increased to 0.97 (Figure 7).

Discussion

This study found that individuals who will eventually seroconvert have higher levels of proinflammatory biomarkers in their plasma compared to individuals who remain HIV-negative, suggesting a novel link between predisposition to HIV infection and systemic biomarker levels

Our findings suggest that pre-existing conditions which induce systemic inflammation can represent a risk factor for HIV acquisition.

An example of a viral infection capable of inducing this is hepatitis C virus. Plasma levels of IL-10 and MIP1ß were positively correlated with HCV RNA levels and may be involved in HCV immunopathogenesis (21), HIV/HCV co-infected women had higher levels of several proinflammatory biomarkers – biomarkers elevated in each infection or disease discussed in this section are summarized in Additional File 11 - Supplemental Table 5. In addition, HCV-positive HIV-negative women had higher levels of IFN-γ and IL-17 compared to other groups (22). However, the prevalence of HCV infections in both Zambia and Rwanda has been reported to be low (23, 24).

Elevated systemic levels of cytokines and chemokines are also seen in multiple infections common in sub-Saharan Africa. Multiple researchers have found that malaria infection has been linked to elevated systemic cytokine responses. HIV and *P. falciparum* co-infected individuals had increased HIV viral loads, a steeper decline in $CD4^+$ T cell counts, and exhibited higher levels of several acute phase proinflammatory cytokines (25). Similarly, symptomatic malaria infections had increased levels of multiple cytokines compared to uninfected controls (26). It has also been found that individuals with greater malaria disease severity had higher levels of CRP, TNFα, and

IFN-γ (27). *P. vivax* infections also are associated with higher levels of proinflammatory cytokines (28).

Elevated systemic biomarkers have also been found in Tuberculosis (TB). Multiple studies found that individuals with acute and latent TB infections had higher levels of cytokines and chemokines compared to healthy controls (29-32). These results show that TB infections can increase several proinflammatory cytokines regardless of the stage of infection.

A separate study looked at proinflammatory cytokine levels in TB-infected individuals co-infected with *S. stercoralis*. *S. stercoralis* is a soil-transmitted helminth that infects about 50-100 million people worldwide (33). Individuals co-infected with TB and *S. stercoralis* have increased levels of several type 2 cytokines compared to individuals only infected with TB (34). One possible explanation for this is an association with microbial translocation. *S. stecoralis* infected individuals exhibited significantly higher plasma levels of microbial translocation markers (i.e. LPS) and this was associated with increased levels of several proinflammatory cytokines (35). Researchers looking at microbial translocation in HIV infected individuals found that LPS was also positively correlated with plasma levels of IL-6, TNFα, and hsCRP (36). This finding is particularly interesting because it is well known that microbial translocation is associated with disease severity in HIV infections (37).

These studies show that several common infections in sub-Saharan Africa are capable of elevating systemic levels of several pro-inflammatory cytokines, which we found as a risk factor for HIV acquisition. However, it is also possible that higher levels of biomarkers prior to infection may have additional impact once an individual becomes

HIV-infected. High levels of several cytokines appear to enhance HIV replication and disease progression (38). Additional studies are needed not only to determine the cause of the elevated levels of biomarkers in individuals prior to HIV acquisition, but also how these elevated levels may impact the disease progression of these patients once infected.

While these common infections in Africa are associated with elevated inflammatory cytokines, the observed biomarker profile of the pre-infection individuals reported on here is not indicative of a classic innate immune response or T cell response. Indeed, the elevated cytokines and chemokines observed play roles in many different arms of the immune system. Our analysis showed that three biomarkers, Fractalkine, ITAC, and IL-7, were highly predictive as risk factors for HIV acquisition if elevated above certain levels. These biomarkers can be elevated from infection, but also from non-communicable conditions. Compared to healthy controls, elevated levels of Fractalkine were found in individuals with type 2 diabetes, systemic lupus erythematosus, and systemic sclerosis, an autoimmune disorder that affects the vascular system and leads to early defective angiogenesis. (39-42). In systemic sclerosis, the higher levels of soluble Fractalkine in the blood were associated with vascular activation and increased disease severity (42). That same study found higher levels of ITAC compared to healthy controls (42). ITAC, also known as IFN-inducible T cell α chemoattractant, was found to be at higher levels in patients with inflammatory bowel disease (Crohn's disease and ulcerative colitis), fibromyalgia, and sarcoidosis (43-45). Sarcoidosis is a systemic inflammatory granulomatous disease that affects lungs of its patients and higher

systemic levels of ITAC were found to be predictive of future pulmonary function test decline (43).

IL-7 is not normally considered an inflammatory cytokine and is more associated with T cell homeostasis. However, higher levels of IL-7 were found to be associated with several diseases, such was fibromyalgia (46) and also in patients with colorectal and esophageal cancers compared to healthy controls (47). Unfortunately, health data on such conditions were not recorded as part of the Heterosexual Transmission Protocol into which discordant couples were enrolled. Nevertheless, the studies reported here indicate that additional studies of cytokine/chemokine levels in at risk individuals is warranted.

There are a number of limitations to the current study. We were only able to analyze the inflammatory biomarkers in a limited number of individuals since the availability of pre-HIV infection plasma samples from the discordant couples studied here are also limited (only 3-7% of individuals in this cohort seroconverted per year depending on the cohort). In addition, we have limited information on other infections and health conditions of individuals in study. This is compounded by the fact that only a small number of studies have reported on systemic biomarkers in other infections or diseases in the context of an African population. As a result, it is difficult for us to identify specific infections or diseases that may be causing the elevated biomarker profile that we observed. Analogous studies of cohorts in the USA or Europe, where additional biomarker data on other infections/diseases may be available, would represent a valuable follow-up study to this one.

The identification of key biomarkers associated with HIV acquisition has important clinical ramifications. Our study identified elevated levels of both ITAC and IL-7 as highly predictive of HIV acquisition risk. If individuals in a HIV high-risk region are found to have elevated levels of one or both of these biomarkers, more intensive HIV-1 prevention approaches could be taken in order to protect those individuals. Additional studies are needed not only to determine the cause of the elevated levels of biomarkers in individuals prior to HIV acquisition and how they facilitate the acquisition event, but also how these elevated levels may impact the disease progression of these patients once infected. Overall, our findings suggest that individuals at risk for infection could be identified by testing for elevated levels of a very limited number of biomarkers.

Conclusion

We show that in a cohort of seronegative partners from serodiscordant couples, individuals who eventually acquire HIV from their partner have higher levels of inflammatory cytokines and chemokines compared to individuals that remain HIV-negative. This was observed in both Rwanda and Zambia where subtype A and subtype C are the predominant HIV-1 serotypes. A Receiver Operator Characteristics analysis showed that the levels of just two cytokines, ITAC and IL-7, were highly predictive of future infection. This suggests high systemic biomarker levels are both a risk factor and a quantitative predictor for HIV acquisition.

Materials and Methods

Study Subjects

All participants were enrolled in the Rwanda Zambia HIV Research Group (RZHRG) discordant couple cohorts in Lusaka, Zambia and Kigali, Rwanda. Subjects from both cohorts were enrolled in human subjects protocols approved by the Emory Institutional Review Board, the Rwanda National Ethics Committee and the University of Zambia Research Ethic Committee and provided written consent. When the participants enrolled in the cohort, they were provided couples counseling and testing, treatment for sexually transmitted infections (STIs), and condoms to reduce transmission of HIV-1. All the subjects tested in this study were the seronegative partner within a serodiscordant cohabitating heterosexual couple. Subjects were selected on their seronegative status, availability of plasma samples, and selected from both Zambian and Rwandan cohorts. The negative partner was tested for HIV every one to three months. In the Zambia cohort, the median days from enrollment to when the sample was taken was 1234 for the uninfected group and 1087 for the pre-infection group. Samples for the pre-infection group was collected a median of 46 days before the estimated date of infection (EDI). In the Rwanda cohort, the median days from enrollment to when the samples were taken was 494 for the uninfected group and 457 for the pre-infection group. Samples for the pre-infection group was collected a median of 45 days before the EDI. The algorithm used to determine the EDI has been previously described (3).

Evaluation of Plasma Cytokines

The plasma cytokine and chemokine levels were measured using a Milliplex Map Human High Sensitivity T Cell Panel (HSTCMAG-28SK). This kit measures the levels of 21 inflammatory cytokines and chemokines. The samples were run in duplicate. In order to eliminate batch to batch variation in the assay, all tests were carried out on the same batch of plates and approximately equal numbers of pre-infection and uninfected plasma were run on the same plate. The plates were quantified and standardized on a Bioplex 2000 at the Yerkes Virology Core and final concentrations were extrapolated from a standard curve and expressed in pg/ml. All plasma samples were stored at -80°C and had undergone a single freeze-thaw for aliquoting prior to use.

Genital Inflammation and ulceration Data Collection

As described in Haaland et al. (3), medical and laboratory signs and symptoms of inflammatory or ulcerative STI, candida, and bacterial vaginosis were recorded systematically at routine study visits and at interim sick visits, with full physical and/or genital exams conducted annually and as clinically indicated; physical and genital exams were routinely conducted on the visit date when lab test results indicated HIV-1 seroconversion. A self-reported symptom was considered present whether or not the patient sought medical treatment and included treatment administered at external clinics. The generation of the composite variables were described in Wall et al (4). Briefly, for each 3-monthly interval, composite variables were created. The genital inflammation composite included inflammatory STIs (clinical or laboratory diagnosis or treatment of gonorrhea, chlamydia or trichomonas) and non-inflammatory STIs (reported discharge, dysuria, dyspareunia; observed discharge or inflammation of

external or internal genitalia; and/or laboratory diagnosis of candida or BV). The composite for genital ulcer included observed or reported genital ulcers and/or incident positive RPR. A subject was considered having positive genital inflammation or ulceration if they had presence of either in the six months prior to biomarker sample collection.

Schistosomiasis Antibody Titer Data Collection

As described in Wall et al. (18), plasma samples were collected at baseline analyzed in an ELISA assay for antibodies to schistosome soluble worm antigen preparation (SWAP). A 4-parameter curve fitting model was used to assign units based on the standard curve to each unknown plasma. The positive cutoff value was set at three standard deviations above the average anti-SWAP IgG in serum from egg negative controls from the US and Europe. A positive schistosomiasis result was defined as having a positive SWAP antibody response.

Data Analysis

Comparison between the uninfected and the pre-infection groups were done with nonparametric Kolmogorov-Smirnov tests in Prism 9. We addressed the multiple testing issue by using the Benjamini-Hochberg False Discovery Rate (FDR) correction (48). The Random Forest model was performed in R 4.0.0 at the default setting. The details were described previously (49).

Partial Least Squares (PLS) analysis was performed using the JMP Pro 15 statistical package. PLS analysis had a variable importance cutoff of 0.8. The Receiver Operating Characteristic (ROC) curve analysis used a cutoff of 0.8 for area under the curve (AUC).

The predictive results were generated with a k-fold validation which included two splits in the model (k=5).

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Author Contributions

Conceived and designed the experiments: SM, RT, EH. Performed the experiments: SM. Provided genital inflammation/ulceration and parasite data: KW. Analyzed the data: SM, TY. Contributed reagents/materials/analysis: SM, KW, TY, RT, JG, WK, SA, EH. Wrote the paper: SM, EH.

Ethics approval and consent to participate

This study was approved by the Emory Institutional Review Board, the Rwanda National Ethics Committee and the University of Zambia Research Ethic Committee. All participants gave written informed consent for their enrollment in the cohort.

Figures

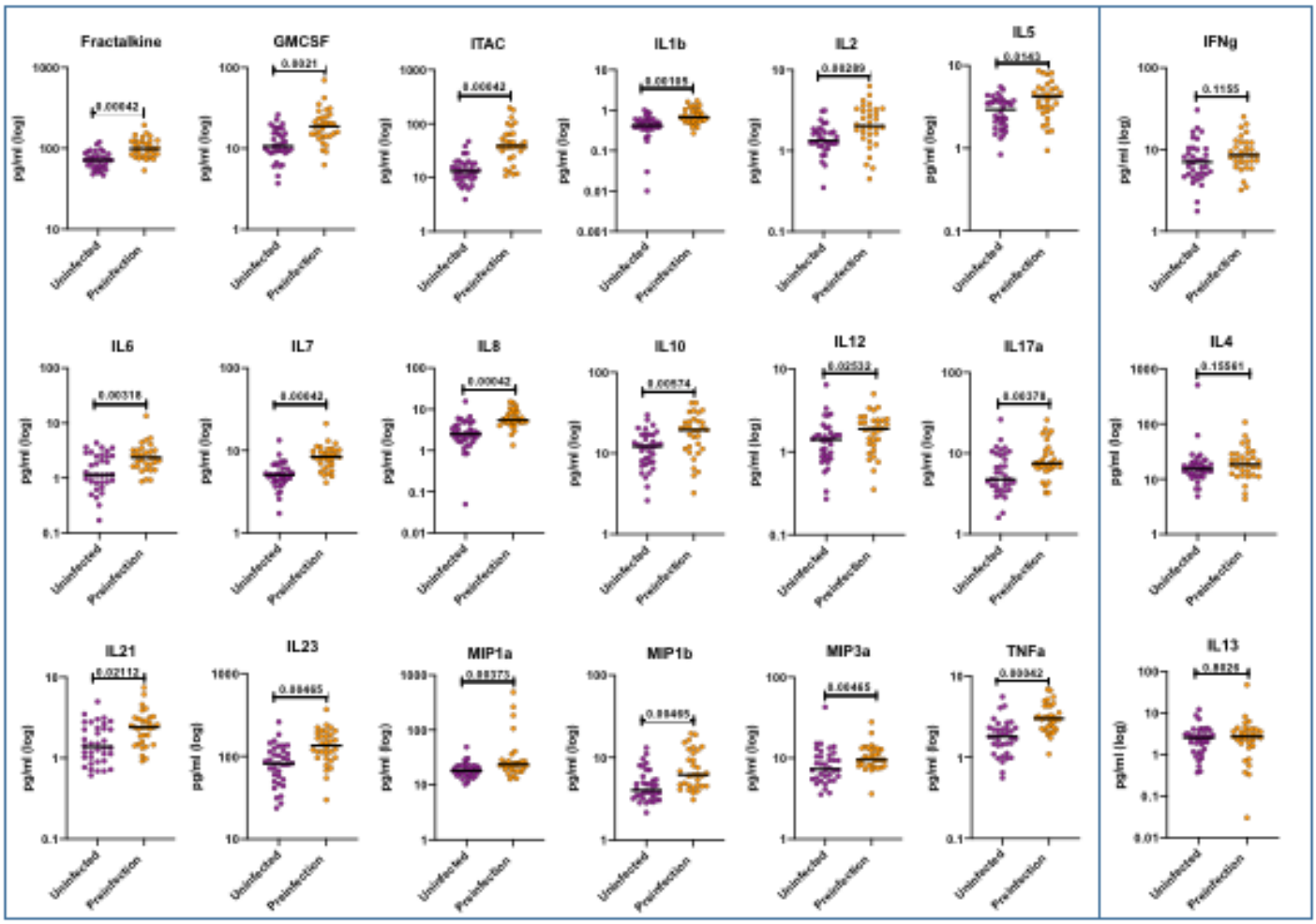


Figure 1. Circulating biomarkers in the combined Rwanda and Zambia cohorts: preinfection individuals have significantly higher cytokines and chemokine levels compared to the uninfected group. Cytokine and chemokines concentrations from plasma from the preinfection and uninfected groups were compared (Kolmogorov-Smirnov test, two-tailed, FDR adjusted p-values). The uninfected (purple) and preinfection (orange) groups contained a mixture of samples from Zambia and Rwanda.

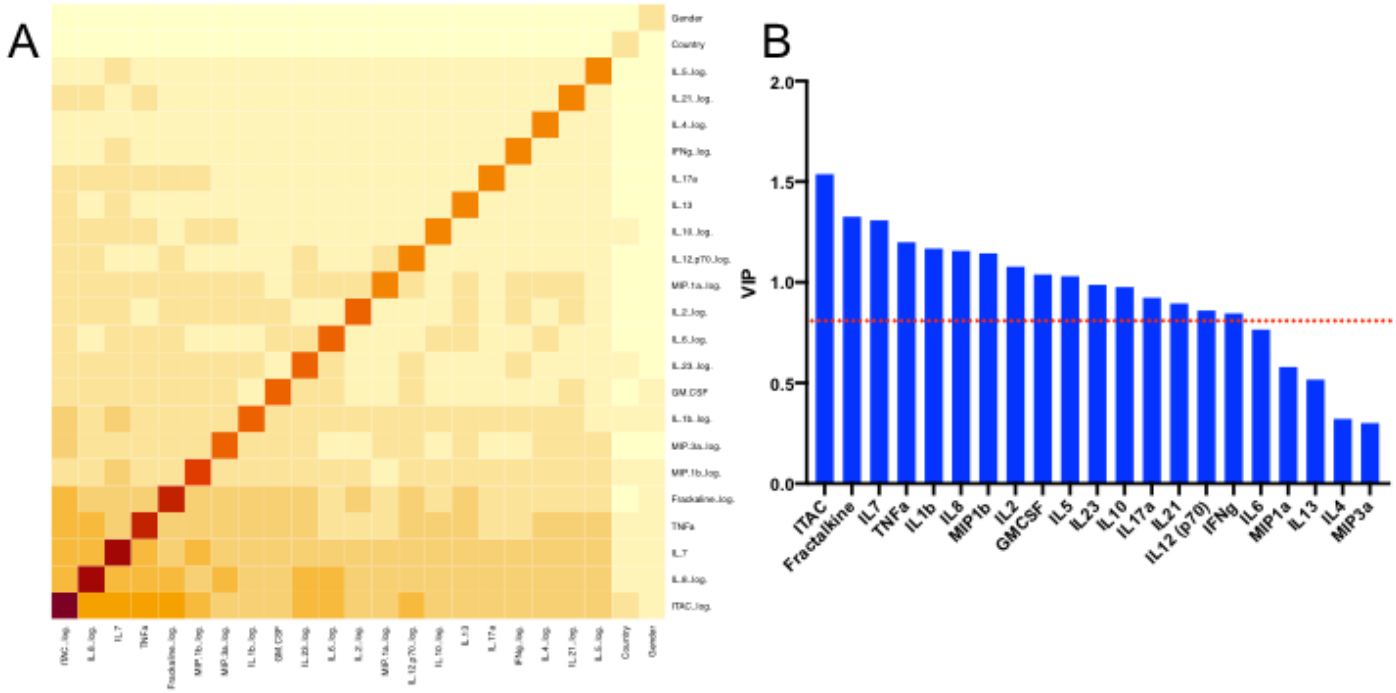


Figure 2. Partial least square (PLS) and Random Forest Analysis supported initial analyses. (A) Partial Least Square (PLS) Analysis for combined Zambian and Rwandan cohorts. Analysis was done with NIPALS Fit with 1 Factor. VIP (Variable Importance Plot) Threshold was set at 0.8. ITAC, GMCSF, Fractalkine, IFN-γ, IL-10, IL-12, IL-17a, IL-1b, IL-2, IL-21, IL-23, IL-5, IL-7, IL-8, MIP-1b, and TNFa were the biomarkers that caused preinfection profile in combined cohorts. (B) Random Forest Analysis used to determine which cytokines and chemokines contributed to the pattern of predisposition observed. In this analysis, the color of the square indicates the importance of the cytokine or chemokine to the profile of predisposition (light yellow is less importance, dark red is high importance).

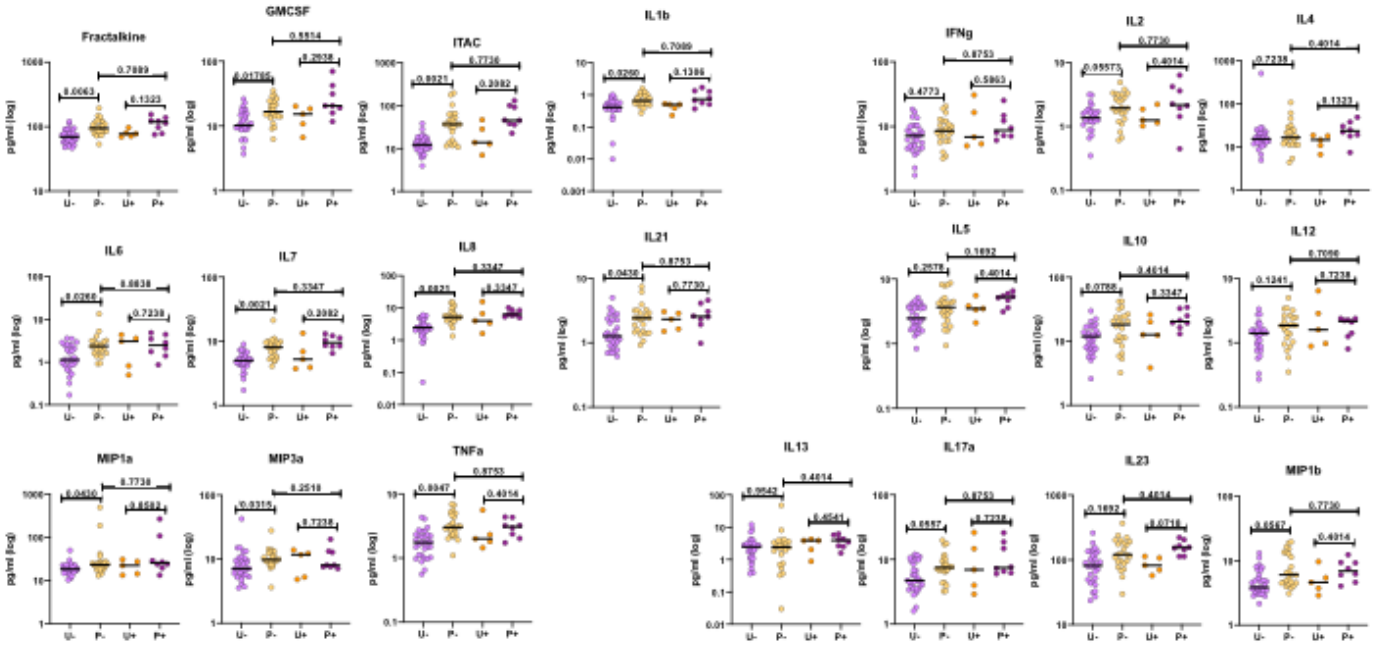


Figure 3. Preinfection individuals have significantly higher cytokines and chemokine levels compared to the uninfected group when individuals exhibiting genital inflammation/ulceration are excluded. Cytokine and chemokines concentrations from plasma from the Zambian and Rwandan preinfection and uninfected groups were compared when individuals were separated based on presence or absence of genital inflammation or ulceration. Forms of genital inflammation included: ulcers, inflammation due to STI, and inflammation not caused by STIs. The levels of cytokines and chemokines were separated by the country of origin (Kolmogorov-Smirnov test, two-tailed, FDR adjusted p-values). Light purple (uninfected individuals without genital inflammation, U-), yellow (preinfection individuals without genital inflammation, P-), orange (uninfected individuals with genital inflammation, U+), purple (preinfection individuals with genital inflammation, P+).

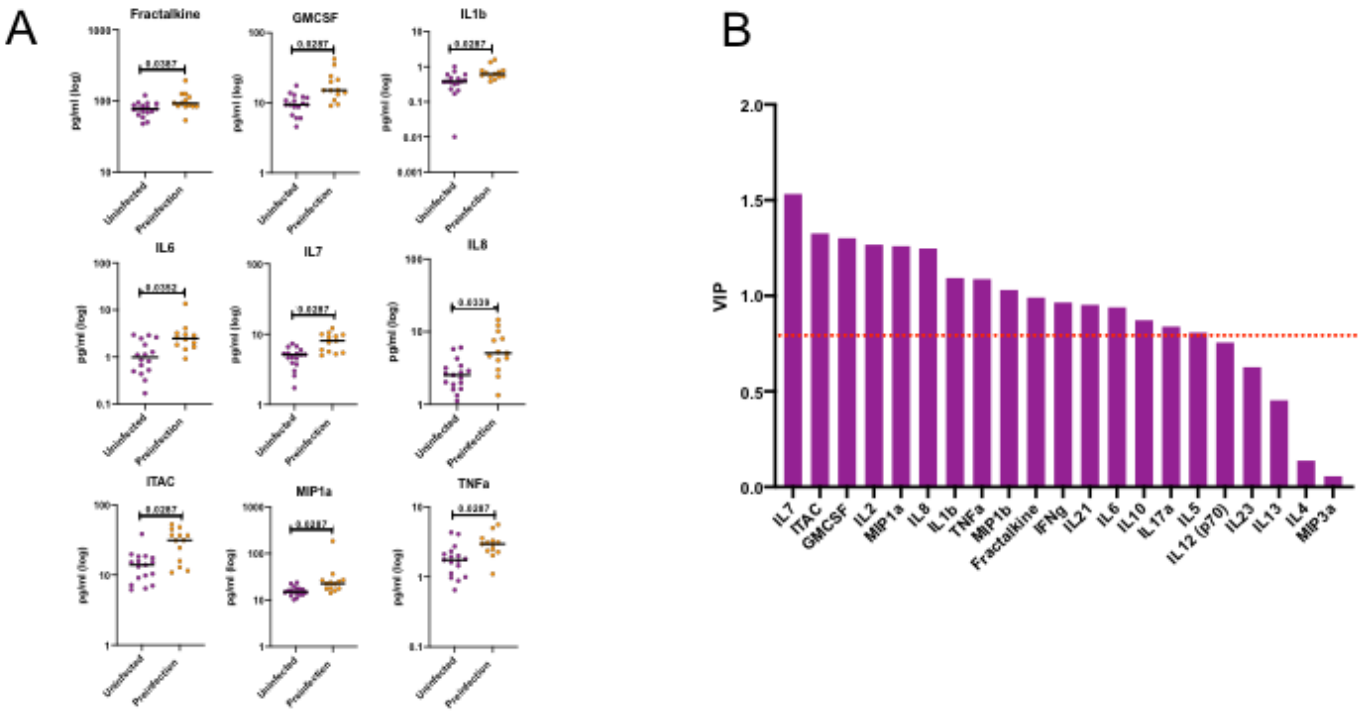


Figure 4. Circulating biomarkers in the Rwanda cohort: preinfection individuals have significantly higher cytokines and chemokine levels compared to the uninfected group. (A) The levels of cytokines and chemokines were separated by the country of origin and the uninfected (purple) and preinfection (orange) groups were compared (Kolmogorov-Smirnov test, two-tailed, FDR adjusted p-values). (B) Partial Least Square (PLS) Analysis for the Rwandan cohort. Analysis was done with NIPALS Fit with 1 Factor. VIP (Variable Importance Plot) Threshold was set at 0.8. PLS analysis for Rwandan cohort. ITAC, GMCSF, Fractalkine, IFN-γ, IL-10, IL-17a, IL-1b, IL-2, IL-21, IL-5, IL-6, IL-7, IL-8, MIP-1a, MIP-1b, and TNFa were the biomarkers that caused preinfection profile in Rwanda.

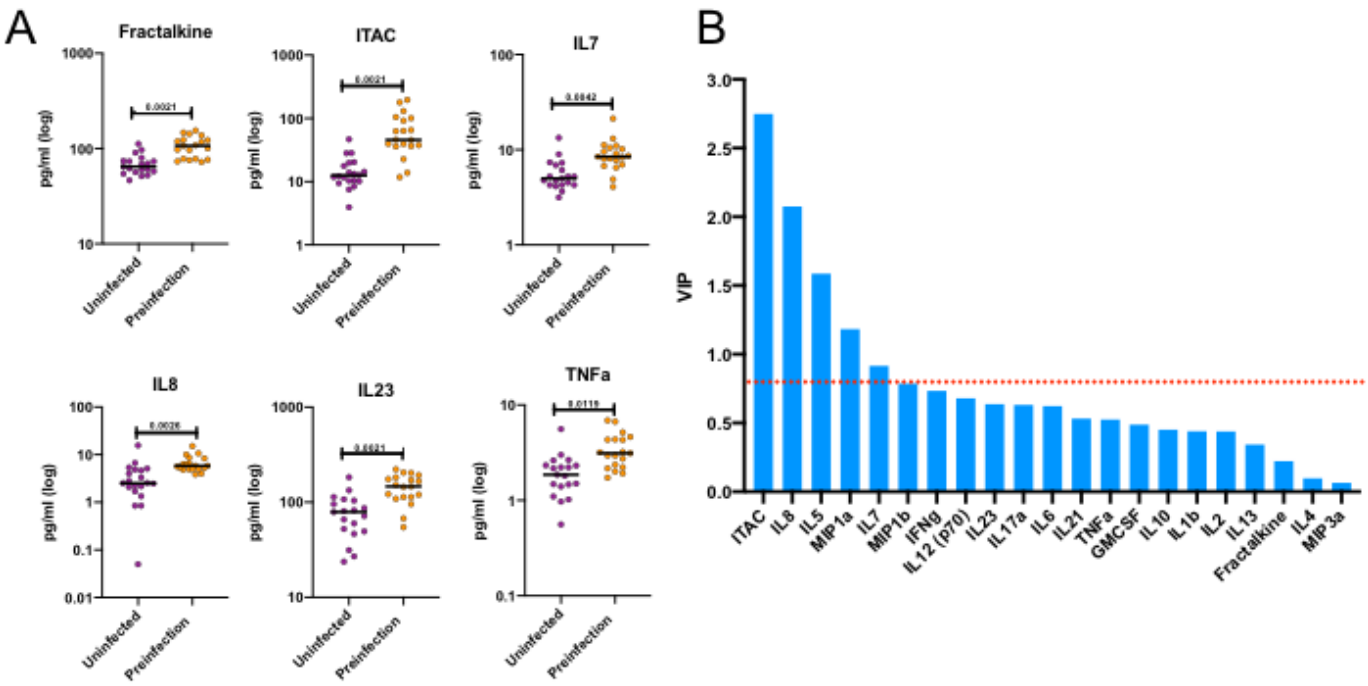


Figure 5. Circulating biomarkers in the Zambia cohort: preinfection individuals have significantly higher cytokines and chemokine levels compared to the uninfected group. (A) The levels of cytokines and chemokines were separated by the country of origin and the uninfected (purple) and preinfection (orange) groups were compared (Kolmogorov-Smirnov test, two-tailed, FDR adjusted p-values). (B) Partial Least Square (PLS) Analysis for the Zambian cohort. Analysis was done with NIPALS Fit with 1 Factor. VIP (Variable Importance Plot) Threshold was set at 0.8. (A) PLS analysis for Zambian cohort. ITAC, IL-5, IL-8, and MIP-1a were the biomarkers that caused preinfection profile in Zambia.

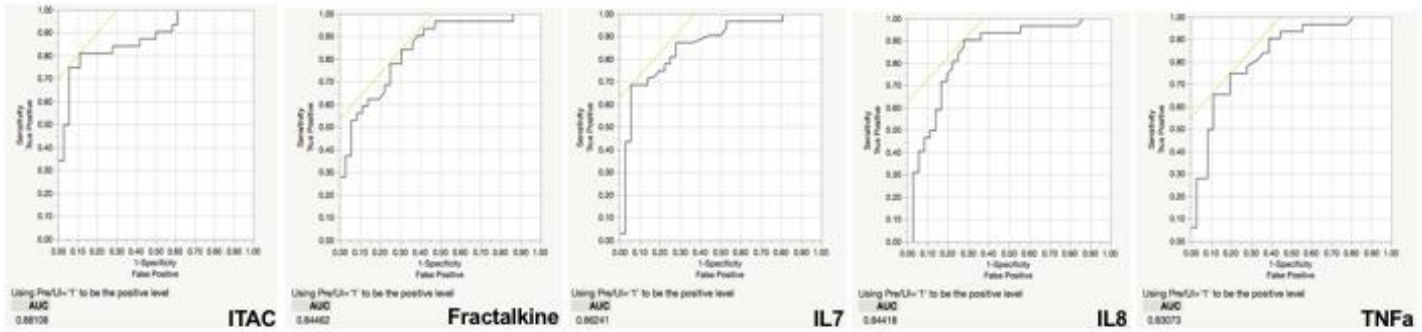
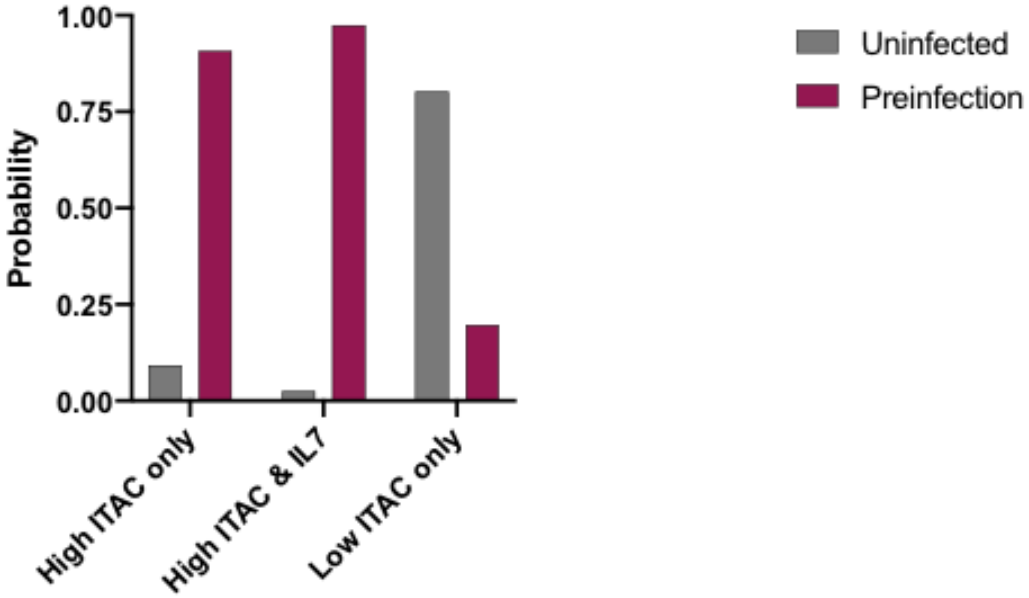


Figure 6. Receiver Operating Characteristics (ROC) curves for combined Zambia and Rwanda cohorts identifies biomarkers that distinguishes preinfection individuals. Elevated levels of ITAC, Fractalkine, IL-7, IL-8, and TNFa identify individuals as risk for HIV acquisition. Area under the curve (AUC) shut off was 0.8 for separating the uninfected and preinfection individuals.



Number of individuals in each split from kfold=5 validation

	Uninfected	Preinfection
High ITAC only	2	24
High ITAC and IL7	0	18
Low ITAC only	34	8

Figure 7. Kfold validation model identifies elevated ITAC and IL-7 levels as markers for preinfection from combined Rwandan and Zambian cohorts. Two splits were performed to find biomarkers associated with preinfection. The bar graph shows the response probability for the multiple splits performed in the model. The model found that elevated ITAC identified preinfection individuals with a probability of 0.9077 and elevated ITAC and IL-7 identified preinfection individuals with a probability of 0.9744. Lower levels of ITAC were found identify uninfected individuals. Uninfected (gray) and preinfection (maroon).

The table shows the number of individuals from the total cohort that fall into each split performed by the model (kfold=5).

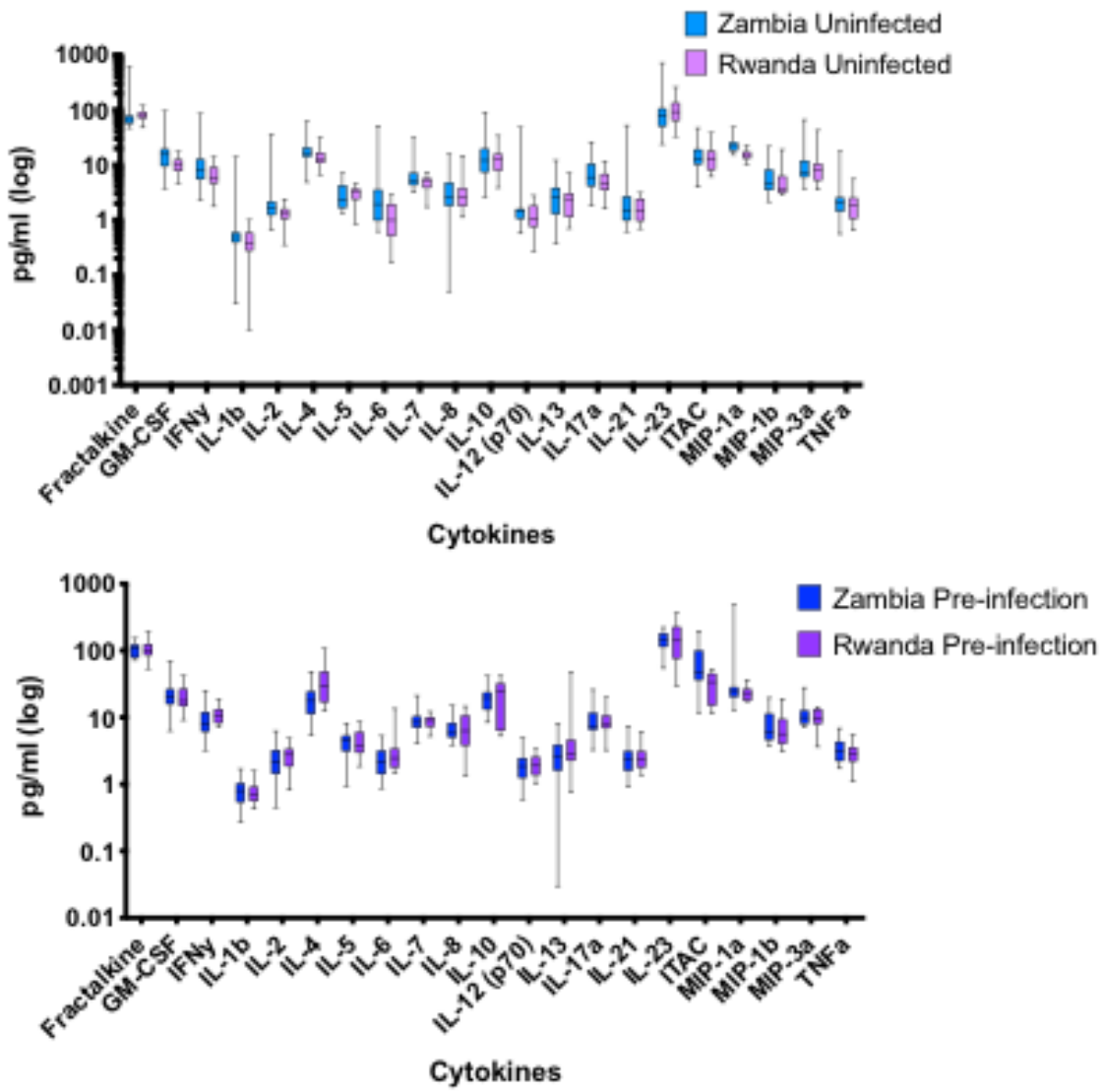
Supplemental Materials

	Zambia	Rwanda
Total # of individuals in cohort	38	30
# in the uninfected cohort	19	17
# of males	8	7
# of females	11	10
# with genital inflammation/ulceration	3	2
# with positive Schistosomiasis titers	1	3
Median days from enrollment	1234	494
# in the preinfection cohort	19	13
# of males	11	7
# of females	8	6
# with genital inflammation/ulceration	7	1
# with positive Schistosomiasis titers	11	2
Median days from enrollment	1087	457
Median days before infection	46	45

Sup. Table 1. Demographics of Zambia and Rwanda cohort.

	Uninfected		Preinfection		
	Median (pg/ml)	IQR (pg/ml)	Median (pg/ml)	IQR (pg/ml)	FDR p-value
Fractalkine	71.42	23.67	99.15	38.73	0.00042
GM-CSF	10.8	6.255	18.76	9.9425	0.0021
ITAC	13.17	7.985	38.67	33.7325	0.00042
IL-1b	0.42	0.205	0.67	0.4575	0.00105
IL-2	1.3	0.56	2.03	1.6275	0.00289
IL-5	3.12	1.81	4.23	2.21	0.01431
IL-6	1.13	1.72	2.37	1.565	0.00382
IL-7	5.01	1.775	8.385	3.3525	0.00042
IL-8	2.52	2.355	5.465	3.33	0.00042
IL-10	12.41	9.2	19.365	13.2275	0.00574
IL-12	1.415	0.8725	1.88	1.1425	0.02532
IL-17a	4.75	5.895	7.475	3.695	0.00378
IL-21	1.43	1.33	2.4	1.5225	0.02112
IL-23	82.23	56.245	134.775	88.9275	0.00465
MIP-1a	18.17	6.965	23.685	7.885	0.00373
MIP-1b	3.85	2.06	6.18	7.885	0.00465
MIP-3a	7.11	5.065	9.54	4.78	0.00465
TNFa	1.8	1.005	3.015	1.985	0.00042
IFNg	7.21	5.24	8.56	5.0825	0.1155
IL-4	15.3	7.22	18.495	16.03	0.15561
IL-13	2.56	2.53	2.705	2.14	0.8026

Sup. Table 2. Biomarkers increased in preinfection group compared to uninfected group in combined Rwandan and Zambian cohort.



Sup. Fig 1. Uninfected and Preinfection levels of cytokines and chemokines compared between Zambia and Rwanda. There were no significant differences observed between levels of biomarkers in Zambia (blue) and Rwanda (purple) in both the uninfected and preinfection cohorts.

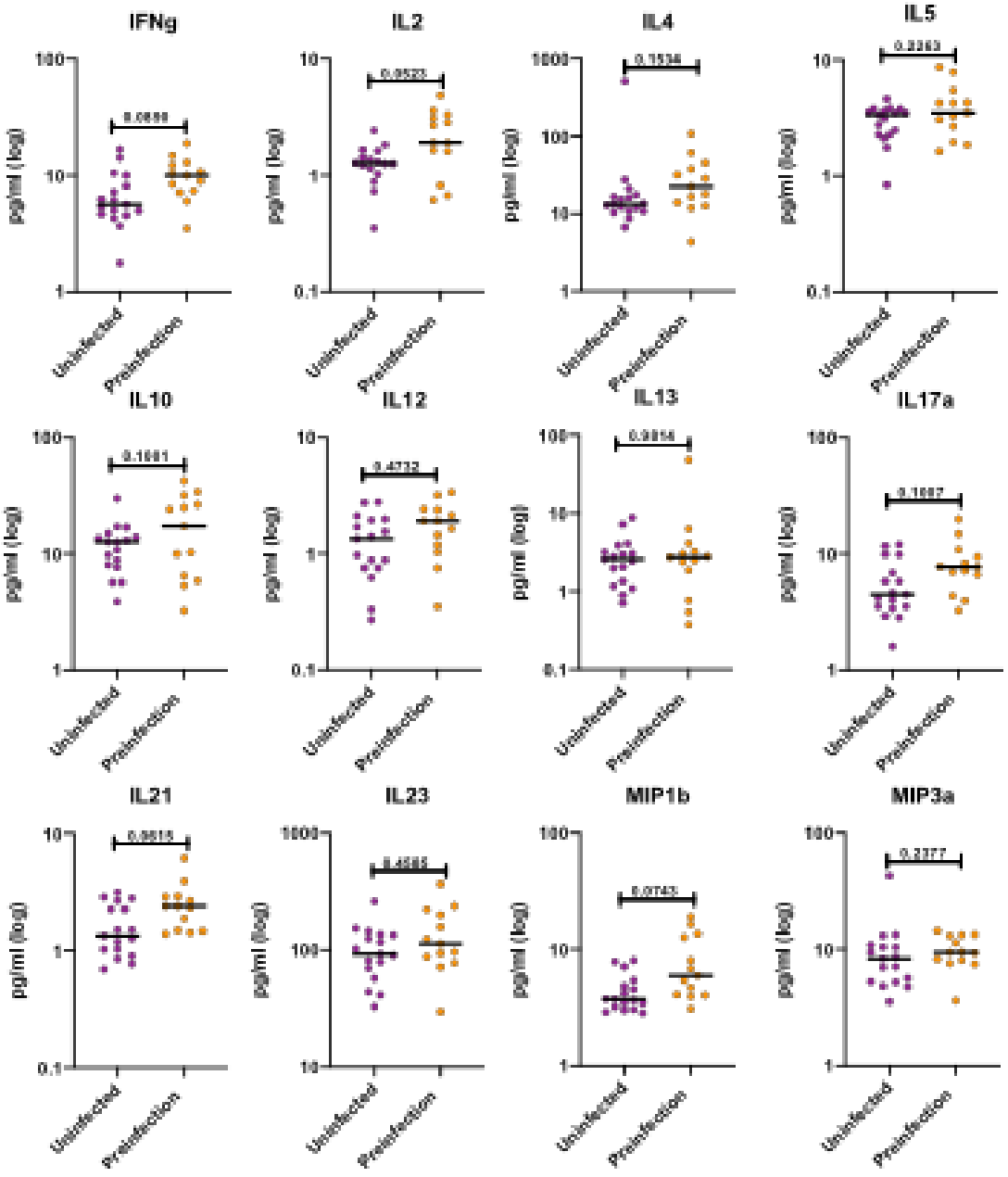
(A) Biomarkers	VIP	(B) Biomarkers	VIP	(C) Biomarkers	VIP
Fractalkine	1.3259	Fractalkine	0.9901	Fractalkine	0.2223
GM-CSF	1.0385	GM-CSF	1.301	GM-CSF	0.4878
IFNy	0.8451	IFNy	0.9651	IFNy	0.7346
IL-1b	1.1681	IL-1b	1.0939	IL-1b	0.4392
IL-2	1.0777	IL-2	1.2672	IL-2	0.4389
IL-4	0.3220	IL-4	0.1382	IL-4	0.0978
IL-5	1.0302	IL-5	0.8081	IL-5	1.5860
IL-6	0.7652	IL-6	0.9391	IL-6	0.6227
IL-7	1.3083	IL-7	1.5326	IL-7	0.9155
IL-8	1.1564	IL-8	1.2471	IL-8	2.0761
IL-10	0.9766	IL-10	0.8721	IL-10	0.4507
IL-12 (p70)	0.8602	IL-12 (p70)	0.7555	IL-12 (p70)	0.6799
IL-13	0.5169	IL-13	0.453	IL-13	0.3421
IL-17a	0.9232	IL-17a	0.8391	IL-17a	0.6320
IL-21	0.8949	IL-21	0.9531	IL-21	0.5320
IL-23	0.9879	IL-23	0.6269	IL-23	0.6380
ITAC	1.5373	ITAC	1.3263	ITAC	2.7488
MIP-1a	0.5792	MIP-1a	1.2595	MIP-1a	1.1830
MIP-1b	1.1439	MIP-1b	1.0304	MIP-1b	0.7871
MIP-3a	0.3005	MIP-3a	0.0568	MIP-3a	0.0651
TNFa	1.1986	TNFa	1.0876	TNFa	0.5248

Sup. Table 3. Variable Importance values for Partial Least Square analyses for combined cohorts (A), Rwandan cohort (B), and Zambian cohort (C).

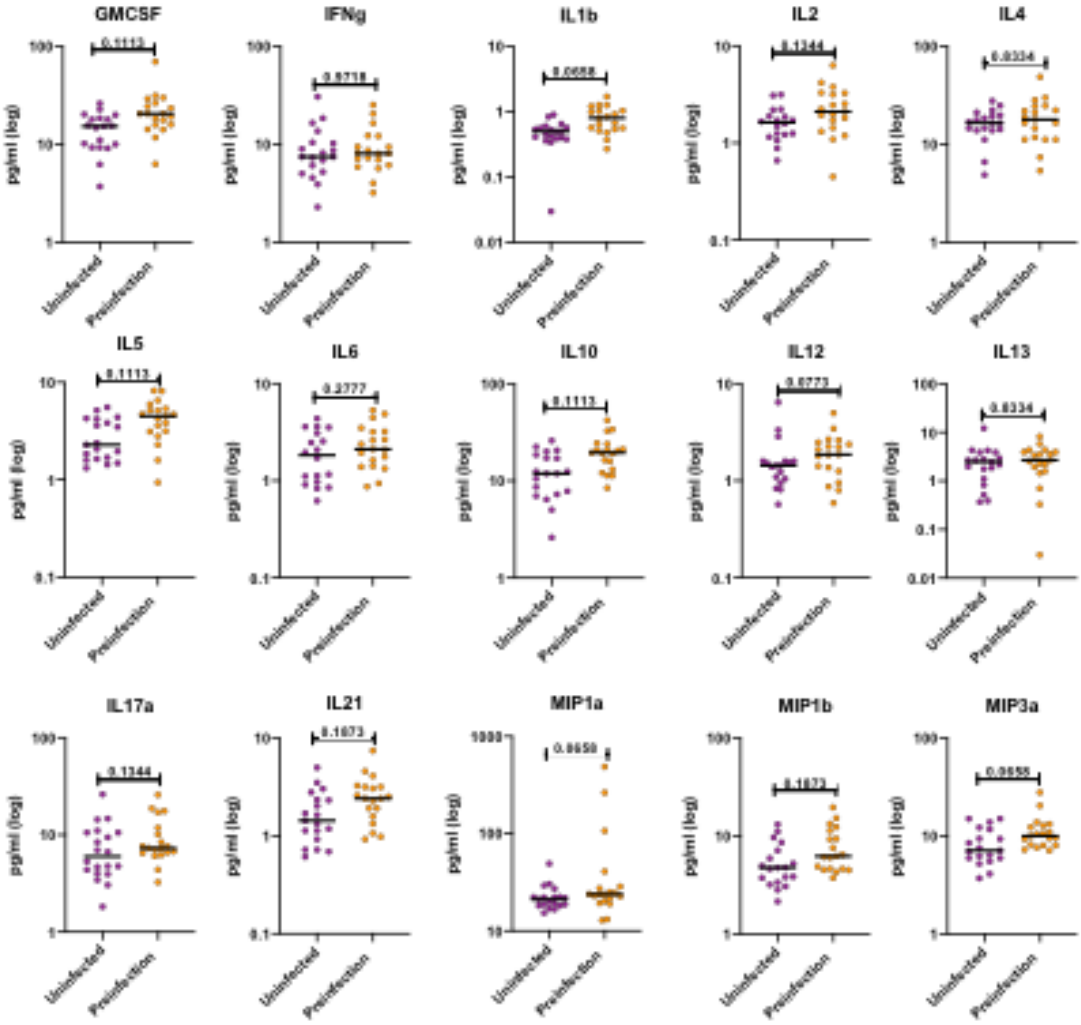
	Uninfected		Preinfection		
A	Median (pg/ml)	IQR (pg/ml)	Median (pg/ml)	IQR (pg/ml)	FDR p-value
Fractalkine	77.48	17.79	92.39	29.16	0.0387
GMCSF	9.37	3.06	15.12	8.08	0.0287
IL-1b	0.38	0.16	0.62	0.26	0.0287
IL-6	0.99	1.4725	2.47	1.295	0.0352
IL-7	5.12	1.46	8.09	4.18	0.0287
IL-8	2.54	1.29	5.07	3.95	0.0339
ITAC	13.87	8.42	31.21	21.18	0.0287
MIP-1a	15.04	4.06	22.54	8.87	0.0287
TNF-a	1.74	0.98	2.95	0.92	0.0287

	Uninfected		Preinfection		
B	Median (pg/ml)	IQR (pg/ml)	Median (pg/ml)	IQR (pg/ml)	FDR P-value
Fractalkine	64.67	15.685	106.78	45.15	0.0021
IL-7	5.01	2.165	8.47	2.955	0.0042
IL-8	2.5	2.9625	5.82	2.395	0.0026
IL-23	79.03	45.565	146.59	65.405	0.0021
ITAC	12.3	5.8075	45.81	59.255	0.0021
TNF-a	1.86	0.9025	3.12	2.045	0.0119

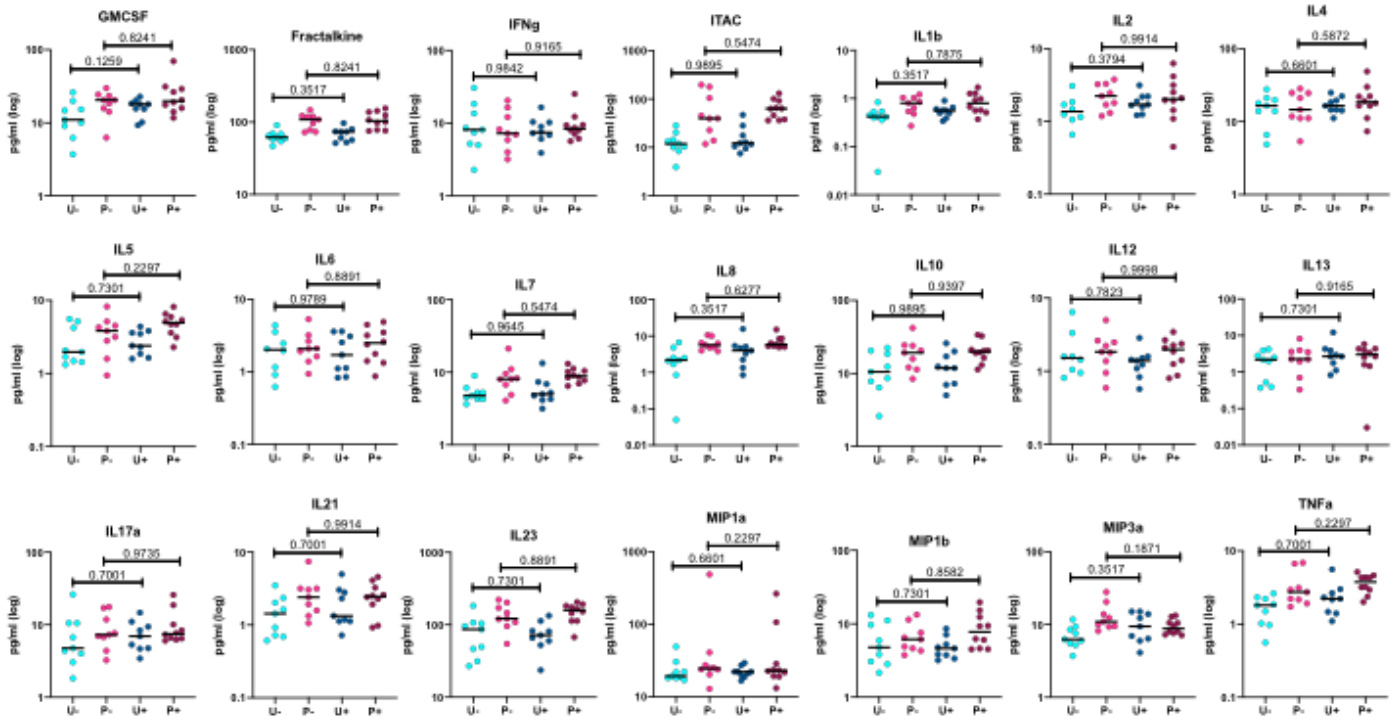
Sup. Table 4. Biomarkers increased in preinfection group compared to uninfected group in Rwandan cohort (A) and the Zambian cohort (B).



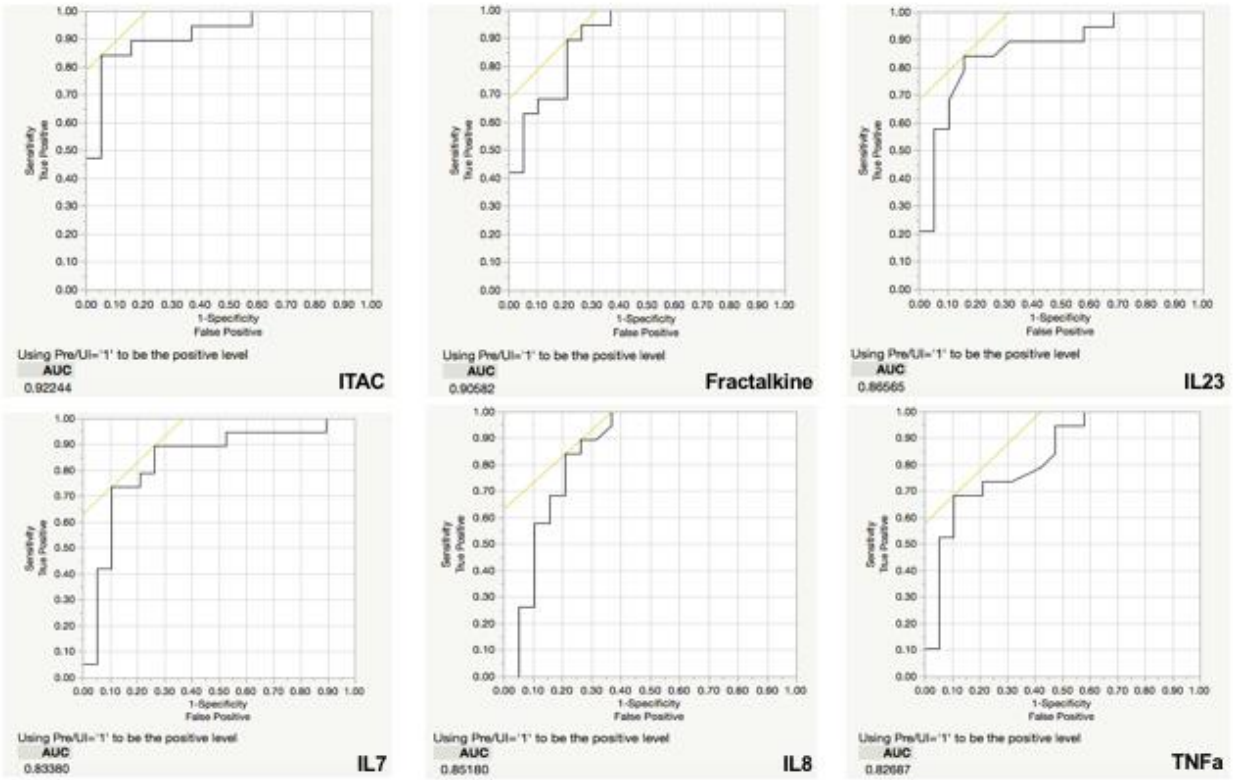
Sup. Fig 2. Nonsignificant circulating biomarkers in the Rwanda cohort: preinfection individuals nonsignificant levels cytokines and chemokine levels compared to the uninfected group. The levels of cytokines and chemokines of the uninfected (purple) and preinfection (orange) groups were compared (Kolmogorov-Smirnov test, two-tailed, FDR adjusted p-values).



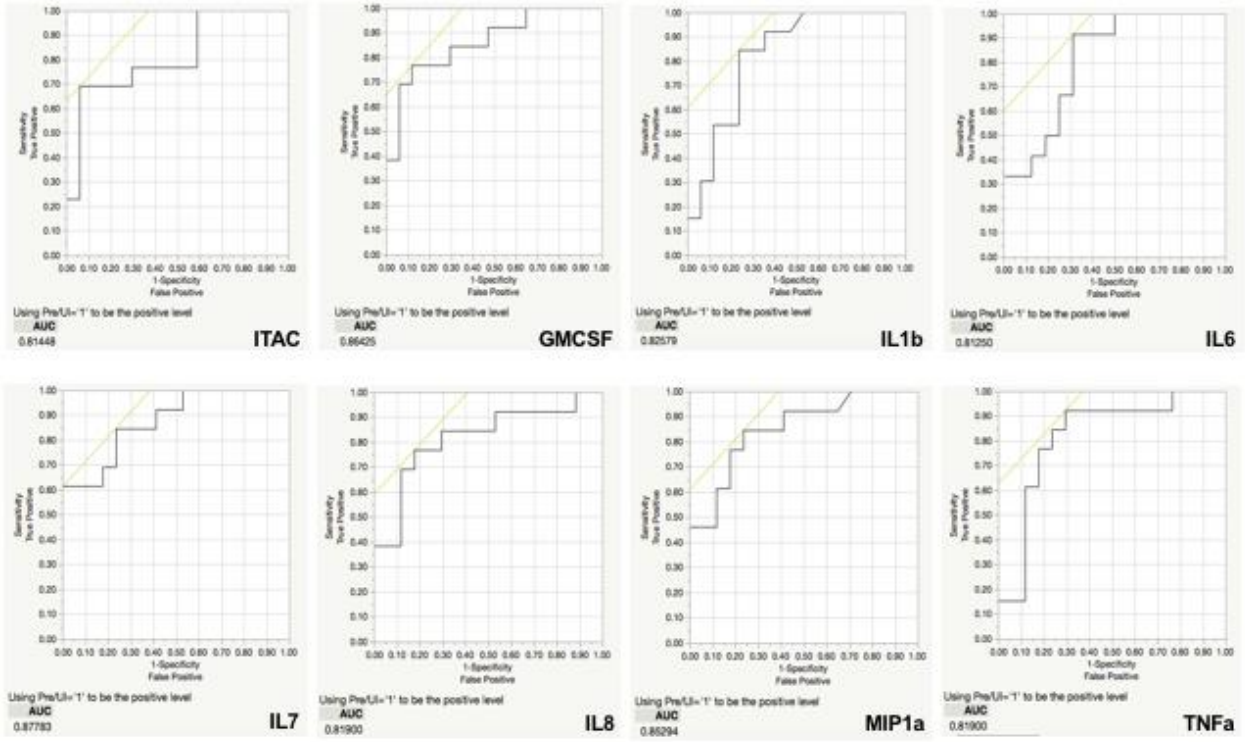
Sup. Fig 3. Nonsignificant circulating biomarkers in the Zambia cohort: preinfection individuals have nonsignificant levels of cytokines and chemokine levels compared to the uninfected group. The levels of cytokines and chemokines were separated by the country of origin and the uninfected (purple) and preinfection (orange) groups were compared (Kolmogorov-Smirnov test, two-tailed, FDR adjusted p-values).



Sup. Fig 4. *Schistosomiasis* infections in Zambian cohort does not appear to a major contributor to elevated biomarker profile. Zambian preinfection individuals with positive *Schistosomiasis* antibody titers do not have significantly higher cytokine levels than individuals with negative *Schistosomiasis* antibody titers regardless of uninfected or preinfection status. Levels of biomarkers were compared between three groups: uninfected individuals with negative antibody titers (U-, light blue), preinfection individuals with negative antibody titers (P-, pink), uninfected individuals with positive titers (U+, dark blue) and preinfection individuals with positive antibody titers (P+, magenta) (Kolmogorov-Smirnov test, two-tailed, unadjusted p-values).



Sup. Fig 5. Receiver Operating Characteristics (ROC) curves for Zambia cohort identifies biomarkers that distinguishes preinfection individuals. Elevated levels of ITAC, Fractalkine, IL-23, IL-7, IL-8, and TNFa identify individuals as risk for HIV acquisition. Area under the curve (AUC) shut off was 0.8 for separating the uninfected and preinfection individuals.



Sup. Fig 6. Receiver Operating Characteristics (ROC) curves for Rwanda cohort identifies biomarkers that distinguishes preinfection individuals. Elevated levels of ITAC, GMCSF, IL-1b, IL-6, IL-7, IL-8, MIP-1a, and TNFa identify individuals as risk for HIV acquisition. Area under the curve (AUC) shut off was 0.8 for separating the uninfected and preinfection individuals.

Disease or Infection	Elevated biomarkers
Hepatitis C Virus and HIV	IL-1b, IL-6, TNFa, IL-8, CXCL10 (22)
Hepatitis C Virus	IFNg, IL-17 (21, 22)
P. falciparum	TNFa, IFNg, IL-1, IL-6, IL-12, IL-4, sTNFRI, sTNFRII (25, 26)
P.vivax	IL-6, IL-17, IL-12, TNFa, MCP1, CRP, IFNg (27, 28)
Tuberculosis	IL-10, IL-1a, IP-10, MCP1, TNFa, IL-2, IFNg, GMCSF, IL-3, IL-13, MIP-1b, IL-5, IL-10, VEGF (29-32)
S. stercoralis and tuberculosis	IL-5, IL-13, IL-17, IL-22, IL-10 (34)
Type 2 Diabetes	Fractalkine (41)
Systemic Lupus Erythematous	Fractalkine (40)
Systemic Sclerosis	Fractalkine, ITAC, CXCL10 (42)
Inflammatory Bowel Disease	ITAC (43)
Sarcoidosis	ITAC (43)
Fibromyalgia	ITAC, IL-7 (46)
Colorectal and Esophageal Cancer	IL-7 (47)

Sup. Table 5. Elevated biomarkers and diseases/infections mentioned in Discussion.

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Chapter III: Comparison of viral phenotype and inflammatory biomarker responses in HIV-1 acute subtype A and C infections

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Abstract

HIV-1 subtype A and subtype C infections have different clinical disease progressions, with subtype C infected individuals having a 60% faster CD4 loss compared to subtype A. In order to investigate whether differences were due to the phenotype of the transmitted founder virus (TFV), or inflammatory cytokines and chemokines, known to drive pathogenesis, we PCR amplified, sequenced and constructed infectious molecular HIV-1 clones from the plasma of 30 acutely infected individuals in Rwanda and Zambia. A comparison of the replicative capacity of 14 subtype A and 16 subtype C TFV showed that they had similar replicative capacity (RC) scores. Nevertheless, high TFV RC scores were linked to more rapid CD4+ T cell loss, and higher inflammatory cytokine levels irrespective of subtype. We next compared the inflammatory plasma cytokine/chemokine profiles of individuals pre- and post- the estimated date of infection of 20 Rwandan individuals infected with subtype A and 34 Zambians infected with subtype C HIV-1. Individuals infected with subtype C exhibited a significant increase in the levels of 10 prominently pro-inflammatory cytokines/chemokines after infection, while, in subtype A infections only 2 cytokines were significantly elevated. Despite these differences, at 3-months post infection individuals infected with subtype A and subtype C viruses have similar overall biomarker profiles, primarily due to higher baseline biomarker levels in Rwanda. In the combined cohort, we found a highly significant association between faster $CD4^+$ T cell decline and higher levels of ITAC, which in turn was linked to higher viral RC. This finding may suggest that high levels of ITAC during acute HIV-1 infection might be a marker for rapid disease progression.

Introduction

Almost 40 years after its initial discovery, HIV-1 remains a major health crisis worldwide with over 37.9 million individuals infected in 2019 and 1.7 million new infections in 2018. While HIV-1 infections occur across the globe, the heaviest disease burden is present in sub-Saharan Africa, harboring over 2/3 of all HIV-1 cases (1).

HIV-1 demonstrates a great potential for generating genetic diversity due to lack of proof-reading ability from the reverse transcriptase and high viral recombination rates (2). In order to organize the amount of genetic diversity, HIV-1 group M is divided into nine different subtypes, A-D, F-H, J, and K (2). Globally, the two most common subtypes observed are subtype C (48% of global infections) and A (12% of global infections); these two subtypes are also the most common subtypes observed in sub-Saharan Africa (2).

Clinical differences between subtypes can be seen across most of the nine subtypes seen globally. Subtype D, a common subtype in sub-Saharan Africa, was found to be associated with increased mortality compared to subtype A and has a 4-fold higher rate of CD4 T cell decline in the absence of anti-retroviral drugs compared to other subtypes (2, 3). Individuals infected with subtype D often also have weaker humoral immune responses to HIV infections compared to individuals infected with other subtypes

While there are numerous studies documenting characteristics of subtype C infections, studies exploring the features of subtype A infections are less common. One of the early studies looking at subtype A infections found that female sex workers infected with non-subtype A viruses were 8 times more likely to develop AIDS compared to those infected with subtype A (4).

A multi-site study of acute-early HIV infections, termed IAVI Protocol C, enrolled over 600 newly infected individuals, who were infected by subtype A, C, D and recombinant viruses, and who we followed for as long as 10 years with regular viral load and CD4 T cell measurements (5, 6). This is suggestive that the viral characteristics of subtype C viruses may result in a more pathogenic outcome compared to other HIV-1 subtypes. Epidemiological analyses found that infections with subtype A and C result in different clinical presentations in the infected patients. Individuals infected with subtype C were found to have a 60% faster disease progression compared to subtype A, and this was based on viral load levels, CD4 T cell decline, and time to AIDS (7). Moreover, subtype C has been associated with higher post-counseling HIV-1 incidence compared to subtype A, when comparing the same counseling and interventional approaches in Zambia and Rwanda (8); this higher incidence is still observed despite the same counseling and interventional approaches in each country. Finally, in individuals infected with subtype A within the IAVI Protocol C cohort, there was a higher proportion of non-progressors, or individuals that never progressed to AIDS without the use of antiretroviral therapy, compared to individuals infected with subtype C (9). In addition, the multivariate analysis from this study found that subtype A viral control was independent of HLA (9). Collectively, these data indicate that virus-specific characteristics may determine a less pathogenic outcome in subtype A infected individuals.

In this study, we sought to investigate possible causes for the observed clinical differences seen between HIV-1 subtype A and C infections and if these differences could be observed during acute HIV-1 infection. To address this question, we compared

the replicative capacity of transmitted-founder viruses from each subtype, since we have shown previously that the trajectory of HIV pathogenesis is defined in part by this trait of the virus. In addition, because inflammation and inflammatory cytokines are linked to more rapid disease progression in HIV-1 infected individuals (10-12), we examined the cytokine/chemokine profiles of individuals pre- and post-infection with either subtype A or C HIV-1 to see if there were any observable differences in the biomarker response in the acute phase of infection between subtypes.

Results

Replicative capacity scores are similar for subtype A and subtype C transmitted-founder viruses.

To investigate subtype differences in disease progression, we first compared the abilities of subtype A and subtype C transmitted-founder viruses (TFV) to replicate *in vitro* in activated PBMCs. We chose 14 individuals acutely infected with HIV-1 subtype A and 16 individuals acutely infected with HIV-1 subtype C for this study. All samples used in the PCR amplification and cloning of TFV in this study were collected a median of less than 25 days post the estimated date of infection (EDI) as shown in Table 1A. When we plotted the viral loads of these individuals over the first two years post infection, we found that individuals infected with subtype A HIV-1 exhibited significantly better viral control compared to individuals infected with subtype C (Figure 1A). This result is consistent with findings obtained in an analysis of more than 400 subtype A and Subtype C infected individuals in the entire Protocol C cohort (13). For each of the 30 individuals, we PCR amplified near full-length genome amplicons from acute infection plasma samples to define the TFV sequence and then constructed infectious molecular clones (IMC) using the methodology described in Deymier et al. (14). We compared the replicative capacity (RC) of virus derived from these TFV IMCs in activated PBMCs over a 12-day period. We calculated the RC score by quantitating every 2 days the amount of reverse transcriptase activity in the culture medium, calculated the area under the curve for each virus, and normalized it to that of the primary isolate MJ4 (15). While RC scores varied over 10-fold for viruses of each subtype, we found no significant difference between the median RC scores of subtype A and C TFVs. Our results

suggest that differences in the *in vitro* replicative capacity of subtype A and C TFVs cannot explain observed differences in clinical outcomes between the two subtypes.

Biomarker responses after acute subtype C infections exhibit a greater increase compared to that of subtype A infections.

We next investigated the possible role that innate immune responses, and specifically inflammatory cytokines/chemokines, might play in the subtype differences in disease progression observed in the Protocol C cohort. To address this question, we identified a total of 20 subtype A individuals and 34 subtype C individuals for whom we had plasma samples collected prior to and shortly after the EDI (Table 1B). Using a multiplex Luminex assay, we compared the biomarker levels in individuals pre- and post-infection with either subtype. The characteristics of the plasma samples used in this study are shown in Table 1B. For both the subtype A and subtype C individuals tested, the pre-infection samples were taken a median of 46 days before the estimated date of infection. For the post-infection samples, samples from both cohorts were collected less than 3 months after the estimated date of infection (Table 1B). We observed that 9 biomarkers (fractalkine, IFNγ, IL5, IL6, IL10, IL12, IL13, IL23, and TNFα) had significantly increased levels post-infection with subtype C HIV-1 compared to the pre-infection levels. In contrast, in subtype A infected individuals, only 2 biomarkers (IL13 and TNFα) had significantly increased levels post-infection (figure 2). To account for the multiplex nature of the assay, we only considered p values less than 0.01 as significant. Despite the fact that subtype A infected individuals exhibited fewer significant increases in biomarker levels, with the exception of TNFα, the absolute levels of biomarkers were not significantly different between individuals infected with the two subtypes. In large

part this appeared to be the result of higher pre-infection biomarker levels in Rwandan subtype A individuals. The median pre-infection biomarker level was higher in each instance, and this difference was significant for IL-6 (p=0.0088) and trending for IL-5 and fractalkine (0.0503 and 0.0533 respectively).

Partial Least Square (PLS) analysis confirms the greater number of biomarkers in subtype C immune response.

When we performed PLS analysis on the pre-post infection cohorts for both subtypes, they were highly consistent with the results from our earlier univariate analyses. In our analysis, we tested the biomarkers changes from pre- to post- infection as the outcome variable, with the biomarkers serving as the factors in the model. In subtype C, we found that the changes in the biomarker profiles post infection compared to pre-infection were due to increases in ITAC, IFNγ, IL4, IL5, IL6, IL10, IL12, IL13, IL17α, IL23, and TNFα (figure 3A). Compared to subtype C, changes in the subtype A biomarker profile were limited to increases in just IFNγ, IL6, IL12, IL17α, MIP1α, and TNFα (figure 3B). For subtype A, most of the variance is due to an increase in TNFα, whereas multiple biomarkers contribute to the variance seen in subtype C acute infection.

Impact of viral RC on disease trajectory and cytokine production in acutely infected HIV-1 individuals

While we observed no significant differences between the RC of subtype A and C TF viruses, the availability of these IMCs allowed us to reexamine the impact of RC on disease trajectory. In a previous study, we examined this through the construction of 127 *gag*-MJ4 chimeric viruses and found that low viral RC was significantly associated with individuals who exhibited slower $CD4^+$ T cell decline, whereas high viral RC was

associated with rapid CD4 loss (10). In the current study, despite the significantly smaller sample size, we observed similar associations between viral RC and $CD4^+$ T cell decline. Viruses that had RCs in the lowest tercile of viral RC values had a slower rate of $CD4^+$ T cell decline compared to viruses with RCs in the upper two terciles of RCs. Although there was clear evidence of a trend (p=0.095, Log Rank), these differences were not significant, presumably because of the small numbers (Figure 4A). Additionally, a Cox proportional hazards model with RC and the protective allele B*81 was significant between the higher RC terciles and the lower RC terciles (p=0.034, HR=4.04).

We also compared the levels of systemic inflammatory cytokines/chemokines of these individuals during acute infection, since our previous study showed a strong inflammatory profile associated with high viral RC. Using a principal component analysis, we again found that there was a separation of cytokine profiles between individuals that were infected by viruses in the lowest tercile of RC compared to those with RCs in the upper two terciles (Figure 4B and 4C). Specifically, figure 4C shows that PC1 loadings for cytokine responses were significantly smaller for the lowest tercile RC viruses compared to upper 2/3 tercile RC viruses.

ITAC is a marker for $CD4^+$ T cell decline in the combined cohort

To identify potential biomarkers associated with disease progression, the 84 acutely infected subtype A and C individuals included in this study were analyzed. All cytokine values were mean centered and normalized to avoid any batch effect that may have existed between the different luminex experiments. ITAC was the one cytokine that stood out as being linked to disease progression; values in the upper two terciles were

significantly associated with more rapid decline of $CD4^+$ T cells to below 300 compared to ITAC values in the lower tercile (p=0.002 Log Rank; p=0.003 Wilcoxon; Figure 5A). When the risk ratios were calculated using a Cox Proportional Hazard model, ITAC values in the upper two terciles were associated with a greater than 5-fold risk of more rapid $CD4^+$ T cell decline compared to those in the lower ITAC tercile (R.R. 5.57 (1.65-18.81), p=0.006). In addition, when the normalized ITAC values were compared within individuals for whom there were TF viruses that had RC values, RCs in the upper two terciles were significantly associated with higher ITAC values compared to individuals in the lower RC tercile (Figure 5B) linking viral phenotype to a diagnostic cytokine.

Discussion

We have previously shown that the phenotype of the virus that initiates infection in an individual, the transmitted founder virus, can significantly impact both viral load and disease trajectory (10, 11, 16). In a previous study of more than 120 individuals newly-infected with subtype C HIV-1, we demonstrated that the replicative capacity (vRC) of the infecting virus was associated both with set-point viral load and the speed of CD4+ T cell decline (11). A number of studies comparing the replicative capacity of viruses, chimeric in either *gag* or *env* genes, have suggested that different subtypes have different replicative capacities (17, 18). In a limited study of 5 subtype A and 5 subtype D authentic molecularly cloned TFVs, we showed that subtype D TFVs replicated better than their subtype A counterparts (19). Because subtype A HIV-1 infected individuals exhibit similar disease characteristics as individuals infected with low vRC viruses of other subtypes, we sought to determine whether authentic transmitted founder viruses derived from acutely infected subtype A and subtype C individuals similarly differed in their replicative capacity.

In this study, where we compared a total of 30 viruses derived from molecularly cloned TFVs; we did not find a significant difference in the replicative capacity of HIV-1 subtype A and C TFVs. Thus, it does not appear that replicative capacity in activated CD4+ T cells *in vitro* can explain the differences in disease progression observed in the two subtypes.

When we examined some of the characteristics associated with disease progression, we saw a lack of association between RC and set point viral load (data not shown), consistent with previous study where the association between the two factors was

weak (11). In contrast, we were able to observe a trending association between RC vs CD4 decline in this study. This supports findings from a previous study that examined 127 gag-MJ4 chimeric viruses; low viral RC was significantly associated with slower $CD4^+$ T cell decline, while high viral RC was associated with rapid $CD4^+$ T cell decline (10). In addition, both this previous study and our current study found that high RC viruses is linked to higher levels of inflammatory cytokines and chemokines (10). Since inflammation is known to be a driver of disease progression and CD4+ T cell loss in HIV-1 infection (10, 20-23), we wanted to determine whether there were differences in inflammatory biomarkers that could explain the differences in CD4+ T cell decline in subtype A and C infected individuals in the Protocol C cohorts (7). We found a greater number of biomarkers were increased as a result of HIV-1 subtype C infection compared to subtype A infections, but, while differences in biomarkers levels were observed within subtypes before and after infection, the overall levels of the biomarkers following HIV-1 acute infection were the same in the two subtypes. The reduced number of cytokines that increased following subtype A HIV-1 infection was due in part to the higher levels of biomarkers observed prior to infection in these individuals, though there may be other factors involved. In addition, while subtype C infections result in the increase in multiple biomarkers post infection, subtype A infection is mostly defined by an increase of TNFa.

The cause for the differences observed between HIV-1 subtype A and C infections remains unclear. One possible explanation is that subtype C viruses are better adapted to the immune system of their new hosts than subtype A viruses. We have previously shown that infections by subtype C TFVs that have a higher fraction of epitopes

preadapted to the HLA-directed immune response of their new host result in higher viral loads and faster CD4+ T cell decline (24, 25). In the study by Monaco et al., viruses from 169 subtype C heterosexual transmission pairs were sequenced and on average one-quarter of the possible HLA-linked target sites in the transmitted virus Gag proteins were already adapted to the new host's HLA types, with transmitted preadaptation significantly reducing early immune recognition of epitopes (25).

While the extent of immune adaptation in subtype A viruses remains unknown, it is possible that the ongoing viral control observed in subtype A infections reflects less adaptation to their Rwandan hosts compared to subtype C infections in Zambian individuals; ongoing studies are aimed at addressing this question.

In an unexpected finding, we were able to find an association between $CD4^+$ T cell count decline and higher levels of the inflammatory marker ITAC when we combined the cohorts in this study. A study from 2005 found that ITAC mRNA levels were upregulated in HIV-1 infected human monocyte-derived macrophages and dendritic cells. Medium from these infected cells were then found to a chemotactic for freshly isolated human $CD4^+$ T cells; however, when the $CD4^+$ T cells were pretreated with an anti-CXCR3 antibody (the receptor that ITAC binds to), the previous observed chemotaxis was abolished (26). This study also found that ITAC mRNA levels were upregulated within a lymph node isolated from a HIV-1 infected individual, which implicates ITAC in the recruitment of susceptible $CD4^+$ T cells to HIV-1 infected lymph nodes (26). Previous studies have identified $CD4^+$ T cell decline and viral replicative capacity are both markers of more rapid disease progression (10, 11, 27), our results raises the possibility that high ITAC levels may be a marker for rapid disease progression. This result falls in

line with our previous observations that high ITAC levels in uninfected partners were associated with HIV-1 acquisition and that the ITAC levels could be used as a predictive variable to identify individuals that would eventually seroconvert within serodiscordant couples (28). The new findings shown in this study show that ITAC not only affects HIV-1 acquisition, but also disease progression in HIV-1 infected individuals. These findings raise the need to further examine the role of ITAC in HIV-1 infection.

Overall, the data presented here demonstrate similar *in vitro* replicative capacities for subtype C and A transmitted founder viruses, arguing against this phenotypic trait as the basis for different pathogenic outcomes in the two subtypes. Moreover, levels of inflammatory cytokines that might drive disease progression were similar during acute infection indicating that additional studies are required to understand the mechanism underlying differences in disease progression between the two subtypes.

Material and Methods

Study Subjects

All participants were enrolled in the Rwanda Zambia HIV Research Group (RZHRG) discordant couple cohorts in Lusaka, Zambia and Kigali, Rwanda. Subjects from both cohorts were enrolled in human subjects protocols approved by the Emory Institutional Review Board, the Rwanda National Ethics Committee and the University of Zambia Research Ethic Committee and provided written consent. When the participants enrolled in the cohort and during each visit, they were provided couples counseling and testing, and condoms to reduce transmission of HIV-1.

Infectious molecular clones (IMC) were derived from seropositive individuals a median of 24-26 days post-estimated date of infection (EDI) (Table 1A). All of the subtype C individuals came from Zambia. In the Zambia cohort, the median days post-EDI was 24. All of the subtype A individuals came from Rwanda. In the Rwanda cohort, the median post-EDI was 26. For biomarker studies, all individuals had samples collected pre-EDI and within three months post-EDI. For the subtype A cohort, samples were taken a median of 47 days before the EDI and 26 days after the EDI. For the subtype C cohort, samples were taken a median of 46 days before the EDI and 45 days after the EDI (Table 1B). The algorithm used to determine the EDI has been previously described (29).

Replicative capacity assays

Generation of IMCs was as described in Deymier et al. 2014 (14). The generation of viral stocks, determination of particle infectivity, and replicative capacity assays are fully described in Deymier et al. 2015 (15). Briefly, Frozen peripheral blood mononuclear

cells (PBMCs) from buffy coats were thawed and stimulated with 20 U/ml of interleukin-2 (IL-2) and 3ug/ml of phytohemagglutinin (PHA) in R10 (Roswell Park Memorial Institute (RPMI)) 1640 Medium supplemented with 10% defined fetal bovine serum (FBS), 1 U/ml penicillin, 1ug/ml streptomycin, 300ug/ml L-glutamine) for 72 hours at 37C. 5×10^5 cells were then infected in 15ml conical tubes by 2 hour spinoculation at 2,200 rpm with an MOI of 0.05 based on the titer in triplicate in TZM-bl cells. Cells were then washed five times in RPMI, resuspended in 300ul of R10 media and plated in a 96 well plate in triplicate. 50ul of supernatant was then sampled every 48 hours starting with a day zero time point taken after spinning the plate at 1,000 rpm for 1 minute to get a baseline reverse transcriptase activity for each infection well using the radiolabeled reverse transcriptase assay. RC score was determined from day 2-6 time points to measure the peak of viral replication and spread. The replication score (RC score) for each variant was calculated using a normalized area under the curve. The median of the replicates were background subtracted using an uninfected control included at each time point and area under the curves (AUC) were divided by the AUC for a standard lab adapted subtype C virus, MJ4, to compare across the different viruses analyzed on different days.

Evaluation of Plasma Biomarkers

The plasma cytokine and chemokine levels were measured using a Milliplex Map Human High Sensitivity T Cell Panel (HSTCMAG-28SK). This kit measures the levels of 21 inflammatory cytokines and chemokines. The samples were run in duplicate. In order to eliminate batch to batch variation in the assay, all tests were carried out on the same

batch of plates and approximately equal numbers of pre-infection and uninfected plasma were run on the same plate. The plates were quantified and standardized on a Bioplex 2000 in the Emory CFAR Virology Core and final concentrations were extrapolated from a standard curve and expressed in pg/ml. All plasma samples were stored at -80°C and had undergone zero or a single freeze-thaw for aliquoting prior to use.

Data Analysis

Plotting of the viral load, CD4 T cell counts, replicative capacity analysis, and comparison between biomarker levels were done in Prism 9. The viral load plot was generated by plotting all the available data points for all individual where data was available. Once plotted, a linear regression analysis was performed to determine if any difference existed between the viral load kinetics between the two subtypes.

Comparison between RC scores of subtype A and C IMCs was done using a nonparametric Mann-Whitney test. For intra-subtype comparisons, we used a nonparametric Wilcoxon matched-pairs signed rank test. For inter-subtype comparison, we used a nonparametric Kolmogorov-Smirnov test, and a cut-off of $p<0.01$ was used for significant findings to address multiple comparisons.

Partial Least Squares (PLS) analysis was performed using the JMP Pro 15 statistical package. PLS analysis had a variable importance cutoff of 0.8 and was performed with a NIPALS fit with 1 factor.

Kaplan-Meier survival analyses and Principal Component Analysis (PCA) were performed with JMP Pro version 15 (SAS Institute). For survival analyses, endpoints

were defined as $CD4^+$ T cells counts falling below 300/mm^3, and significance is reported using the log-rank test. Risk-ratios were calculated through proportional hazards models. For PCA, cytokine data was preprocessed to winsorize extreme high values to the 90^{th} percentile of the data distribution, and missing values were imputed through linear regression.

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Author Contributions

Conceived and designed the experiments: SM, EH. Performed the experiments: SM. Analyzed the data: SM. Contributed reagents/materials, analysis: SM, LY, EEB, RX, QQ, ZE, MD, DC, WK, JG, EK, SA, EH. Wrote the paper: SM, EH.

Figures

A	Individuals included	Gender	Days Post infection
	30 total		
Subtype A	14	6 male/8 female	Median 24 (Range 14-65)
Subtype C	16	8 male/8 female	Median 26 (Range 8-66)

B	Individuals included	Gender	Days Pre infection	Days Post infection
	54 total			
Subtype A	20	11 male/9 female	Median 46.5 (Range 19-107)	Median 25.5 (Range 11-72)
Subtype C	34	19 male/15 female	Median 46 (Range 12-115)	Median 44.5 (Range 14-74)

Table 1. Demographics of the cohorts used in experiments in this paper. (A) Demographics of plasma samples used to generate the infectious molecular clones (IMC) used in the replicative capacity assays. (B) Demographics of plasma samples of pre and post HIV-1 subtype A and C infection.

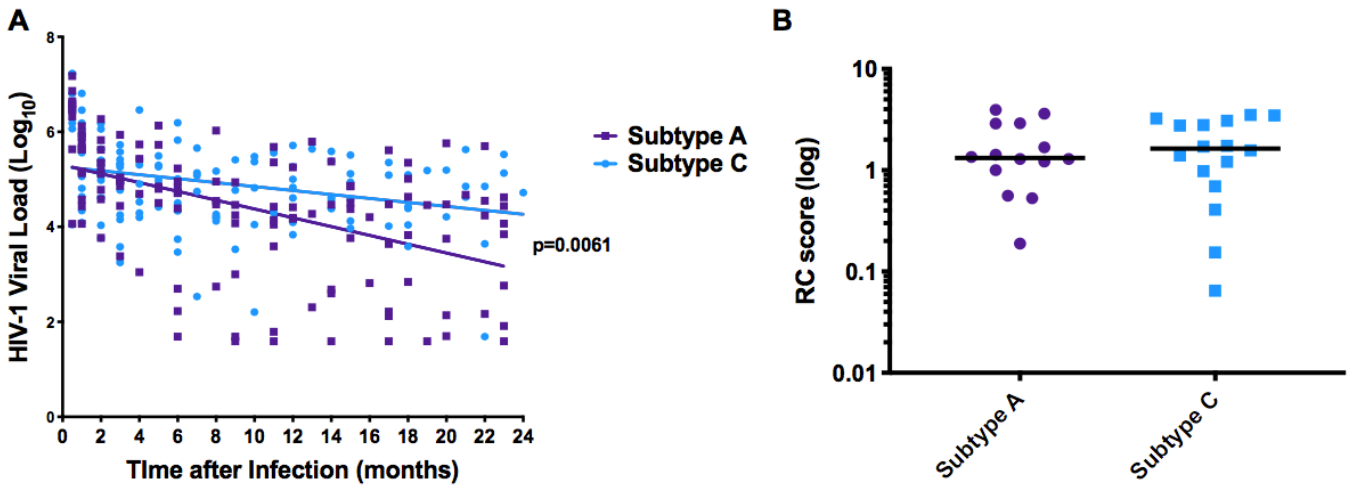

Figure 1. Comparison of viral phenotypes during acute infection of HIV-1 subtype A and C transmitted-founder (TF) viruses. (A) Viral loads of the individuals from which the IMCs were generated from the plasma over the first two years post infection. Purple indicates subtype A viral loads and light blue indicates subtype C viral loads. Linear regression. (B) Replicative capacity score of the subtype A and C TF viruses tested. Purple indicated subtype A RC scores and light blue indicates subtype C RC scores. Mann-Whitney, two-tailed test.

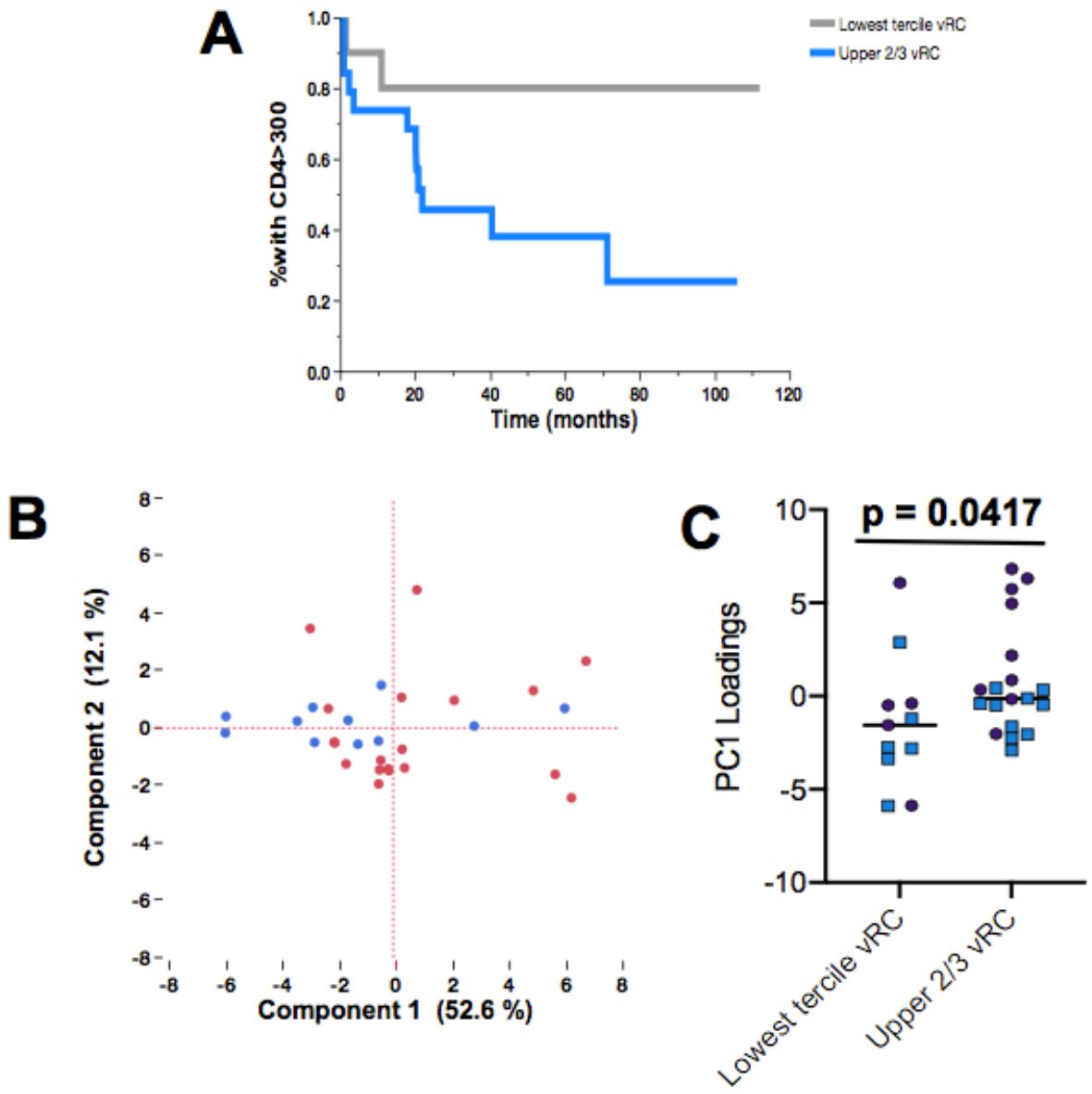

Figure 2. In vitro replication capacity of full-length TF IMCs predicts predicts CD4 loss and inflammatory cytokine profiles. (A) Replicative capacity assays with 30 total Infectious Molecular Clones (IMCs) of both HIV-1 subtype A and C showed that IMCs that have viral replicative capacities (RCs) in the upper 2/3 terciles (blue line) lose $CD4^+$ T cell counts at a faster rate compared to IMCs with RCs in the lowest tercile (gray line). (B) Principal component 1 (PC1) and principal component 2 (PC2) scores are shown in

a two-dimensional scatter plot for all 30 individuals used in the RC assays. PC1 showed the greatest variation in the dataset. The analysis showed separation of cytokine responses based on RC terciles (lowest RC tercile, blue dots; upper 2/3 terciles, red dots. (C) The lowest RC tercile showed lower PC1 loadings compared to the upper 2/3 RC terciles. Subtype C samples: blue squares; subtype A samples: purples circles. Mann-Whitney, two–tailed.

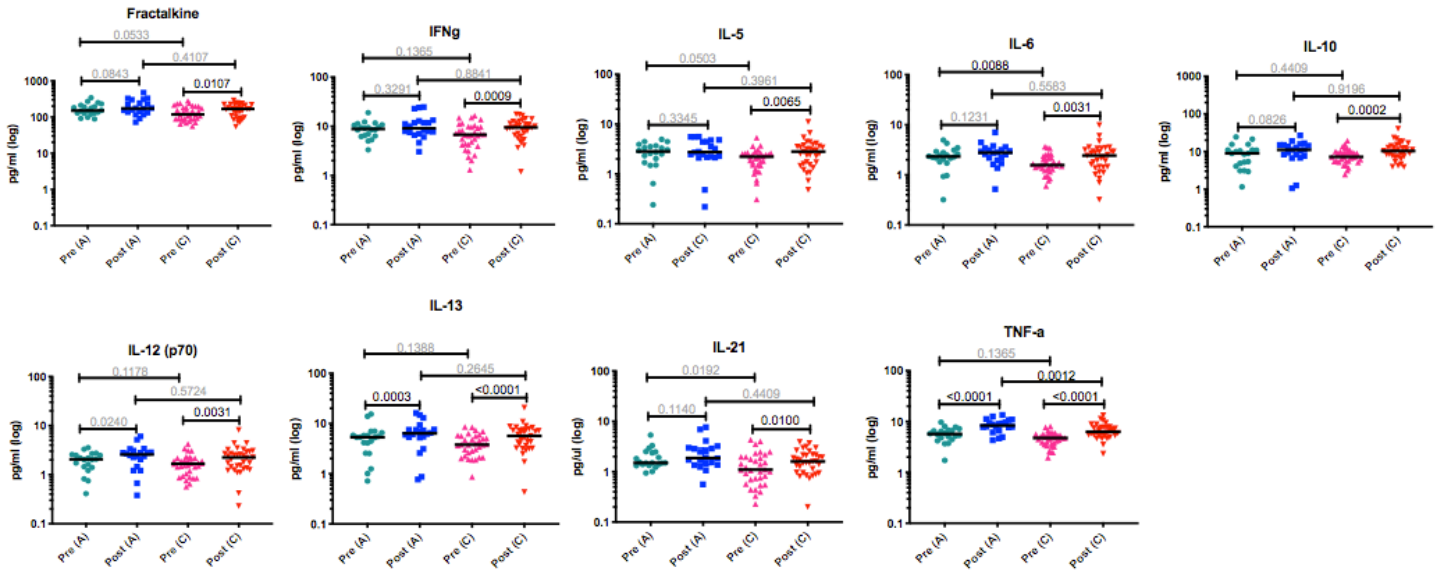

Figure 3. More biomarkers are significantly increased post infection in HIV-1 C compared to subtype A. Nine biomarkers are increased post infection in acute subtype C infection compared to only two in subtype A infection. Teal is biomarker concentrations pre subtype A infection, blue is post subtype A infection, pink is pre subtype C infection, and red is post subtype C infection. Wilcoxon matched-pairs signed rank test, two-tailed was used between pre and post levels within a subtype. Kolmogorov-Smirnov test, two tailed was used for remaining comparisons.

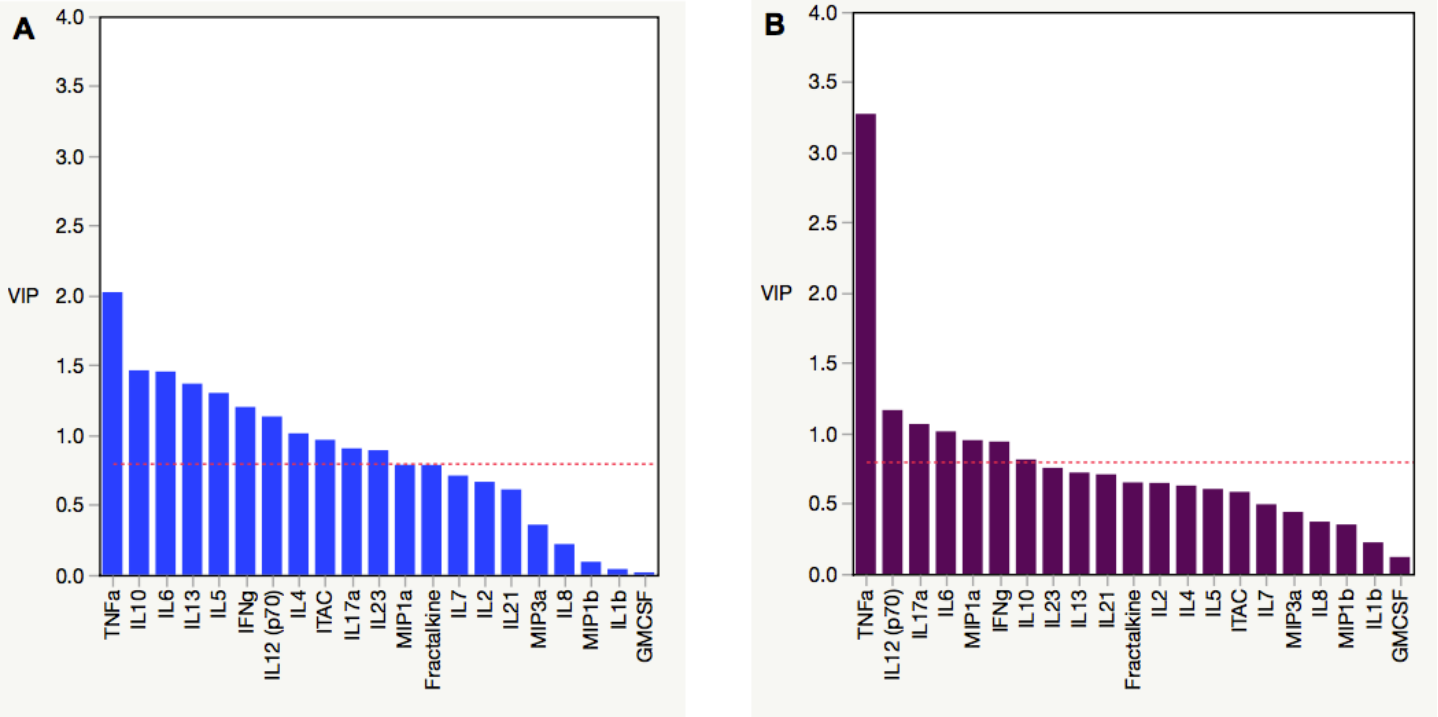

Figure 4. Partial Least Square (PLS) analysis of changing biomarker profiles pre and post infection in HIV-1 subtype C and A acute infection cohort. Analysis was done with NIPALS Fit with 1 Factor. VIP (Variable Importance Plot) Threshold was set at 0.8. (A) PLS Analysis for Zambian subtype C cohort. TNFa, ITAC, IFN-γ, IL10, IL12, IL13, IL17a, IL23, IL4, IL5, and IL6 were all increased post infection. (B) PLS analysis for Rwandan subtype A cohort. TNFa, IFN-γ, IL12, IL17a, IL6, and MIP1a were all increased post infection.

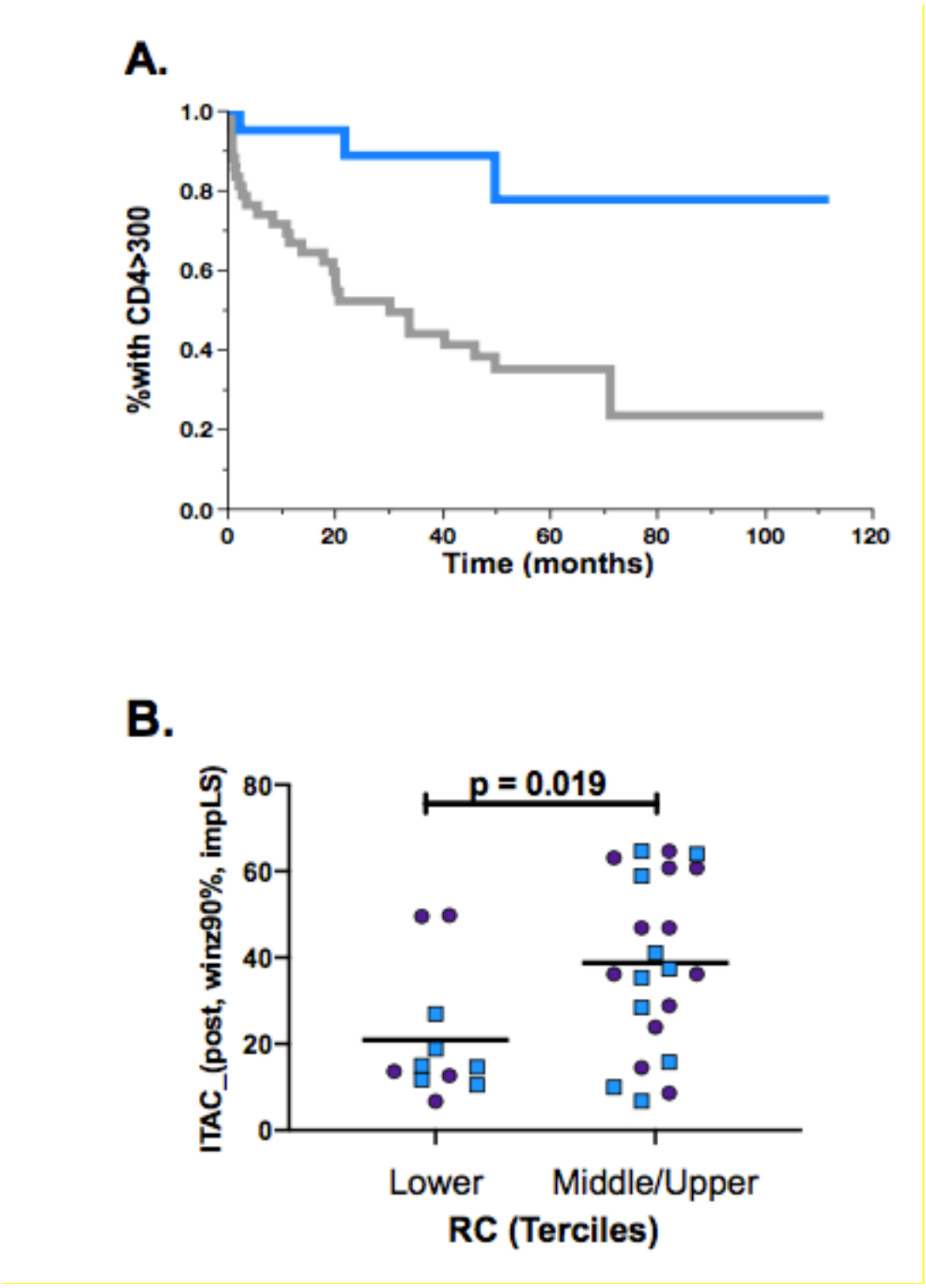

Figure 5. ITAC is a biomarker associated with HIV pathogenesis and higher vRC.

(A) 84 post infection ITAC values were mean centered and normalized minimize batch effects in luminex assays. Combined subtype A and C cohorts showed that individuals

with ITAC values in the upper 2/3 terciles (blue line) had a more rapid $CD4^+$ T cell count decline compared to low ITAC tercile (gray line). (B) Risk ratio analysis showed that individuals in the upper 2/3 ITAC terciles had over 5.5 higher risk of losing $CD4^+$ T cell counts compared to individuals in the lower ITAC tercile. (B) Comparison of the lowest and the upper 2/3 vRC terciles showed that the upper 2/3 vRC terciles had significantly increased ITAC levels compared to vRC values in the lowest tercile. Subtype C samples: blue squares; subtype A samples: purple circles. Student's t-test, two tailed.

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Chapter IV: Discussion

Chapter I of this thesis found that elevated systemic plasma biomarker levels can identify individuals who may be at increased risk for HIV acquisition and identified specific biomarkers associated with increased HIV acquisition despite not being classic inflammatory biomarkers. In chapter II, the study described was one of the first to examine the biomarker profiles between acute subtype A and subtype C HIV-1 infections, with the hope of identifying overlying causes for the observed disease progression (1-3). Additional studies outlined in the Appendixes are some of the first to analyze sequences of subtype A transmission pairs, identify whether a consensus bias may exist in subtype A transmission, and develop *in vitro* subtype A infectious molecular clones (IMCs) that can be used in future studies.

Chronic diseases or other infections: Potential increased risk for HIV-1 acquisition

In Chapter I of this thesis, we found that elevated systemic biomarkers increased an individuals' risk for HIV-1 acquisition. In addition to HIV-1, many other infections cause elevated systemic biomarkers, such as tuberculosis, Hepatitis C Virus, Malaria, etc (4-13). In additional to inflammatory infections, there's also a number of chronic infections that result in elevated levels of biomarkers that we identified as predictors for HIV-1 acquisition, such as type II diabetes, systemic sclerosis, inflammatory bowel disease, sarcoidosis, fibromyalgia, and colorectal and esophageal cancers (14-22). What is unusual about the elevated biomarkers identified, ITAC, Fractalkine, and IL-7, is that they are not considered classic inflammatory biomarkers.

Using the knowledge gained from this study, it is possible that future testing on systemic biomarker levels could be implemented to test individuals with chronic diseases or other non-HIV related infections to see if they may be at an increased risk for HIV-1 acquisition. This potential testing would only require measuring the levels of a select few biomarkers shown to increase risk for HIV-1 acquisition (i.e. ITAC, Fractalkine, IL-7, IL-8, or TNF-a). This testing could allow people to be informed of their increased risk; they could use that information to modify their behavior, such as adopting safe-sex habits or using clean needles for drug use.

While this research presented novel findings for potential intrinsic HIV-1 risk factors, additional research is needed into other infections and chronic diseases that may result in elevated systemic biomarker levels and if those elevated levels can result in an increased risk for HIV-1 acquisition. These types of studies are especially needed in sub-Saharan Africa, where relatively little is known about the biomarker profiles of chronic diseases or other infections.

Role of ITAC in HIV-1 acquisition and infection

ITAC, or CXCL11, is a chemotactic cytokine. It is induced by IFNγ and produced by neutrophils, monocytes, macrophages, astrocytes, fibroblasts, endothelial cells, epithelial cells, and keratinocytes (23, 24). ITAC binds to the chemokine receptor CXCR3 to induce cellular migration; CXCR3 is preferentially expressed on Th1 cells but can also be seen on other forms of T cells, B cells, and natural killer cells (23, 24). While CXCR3 has three ligands (CXCL9, CXCL10, and CXCL11), however, the latter binds to CXCR3 with the highest affinity and is the most potent and efficient of the three

(24, 25). In addition to its chemotactic function, ITAC has also been shown to have antimicrobial activity against *E. coli* and *L. monocytogenes* (26). This activity is similar to α-defensin, due to its C-terminal being rich in positively charged amino acids which can form holes in bacteria surfaces (26).

While ITAC plays an important role in attracting cells to sites of infection or injury, regulating chemotaxis is important to prevent excessive cell recruitment and resulting pathology (23). Researchers looking at this question found that ITAC can be cleaved. by CD26/DPP IV, a membrane-bound and cell-free protease (27). As with processing by matrix metalloproteinases (MMPs), cleavage of ITAC by CD26/DPPIV results in ITAC gaining antagonistic properties that reduce CXCR3-binding of other chemokines, loss of calcium-signaling capacity through CXCR3, and reduced chemotactic potency (27). ITAC can also be cut by multiple MMPs (23). Researchers found that cleavage of ITAC at the N-terminal causes antagonist binding of CXCR3, but this antagonism is lost upon C-terminal truncation (23). The researchers theorized that the “truncation of ITAC’s C-terminus may be a new mechanism that results in dispersal of chemotactic gradients and may contribute to regulation of Th1 cell recruitment and cell accumulation” (23).

ITAC has also been found to be an antagonist for other chemokine receptors. One such example is CCR3. ITAC competes with eotaxin for binding to CCR3 and was able to inhibit migration induced by eotaxin (28). As a result, ITAC can block migration of Th2 cells in response to CCR3 ligands (28). In addition, ITAC has been found to inhibit binding of MIP1α to cells expressing CCR5 and reduced CCR5 activity levels within cells (24). This paper also found that ITAC caused a significant reduction of

migration of CCR5 expressing cells in the presence of another CCR5 ligand, RANTES (24). As a result, ITAC serves an important function in regulating not only the migration of CXCR3-expressing cells, but also CCR3 and CCR5-expressing cells. The multiple functions ITAC has for regulating chemotaxis is shown in Figure 1.

While ITAC serves as a chemoattractant for multiple cell types, it has been shown to have potent chemoattractant activity of IL-2 activated T cells (25). In a co-culture of IFNγ-activated human saphenous vein endothelial cells and T cells, ITAC was shown to be the most potent inducer of trans-endothelial migration of T cells (29). In a similar study done in unstimulated human umbilical vein endothelial cell monolayers in a transwell chamber found that ITAC induced a 4-6 fold increase in trans-endothelial migration of T cells (30). This study found that ITAC induced migration of $CD4^+$ and $CD8^+$ T cells and that memory T cells migrated better than naïve T cells (30). Moreover, ITAC was found to be one of the most potent chemoattractants of $CD4^+$ and $CD8^+$ T effector memory cells (30). This finding raises the question about the possible connection between ITAC and HIV since HIV preferentially infects $CD4^+$ cells expressing both CD4 and CCR5.

While it's general function in the body is known, it is unclear the role ITAC plays during HIV-1 infection. In chapter II and III of this thesis, we found that high levels of ITAC are associated with increased risk for HIV acquisition in HIV-negative individuals and that higher levels of ITAC post HIV infection is associated with steeper $CD4^+$ T cell decline. We clearly found that high levels of ITAC have adverse effects on patients both pre and post infection; however, we don't know how ITAC mediates these effects.

Other studies of ITAC in the context of HIV infection have done so in combination with other CXCR3 binding chemokines. A study from 2019 found that using a panel of three chemokines, CXCL9, CXCL10, and CXCL11, measured during primary HIV-1 infection could predict long-term HIV disease prognosis (31). Another study from 2005 found that CXCL10 and CXCL11 mRNA expression levels were upregulated in HIV-exposed ectocervical epithelial cells (32).

One of the most complete studies focused on the relationship between ITAC and HIV-1 infection is that published in 2005 by Foley et al.. This study found that CXCL10 and CXCL11 mRNA levels were upregulated in HIV-infected human monocyte-derived macrophages and dendritic cells (33). When medium from these infected cells were tested, it was found to be chemotactic for freshly isolated human $CD4^+$ T cells; however, when the $CD4^+$ T cells were pretreated with an anti-CXCR3 antibody, the observed chemotaxis was abolished (33). In addition, CXCL10 and CXCL11 mRNA was found to be expressed in the paracortex of a lymph node isolated from a HIV-infected individual (33). This paper points out that CCR5 on $CD4^+$ T cells is found predominantly on cells that also express CXCR3 and implicates CXCL10 and CXCL11 in the recruitment of susceptible T cells to HIV-1 infected lymph nodes, macrophages, and dendritic cells (33). The paper hypothesizes that “this recruitment might enhance the sequestration of T cells in infected lymphoid organs and spread of infection between cells and contribute to the immunopathology of AIDS” (33).

The hypothesis stated in this paper could provide an explanation for our findings in regard to ITAC. Having a high level of ITAC post infection could recruit uninfected susceptible $CD4^+$ T cells to infected cells or lymphoid organs and the recruitment might

explain the more rapid loss of $CD4^+$ T cells observed. In addition, having high level of ITAC before HIV acquisition, could increase the number of susceptible cells available in peripheral tissues, which could then result in a successful HIV transmission in the case of a possible HIV exposure (as shown in Figure 2).

HIV-1 preadaptation and possible HIV-1 subtype A preadaptation

In chapter II of this thesis, we examine the different clinical progressions of HIV-1 subtype C and A infections and try to identify the overlying causes. Our research showed subtle differences in the biomarker profiles during acute infection, but nothing that would explain the differences seen in disease progression between these two subtypes. One possible explanation not explored extensively in that chapter is the role that preadaptation may play in regard to the different subtypes.

In 2016, the Hunter Laboratory demonstrated that transmission of viruses that were preadapted to the HLA molecules expressed in the newly-infected partner was associated with impaired immunogenicity, elevated viral load, and accelerated $CD4^+$ T cell decline (34). There are a number of HLA alleles that are protective against HIV (35) and this study found that “allele-specific circulating adaptation explained much of the variation in HLA-specific viral load and $CD4^+$ effects” (34). The results of the study suggest that the protective alleles, which have been identified, are those to which the circulating virus is not well adapted (34).

In a separate study from the Hunter Laboratory also in 2016, 169 subtype C heterosexual Zambian transmission pairs were examined and it was found that 25.8% of possible HLA-linked target sites in the transmitted virus gag protein were already

adapted; these transmitted preadaptations significantly reduced early immune recognition of the viral epitopes (36). Consistent with the study by Carlson et. al., this study also found that transmitted preadaptation resulted in higher viral loads and it was significantly associated with faster $CD4^+$ T cell decline (36).

These findings raise the possibility that subtype C may be a more ancient infection and may be more adapted to individuals' HLAs compared to subtype A. In addition, subtype C may be more preadapted to the African population and the common HLAs present in that population compared to subtype A, which may explain the differences observed in disease progression between the two subtypes. While we were not able to examine this possibility in this thesis, current work in the Hunter Laboratory is repeating the analysis done in the Carlson 2016 paper in order to understand the extent of preadaptation observed in HIV-1 subtype A infections.

Addressing different HIV-1 subtypes in future treatment and vaccine efforts

While we were not able to find a main contributor or cause to explain the observed subtype difference in chapter II of this thesis, acknowledging that these differences exist is important in the development of future vaccines or treatments. When looking specifically at vaccine development, it is important that vaccines are able to generate cross-clade, or cross-subtype, antibodies when trying to make a universal vaccine.

One set of researchers studied the ability of different antibodies to neutralize different study viruses using different pools of antibodies generated against different subtype viruses (37). When tested in PBMCs, the study found that pairing antibody pools with homologous clade viruses generated the highest neutralizing antibody titer in

4 out of the 6 subtypes tested. When pseudoviruses were then tested, the researchers found that cross-clade neutralization was more limited compared to the neutralization with homologous antibody pools and virus (37). Despite the limited neutralization, the researchers saw that the clade C antibody pools were broadly cross-reactive, neutralizing the greatest number of viruses in both the PBMC and pseudovirus assays (37). This suggested that the subtype C envelope may merit more research for the elicitation of broadly neutralizing antibodies (37).

The holy grail of HIV-1 vaccine research is the development of an immunogen that can induce broadly neutralizing antibodies. Recent studies have shown that it is possible to develop vaccine strategies that can generate cross-clade antibodies. In 2018, a strategy was developed that targeted the exposed N-terminus of the HIV fusion peptide through priming with the free peptide and boosting with pre-fusion stabilized envelope trimers. The monoclonal antibodies elicited from immunization with that approach were capable of neutralizing up to 31% of a cross-clade panel of 208 HIV-1 strains (38). Another group of researchers used a prime/boost strategy using phage-displayed peptides that were similar to the V3 loop of the envelope and the conserved linear region of the C-terminus gp120 from subtype C viruses. This strategy induced not only gp160 binding antibodies to HIV-1 subtype C viruses, but also cross-clade neutralizing antibodies (39). These studies show that it is possible for a singular vaccine series to develop cross-clade antibodies, albeit at low titers.

With the genetic differences that exist between the different HIV-1 subtypes, it may be more effective to design subtype specific vaccines that are administered based on geographical distribution of the subtype. Another vaccination method would be to

design a vaccine series in which different vaccines in the series contain proteins of different HIV-1 subtypes, which potentially could allow for more comprehensive protection against multiple HIV-1 subtypes. In addition, similar lines could be followed in designing a preventative antibody treatment in which a combination of antibodies generated against multiple subtypes of HIV-1 are given simultaneously to provide broader protection.

Presence or absence of HIV-1 subtype A consensus bias

While not discussed in the main chapters of this dissertation, a large amount of work went into the examination of the role of consensus bias in HIV-1 subtype A infection (see Appendix I). While the role of consensus bias in subtype A infections has yet to be confirmed, a number of previous studies have examined whether a consensus bias existed for subtype C transmission.

In 2009, a group examined 10 transmission pairs infected with HIV-1 subtype D and 3 infected with subtype A and analyzed the presence of any sort of consensus bias. This limited study found that the transmitted variants had less diversity and divergence and were more closely related to ancestral sequences (40).

In 2014, a study from the Hunter Laboratory examined 127 linked subtype C transmission pairs from Zambia. The plasma from these individuals was examined a median of 46 days after the estimated date of infection. When variants from the consensus were examined in the amino acid sequences of gag, pol, and nef, this analysis found a clear bias for transmission of cohort consensus residues compared to variants that were defined as polymorphisms (41).

The previous study looking at consensus bias did not examine the near full-length genome in their analysis. In a study in 2015, researchers in the Hunter Laboratory looked at six subtype C transmission pairs and were able to find that transmitted-founder variants had significantly shorter pairwise distance to the subtype C consensus node than the median of the corresponding non-transmitted variants for both the nucleotide and amino acid sequences (42). This confirms the existence of a selection bias for consensus-like nucleotides or amino acids across the viral genome during subtype C transmission.

During my dissertation work, I isolated and sequenced non-transmitted donor viral sequences for 8 chronically infected subtype A individuals to develop a total of 10 subtype A transmission pairs, which contained sequences from both the transmitted-founder virus and non-variant donor variants (sequences for two subtype A transmission pairs were already generated). Using a subtype A cohort consensus newly generated in the Hunter Laboratory, I was able to complete a consensus bias analysis for the sequences from those 10 transmission pairs. Doing both a near full-length genome nucleotide and amino acid analysis of viral genes of gag, pol, env, and nef, I did not observe that the transmitted-founder variants had a shorter pairwise distance to the subtype A cohort consensus compared to the non-transmitted donor viral variants. In addition to the subtype A transmission pairs I generated, I generated four new subtype C transmission pairs and was able to repeat this analysis with 10 subtype C transmission pairs (which included the six pairs previously made in the laboratory). In this analysis, I found that the transmitted-founder variant had a shorter pairwise distance to the subtype C consensus sequence compared to the non-transmitted

variants; this was significant for the near full-length genome nucleotide sequences and the pol amino acid sequences. This analysis further confirmed the existence of a consensus bias in subtype C transmission. However, the presence of a consensus bias in subtype A transmission could not be shown in this analysis.

Currently in the Hunter Laboratory, additional studies are underway to repeat the Carlson study (41) with a large number of subtype A transmission pairs to confirm or deny the presence of a consensus bias in subtype A transmission.

The interferon-debate: What is its role in HIV-1 transmission?

While not discussed in the main chapters of this dissertation, a large amount of work went into making brand new *in vitro* transmission pairs in order to test the role of IFN resistance in HIV transmission (see Appendix II).

Much is known about the effect of IFN early in HIV-1 infection. IFN-α, a type I IFN, is usually induced early and its expression is often transient during the first few weeks of acute HIV-1 infection (43, 44) and it has been shown to be able to inhibit HIV replication in primary macrophages and $CD4^+$ T cells (45-48). The upregulation of type I IFNs (IFN-α and IFN-β) leads to the cellular expression of IFN-stimulated genes (ISGs), which promotes an antiviral environment in infected cells and bystander cells (49).

Due to its ability to inhibit HIV-1 replication and induce antiviral genes, many studies have examined IFN as a possible treatment for HIV-1 infection. Clinical trials in patients with HIV-1 showed that IFN-α, alone or in combination with ART drugs, significantly decreased HIV viral loads (50, 51). In another clinical trial, IFN-α was given during a drug-free period in an ART interruption study; this was shown to result in a reduction of the magnitude of viral rebound, as well as a delay in the time to rebound.

However, there was no difference observed in the overall size of the reservoir (52). Studies with IFN-β and zidovudine (an ART drug) were able to reduce the level of HIV p24 *in vitro*. However, this finding was not confirmed in *in vivo* studies (53). However, there are risks associated with IFN monotherapy; it has been observed that when high doses of IFN-α are used, there is accelerated $CD4^+$ T cell loss (54).

While IFN is able to help inhibit the virus, HIV-1 has also evolved ways to escape from the antiviral properties induced by IFN. HIV viral genes, Vif and Vpu, have been shown to directly block the effects of IFN-induced restriction factors APOBEC and tetherin, respectively (55-60). Other studies have shown that HIV is able to escape the intrinsic IFN-β response activation by limiting the replication of viral DNA or actively blocking virus sensing by PRRs (61, 62).

With the dual role of IFN both helping and hindering HIV infection, understanding its effect on the transmitted-founder virus is important since this is the virus that initiates new infections. In recent years, there have been conflicting findings about the role of IFN in HIV-1 transmission, as we discuss in the Introduction. A paper from Beatrice Hahn's group found that the transmitted-founder virus was significantly more resistant to both IFN-α and IFN-β than the corresponding donor isolates (63). In contrast, a study from the Hunter Laboratory found that resistance of the transmitted-founder variant to IFN-α was not significantly different from that of non-transmitted variants from the same transmission pair (42) and others have reported similar findings (64-67).

As a result of these different studies, uncertainty exists about the role that IFN plays in heterosexual transmission bottleneck. To help address this question, I generated seven new transmission pairs (four subtype A and three subtype C) to

increase the number of viruses that we can use to test the role of IFN resistance, both IFN-α and IFN-β. In addition, no subtype A transmission pairs had been generated before this point, so these new pairs will allow us to test the role of IFN resistance on subtype A transmission pairs, which has not been tested before.

Summary

Much information has been discovered involving the different stages of HIV-1 infection. Previous research has identified a number of practices that can increase risk for HIV infection, such as intravenous drug use and unsafe sex practices, along with people at higher risk of infection, such as homosexual mean and sex workers. In an unexpected turn of events, we discovered that HIV-uninfected individuals with elevated systemic biomarkers also are at a higher risk for HIV-1 acquisition. This means that individuals with chronic diseases or other infections may have a higher risk of HIV infection. This finding requires additional research in order to fully understand the role these biomarkers play in HIV acquisition. Once an individual becomes HIV-positive, additional factors such as HIV-1 subtype play an important role in future disease progression. Questions still remain as to why these clinical differences exist between infections of different subtypes. Ongoing research into the roles of consensus bias and preadaptation in subtype A transmission may help to answer some of these lingering questions. Additional questions still remain about the role of IFN resistance in HIV transmission. Understanding these unresolved questions will be important to understand early risk factors for HIV infection and how to address the differences in HIV-1 infections in regard to treatments and vaccine development.

Figures

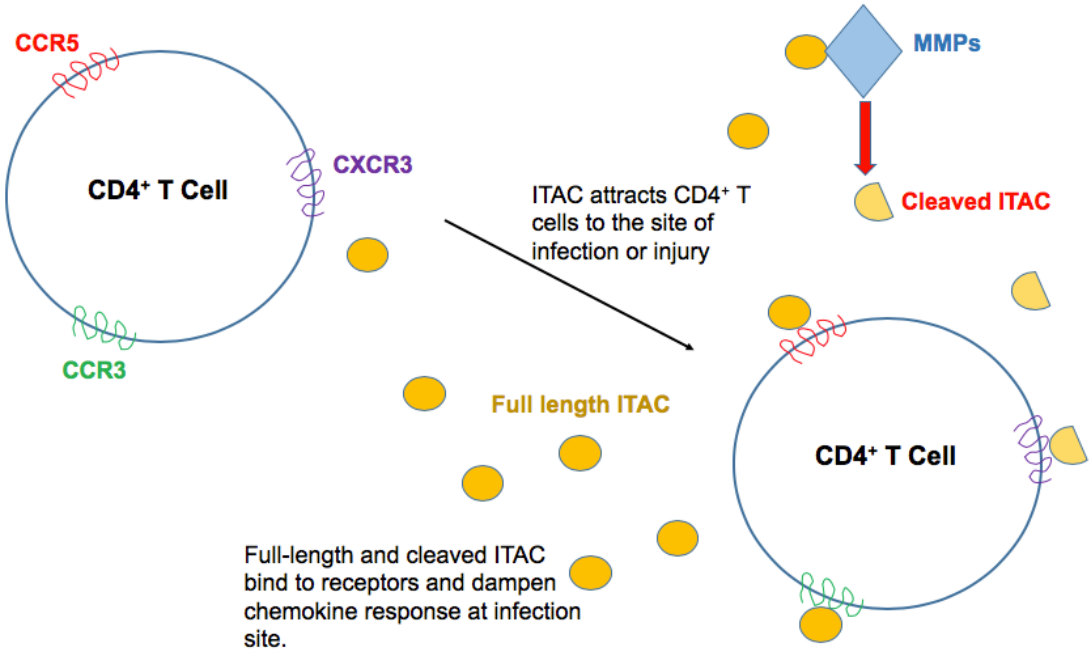

Figure 1. ITAC regulates chemotaxis to sites of infection or injury. ITAC binds to CXCR3 and attracts CXCR3 expressing cells, such as $CD4^+$ T cells, to sites of infection and injury via chemotaxis. Full-length ITAC binds to CCR3 and CCR5 and functions as antagonists to prevent further chemotaxis due to CCR3 and CCR5 ligands; antagonism against CCR3 can block migration of Th2 cells to the infection site. Matrix metalloproteinases (MMPs) cleave full-length ITAC into truncated forms that serve as antagonists to CXCR3 to further dampen chemotaxis due to CXCR3 ligands. The combined antagonism of the different forms of ITAC reduce chemotaxis and regulate which cell types are recruited.

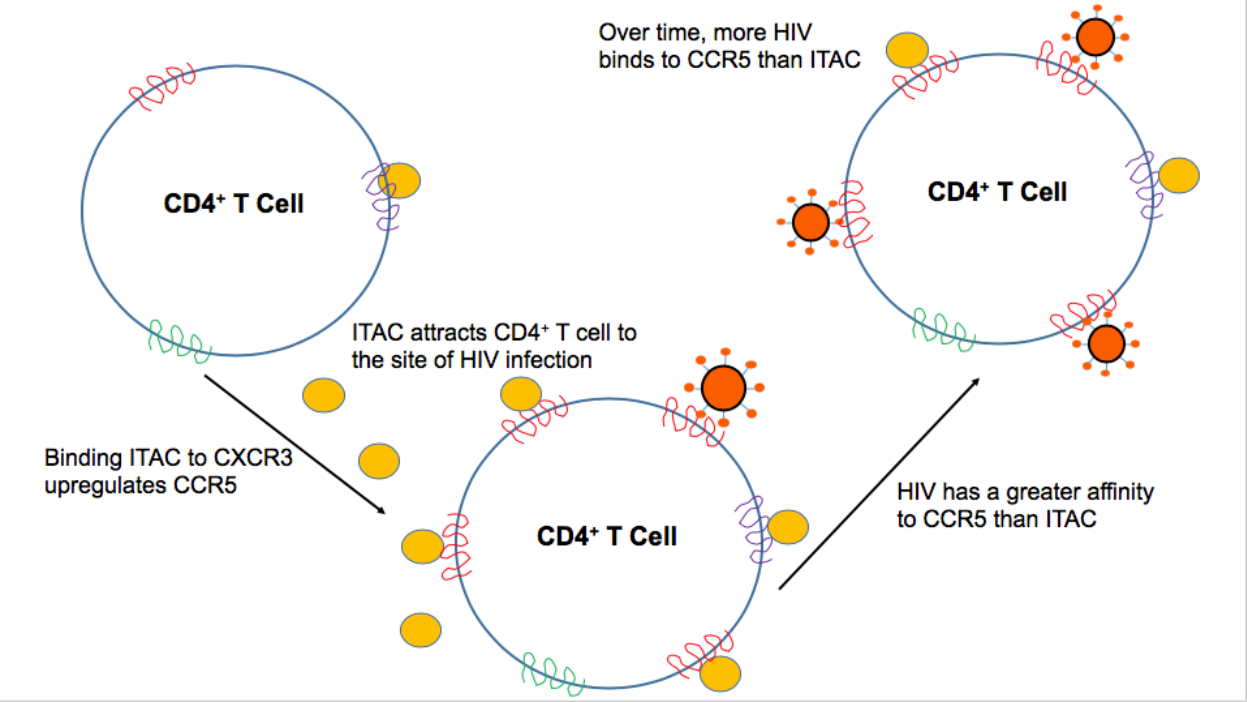

Figure 2. Binding of ITAC to CXCR3 on CD4$^+$ T cell upregulate surface expression of CCR5. ITAC binds to CXCR3 on CD4$^+$ T and attracts the cell to the site of HIV-1 infection. Binding of ITAC to CXCR3 upregulates the surface expression of CCR5 on susceptible CD4$^+$ T cells. HIV-1 virions have a higher affinity to CCR5 than ITAC; as a result, HIV-1 displaces ITAC to bind to more CCR5 compared to ITAC.

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Appendix

Introduction

The figures included in the appendix are not enough for a complete story that can be published in a manuscript, however they represent an important part of this dissertation. These sections show new findings in subtype A transmission and the development of new materials that can be used in future experiments, some of which are mentioned in the discussion. This section includes a discussion of the results and a brief description of the methods. Each figure has a corresponding legend, The findings are broken up into two sections.

The first section is on the development of 10 subtype A and 10 subtype C HIV-1 transmission pairs. This section details the sequencing data from both subtype A and C transmitted-founder viruses and non-transmitted donor viruses. This section is made up of six figures and relates to data previously published by the Hunter Laboratory (1).

The second section shows the titers of IMCs for seven new transmission pairs, four subtype A and three subtype C. The results shown in this section are related to section one of the appendix, as the sequences shown in that section, were used to generate these IMCs.

Appendix A: Isolation of genetic sequences of 10 subtype A and 10 subtype C transmission pairs

The Hunter laboratory has previously developed and published data on 6 subtype C transmission pairs (1) and 2 subtype A transmission pairs (2). While limited studies were done with the genetic sequences from the subtype A transmission pairs, the 6 subtype C transmission pairs were used to test multiple characteristics of subtype C HIV-1 transmission and characteristics of the transmitted-founder virus. Specifically, one characteristic that was tested was consensus bias in the transmission bottleneck.

Previous studies have shown that a transmission bottleneck exists in HIV-1 infections. In heterosexual HIV-1 transmission, about 80% of new HIV-1 infections are caused by a single genetic variant of the virus (3). With this single genetic variant, other studies have found that viruses that are closer to consensus are preferentially transmitted over viruses that have polymorphisms that distance that seqeuence from the consensus (4). A previous study done in the Hunter laboratory with 6 subtype C transmission pairs found that the transmitted-founder virus was closer to the subtype C LANL consensus sequence than the non-transmitted donor viruses (1). Consensus bias studies have yet to be done for subtype A infections.

In order to address the possible consensus bias in subtype A transmissions, the number of subtype A and subtype C transmission pairs were increased to 20, 10 per subtype. Figures 1 and 4 show the phylogenetic trees for the 10 transmission pairs generated for subtype A and subtype C, respectively. Each node is labeled by the coded ID of the transmission pair and the transmitted-founder viral sequence is showed with a red dot. For this analysis, a subtype A cohort consensus sequence with over 300 subtype A sequences was generated in order to performed consensus bias analyses.

Consensus bias analyses for subtype A are shown in figure 2 and 3; figure 2 shows the analysis using nucleotide sequences of near full-length HIV-1 nucleotide sequences and figure 3 shows the analysis of amino acid sequences for four viral genes, gag, pol, env, and nef. This analysis showed that the transmitted-founder sequence, either nucleotide or amino acid, were not closer to consensus compared to the non-transmitted donor sequences. Figures 5 and 6 repeated the nucleotide and the amino acid analysis for subtype C, respectively. The nucleotide analysis found that the transmitted-founder virus was closer to the subtype C LANL consensus sequence compared to the non-transmitted donor viruses. In the amino acid analysis, only pol sequences of the transmitted-founder viruses were significantly closer to the consensus sequences compared to the non-transmitted donor viruses.

Method: Full methodology of RNA extraction, cDNA, generation, and DNA amplification is described in Deymier 2014 (5). Briefly, viral RNA was extracted and converted to full-length cDNA. The plasma chosen for extraction was taken within 90 days of the EDI (estimated date of infection) for the linked recipients, and as soon as possible to the EDI of the linked recipient when isolating RNA from the donor plasma. This allows us to identify two important genotypic characteristics: 1) the sequence(s) of the viral variants that established the infection in the linked recipient and 2) the viral quasispecies of the chronically infected individuals around the time of transmission.

cDNA then undergoes two rounds of single genome amplification PCR to generate a PCR product. The PCR product is then run on a 1% agarose-TAE gel at 200 V for 30 minutes to determine the presence of a 9kb band on the gel. Correct size PCR

products were then purified with a Wizard SV Gel and PCR Clean-Up System (Promega). The purified PCR products are then made into a library to prepare for PacBio SMRT sequencing on the PacBio RSII instrument. The protocol for PacBio library preparation and subsequent analysis of the sequences are described in Dilernia 2015 (6). Transmitted-founder viruses and non-transmitted donor viruses were sequenced in separate libraries. In addition, non-transmitted donor variant sequences were identified from gp41 that were determined via Sanger sequencing.

Phylogenetic trees for each of the two subtypes were made using the Maximum-likelihood model in Geneious. Each of the nodes in the generated tree were then extracted and used for consensus bias analysis. Details for this analysis is described in Deymier, Ende 2015 (1). Briefly, the sequences for the transmitted-founder virus and the non-transmitted founder viruses were combined into one file; this file also included the consensus sequences for both HIV-1 subtype A and C. Depending which subtype was being examined, the non-matching subtype served as a negative control; the other one would be used to determine the distance to the matching subtype consensus sequence. For subtype C, the consensus sequence used was the 2002 LANL subtype C sequences. For subtype A, a cohort consensus sequence was generated from over 300 chronic HIV-1 sequences isolated by the Hunter Laboratory. The combined file was then submitted to the DIVEIN server to determine the pairwise distance values (indra.mullins.microbiol.washington.edu/DIVEIN/diver.html) (7). Amino acid gene-specific distance analysis required an additional step before submission to the DIVEIN server. The combined file was submitted to the gene cutter tool on the Los Alamos National Laboratory (LANL) database to isolate the specific gene of interest. The output

of the amino acid sequences for the gene was then submitted to the DIVEIN server for distance analysis. The generated distance values were then plotted in Prism statistical program and tested for differences in the distance to consensus between the transmitted-founder virus and the median distance of the non-transmitted donor viruses.

Figures

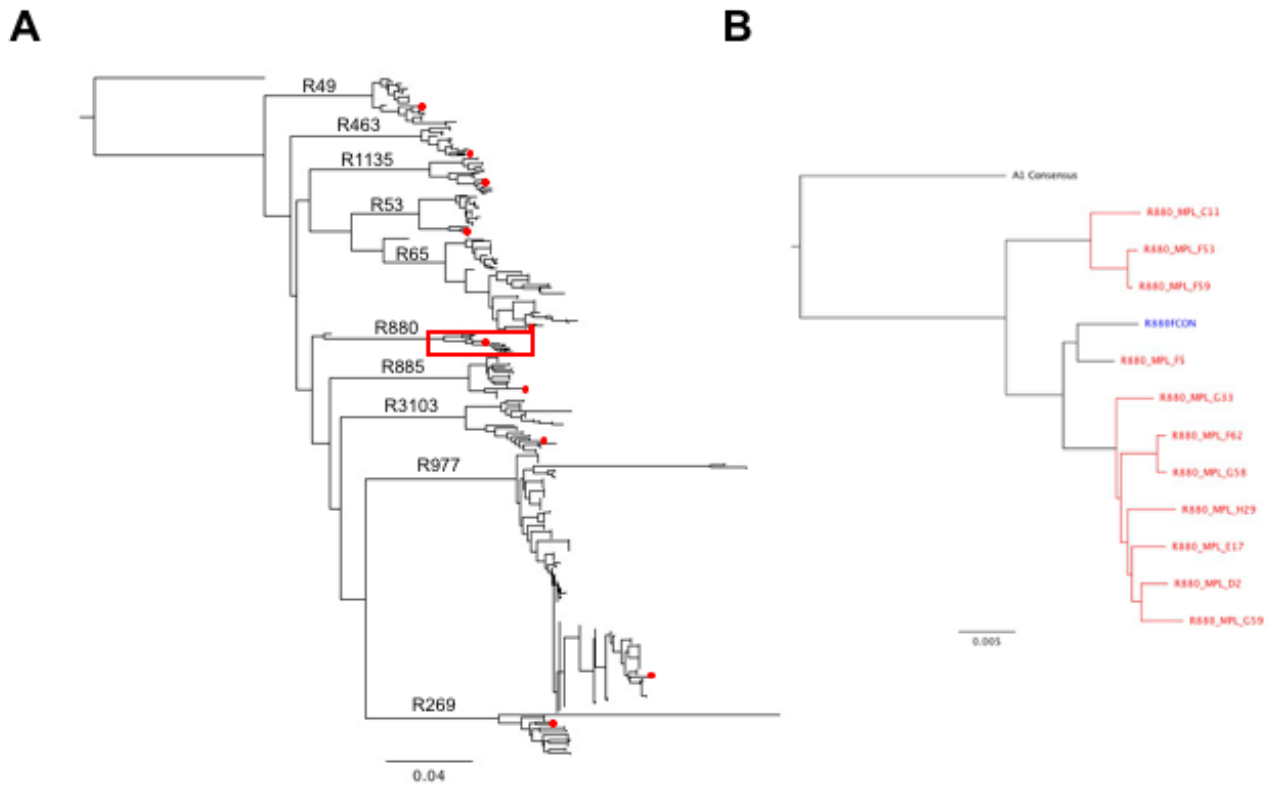

Figure 1. Phylogenetic analysis of 10 subtype A transmission pairs. (A) Sequence of the transmitted-founder virus is shown with a red dot, while the rest of the branches are the non-transmitted donor viral sequences. The phylogenetic tree was generated in Geneious 9 using maximum likelihood mode and rooted on a A1 consensus sequence generated from LANL. (B) Inset shows a zoomed in region of one of the transmission pairs, R880 (the transmitted-founder sequence labeled in blue and the donor sequences labeled in red). The red boxed area in (A) shows where on the tree the inset came from. Inset taken from Yue *PLOS Pathogens* 2015 (2).

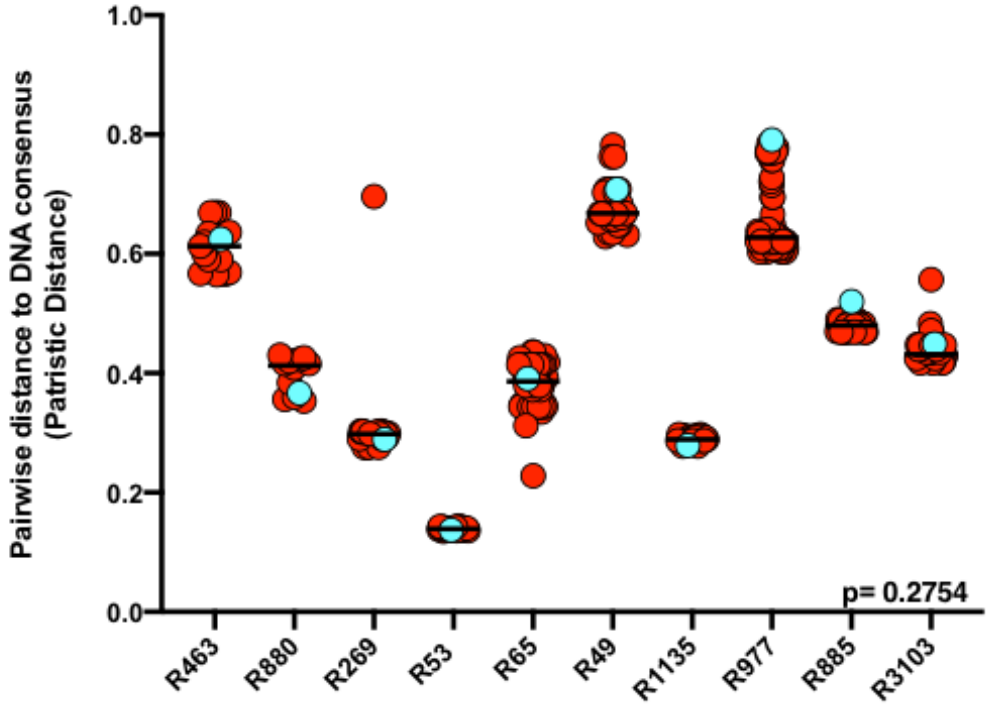

Figure 2. Nucleotide sequence of full-length subtype A transmitted-founder viruses are not closer to the subtype A cohort consensus compared to the sequences of non-transmitted donor viruses. The transmitted-founder viral sequence shown in light blue and non-transmitted donor viras sequences shown in red. Patristic distances were generated using the DIVEIN tool at http://indra.mullins.microbiol.washington.edu/DIVEIN (7). Wilcoxon Signed-rank test, two-tailed.

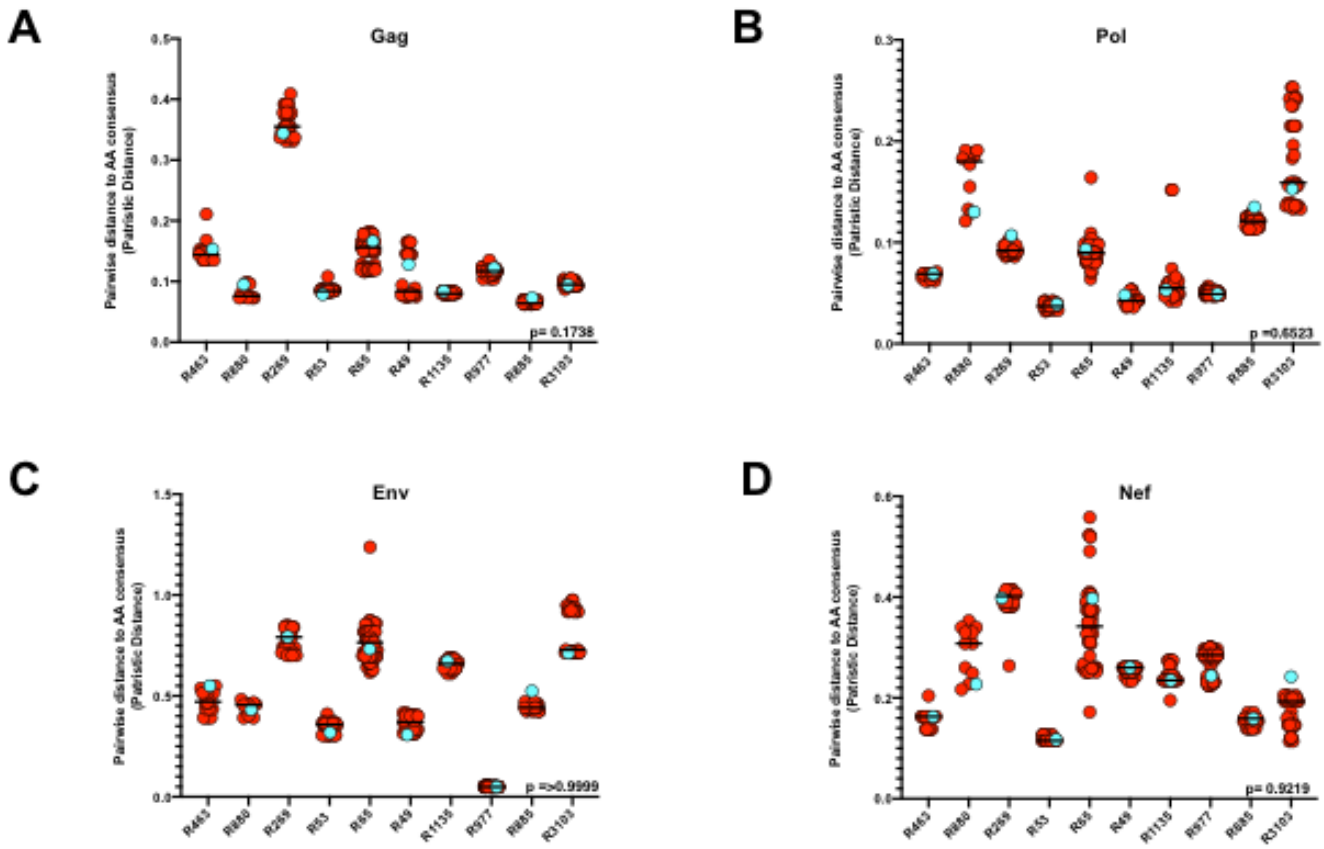

Figure 3. Amino acid sequences of HIV-1 genes of subtype A transmitted-founder viruses are not closer to the subtype A cohort consensus compared to the sequences of non-transmitted donor viruses for viral genes (A) gag, (B) pol, (C) env, (D) nef. The transmitted-founder viral sequence shown in light blue and non-transmitted donor viral sequences shown in red. Patristic distances were generated using the DIVEIN tool at http://indra.mullins.microbiol.washington.edu/DIVEIN (7). Wilcoxon Signed-rank test, two-tailed.

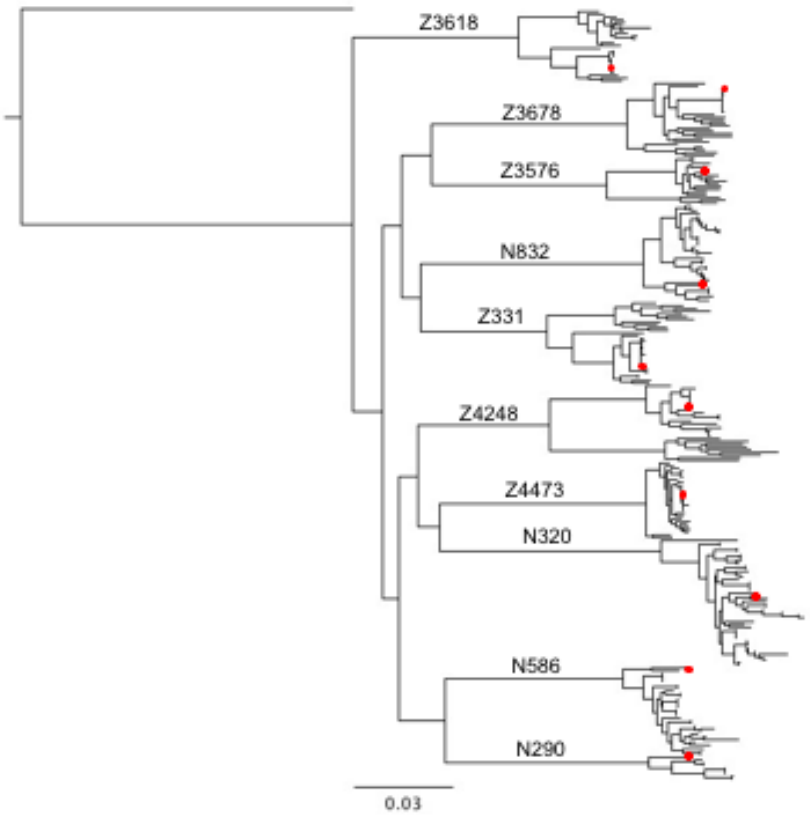

Figure 4. Phylogenetic analysis of 10 subtype C transmission pairs. Sequence of the transmitted-founder virus is shown with a red dot, while the rest of the branches are the non-transmitted donor viral sequences. Phylogenetic tree was generated in Geneious 9 using maximum likelihood mode and rooted on a subtype C consensus sequence generated from LANL.

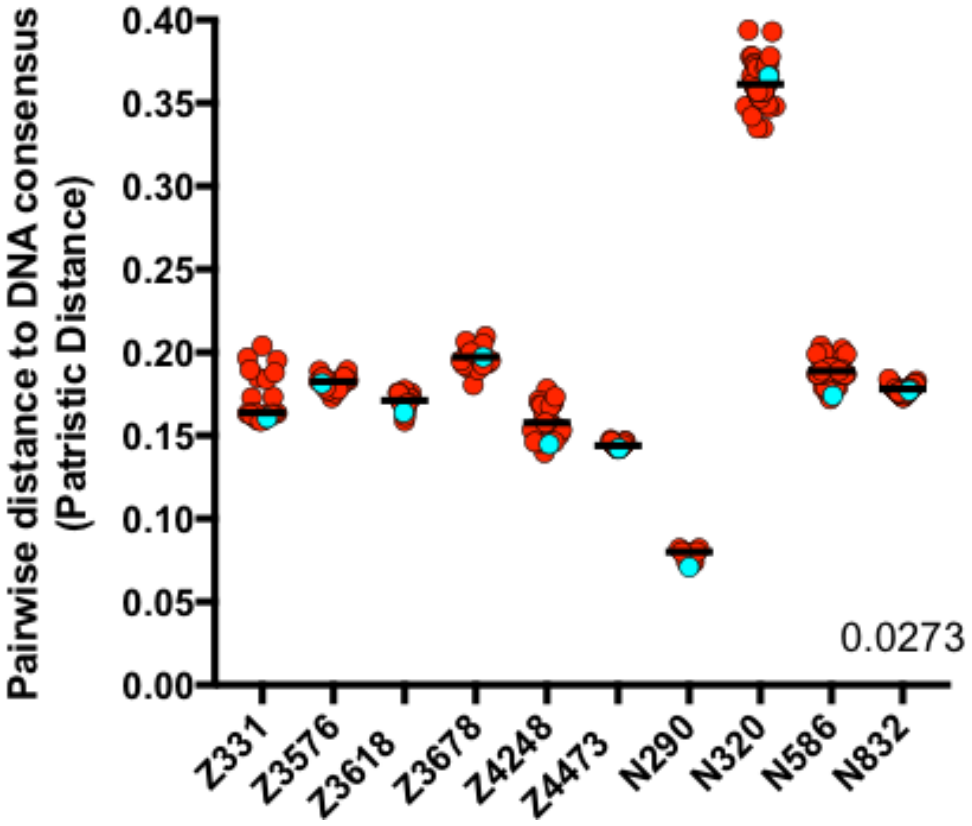

Figure 5. Nucleotide sequence of full-length subtype C transmitted-founder viruses are closer to the subtype C consensus sequence compared to the sequences of nontransmitted donor viruses. The transmitted-founder viral sequence shown in light blue and non-transmitted donor viral sequences shown in red. Patristic distances were generated using the DIVEIN tool at http://indra.mullins.microbiol.washington.edu/DIVEIN (7). Wilcoxon Signed-rank test, two-tailed.

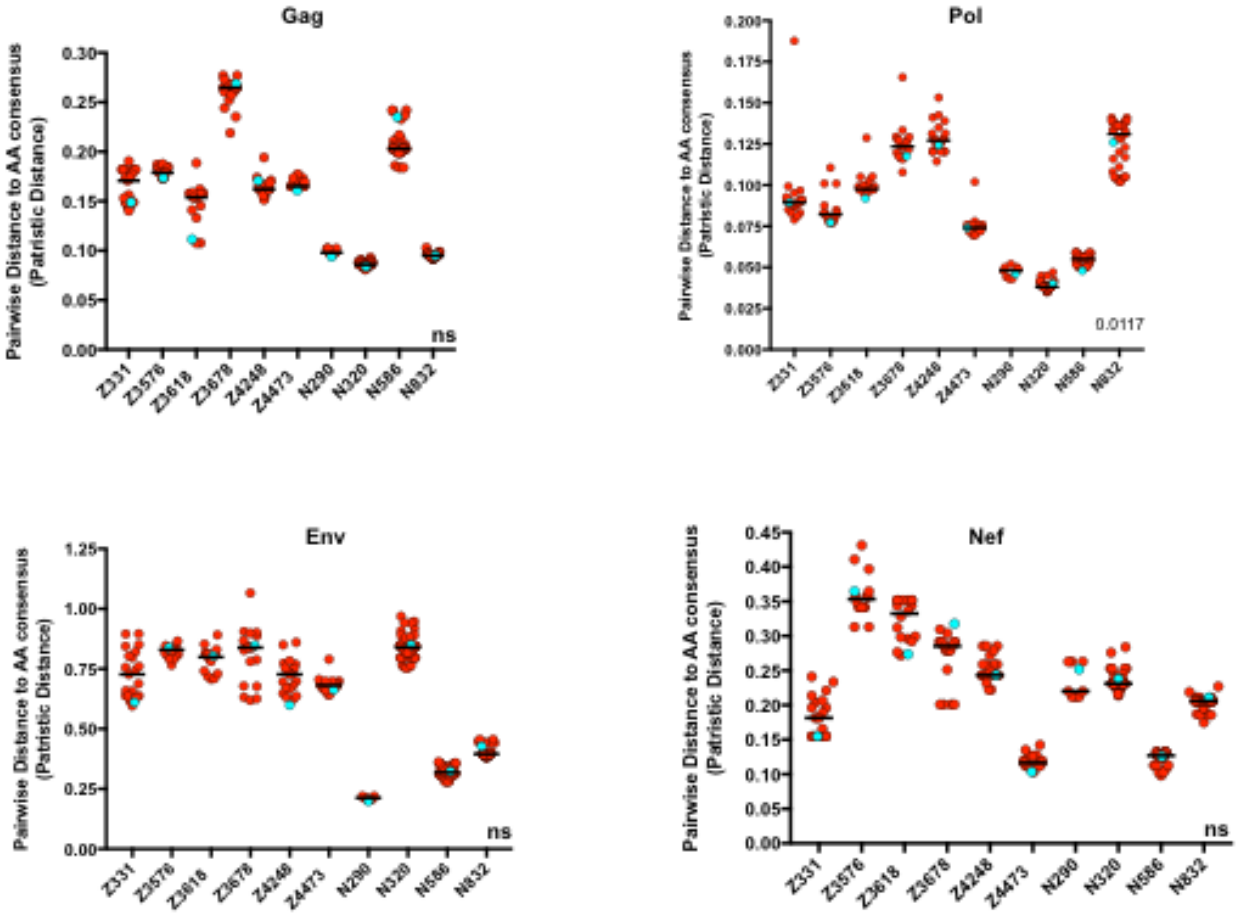

Figure 6. Amino acid sequences of HIV-1 genes gag, env, and enf of subtype C transmitted-founder viruses are closer not to the subtype C consensus compared to the sequences of nontransmitted donor viruses. The four viral genes analyzed were (A) gag, (B) pol, (C) env, (D) nef. Amino acid sequence of pol (B) from subtype C transmitted founder viruses is closer to consensus compared to non-transmitted donor virus sequences. The transmitted-founder virus sequence shown in light blue and non-transmitted donor virus sequences shown in red. Patristic distances were generated using the DIVEIN tool at http://indra.mullins.microbiol.washington.edu/DIVEIN (7). Wilcoxon Signed-rank test, two-tailed.

Appendix B: Generation of infectious molecular clones for seven new transmission pairs

One question that the Hunter Laboratory examines is the role of IFN resistance in the role of HIV-1 transmission. Previous studies with 6 subtype C transmission pairs found that transmitted-founder viruses were not more resistant to IFN compared to non-transmitted founder viruses (1). This contradicts studies found from another group that showed that the transmitted-founder virus is more resistant to both IFN-α and IFN-β (8).

To further address this question, we wanted to test IFN resistance in both subtype A transmission pairs. Using the sequences isolated for the consensus bias analysis, we were able to make three new subtype C and four subtype A *in vitro* transmission pairs. Figure 1 shows the titers of the newly made *in vitro* infectious molecular clones (IMCs). Every newly made transmission pair has at least 3 non-transmitted donor virus IMCs. The titers of the transmitted-founder virus shown in blue and non-transmitted donor viruses are shown in red.

Method: Methodology is fully described in Deymier 2014 (5). Briefly, near full-length HIV-1 genomes used in the sequence analysis were re-amplified with cloning primers. In addition, LTRs were isolated from the linked-recipient, cloned into a pbluescript backbone, and re-amplified with cloning primers. For each transmission pair, the LTR from the linked-recipient was used to generate both the transmitted-founder virus IMC and the non-transmitted donor virus IMCs. The near full-length genome and LTR cloning pieces were attached using the Clontech In-Fusion HD cloning kit and then added to component cells for a heat shock transformation. After 48 hours growing at room temperature on LB/agar-ampicillin plates, colonies were picked and grown at 30°

C for 48 hours and the DNA plasmid was prepared using the Pureyield Plasmid Miniprep System (Promega). Cloning was confirmed to be successful by restriction digest and gel visualization to ensure that the generated IMCs was the correct size (around 12 kb). IMCs that were the correct size were then reamplified and grown on LB/agar-ampicillin plates to enhance purify and increase the DNA yield with Pureyield Maxiprep System (Promega). 1.5 ug of IMC plasmid was then transfected in 293T cells with Fugene-HD Transfection reagent (Promega). 293T cell supernatants were collected 48 hours after transfection and clarified by centrifugation. Viral stocks were titered on tzm-bl reporter cell line as described in Prince et al and Claiborne et al (9, 10).

Figures

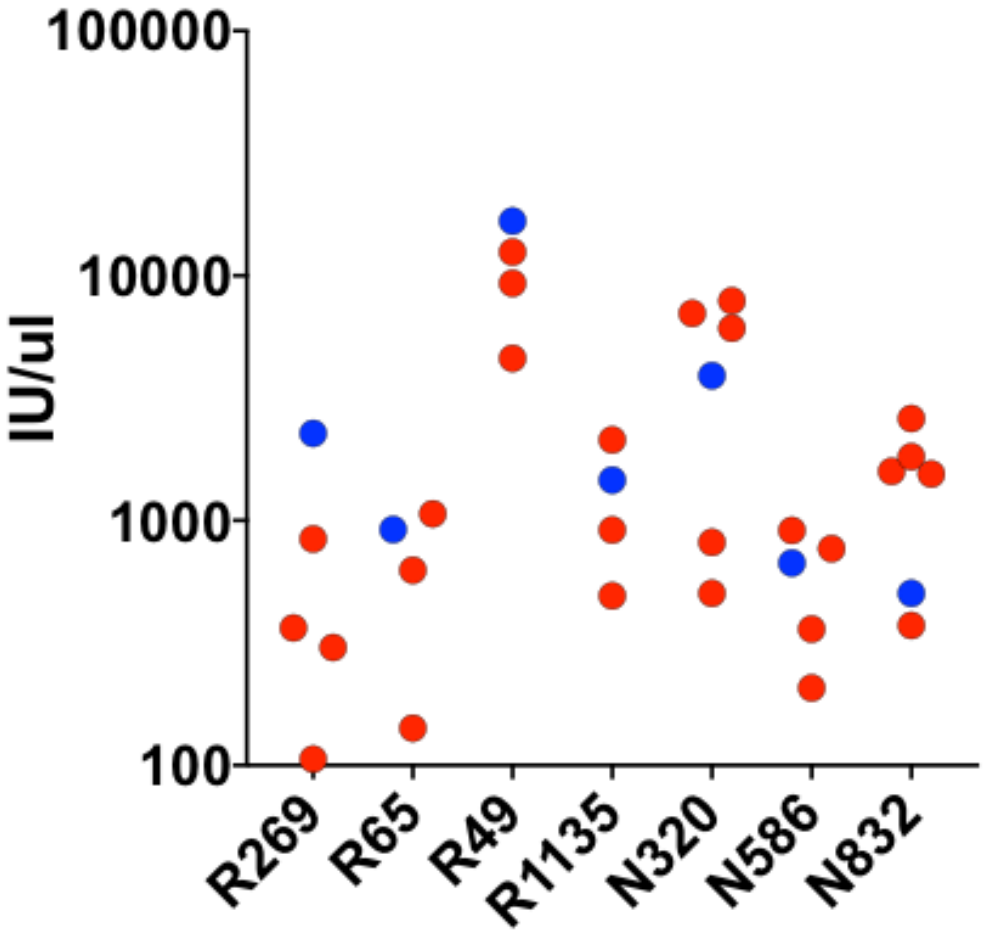

Figure 1. Titers of newly generated subtype A and C transmission pairs. Four new subtype A transmission pairs and three new subtype C transmission pairs were generated using the methodology described in Deymier *Virology* 2014 (5). Titers of the transmitted founder virus are shown in blue and titers of the non-transmitted donor viruses are shown in red. All titers shown were tested in triplicate.

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