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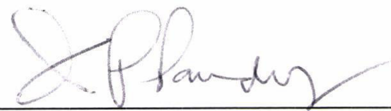
Immunoglobulin and Fcγ Receptor Genes Influence the Control of HIV Replication and the Progression of HIV Infection

Ray Deepe
Mentor: Dr. Janardan Pandey

A Thesis submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Master of Science in the College of Graduate Studies

Department of Microbiology and Immunology
2011

Approved by:

Chairman, Advisory Committee 
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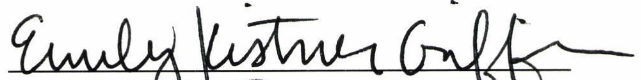
Emily Kistner-Griffin, PhD

Carl Atkinson, PhD

Natalie Sutkowski, PhD

Shashidhar Pai, MD

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List of Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
ADE	Antibody-dependent enhancement
AIDS	Acquired immunodeficiency syndrome
APC	Antigen-presenting cell
CTL	Cytotoxic T-lymphocyte
CRF	Circulating recombinant form
DC	Dendritic cell
FcγR	Fc gamma receptor
FDC	Follicular dendritic cell
GWAS	Genome-wide association study
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
KIR	Killer-cell immunoglobulin-like receptor
KS	Kaposi's sarcoma
LTNP	Long-term nonprogressor
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
NK	Natural killer cell
PIC	Pre-integration complex
rgp120	Recombinant gp120
SIV	Simian immunodeficiency virus

Abstract

HIV is an infection affecting approximately 33 million people worldwide especially in Sub-Saharan Africa and Southeastern Asia. HIV infection is marked by the loss of CD4+ T-cells and is the causative agent of AIDS. Host genetic factors have been shown to influence the progression and outcome of HIV infection, but the genes identified thus far account for approximately 15% of the variance observed in viral load and progression, suggesting involvement of additional genes in HIV pathogenesis. FcγRIIa and FcγRIIIa genotypes have been shown to be influential in the transmission, control, and progression of HIV. These receptors contain polymorphisms that influence binding affinity for their ligand, the Fc region of IgG. The Fc region is also highly polymorphic and could potentially contribute to the differences seen in control and progression. Yet, these polymorphisms, known as GM allotypes, have not been investigated. Determinants expressed on Fc (GM) and FcγR are probably some of the most likely ligand-receptor candidate pairs for gene-gene interactions in the human genome. Thus, the aim of this investigation was to determine whether particular GM and FcγRIIa, genotypes were individually or epistatically associated with the host control of HIV replication and progression of HIV to a low CD4+ T-cell count. This study suggests that while no GM allotype is influential by itself, particular combinations of FcγR-GM are influential in the control of HIV replication as well as the progression of HIV to a low CD4+ T-cell count.

Chapter 1: Introduction

Section 1: History

Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), was first transferred to humans when HIV-1 jumped from simian immunodeficiency virus (SIV) infected chimpanzees [1,2] to humans between 1884 and 1924 [3] in southern Cameroon [4]. SIV is thought to have infected primates at least 32,000 years ago [5], and the most plausible explanation for the transmission from chimpanzees to humans is that hunters in Africa most likely killed and ate infected chimpanzees or that infected blood from butchered chimpanzees got into open wounds or cuts on the hunter [6]. From that point, HIV then spread through Africa and was then likely introduced to Haiti around 1966 which then spread to the United States around 1969 [7].

Before HIV was known to be the cause of AIDS, the disease first appeared in early 1981 in San Francisco [8] when unusual numbers of gay men and injection drug users presented symptoms of a rare opportunistic infection, *Pneumocystis carinii* pneumonia. A rare skin cancer mainly seen in the elderly, Kaposi's sarcoma (KS), was additionally reported in young gay men in New York [9]. Numerous theories were presented as to the possible cause of the opportunistic infections [10-12]. Initially, the condition seemed to be contained to homosexual males and was referred to as the "gay cancer" or gay-related immune deficiency [13,14]. However, this disease was also occurring in non-homosexual populations [15,16] and the name of the disease was officially set as AIDS by the CDC in 1982 [17]. Prior to the discovery of HIV, several modes of transmission were observed including blood transfusions, mother-to-child transmission, and heterosexual contact which was clear evidence that AIDS was caused by an unknown infectious

agent [18-20]. In 1983, both Robert Gallo and Luc Montagier isolated HIV that was later identified as the causative agent of AIDS [21,22] which led to the development and implementation of a commercial blood test for HIV in 1985 [23].

From the time when AIDS was first recognized in 1981, HIV has resulted in over 60 million infections and 25 million deaths [24]. HIV is mainly concentrated in Africa, especially Sub-Saharan Africa, and Southeast Asia where upwards of 28% of the population in some countries are infected (Figure 1) [24]. Currently, there are approximately 1.1 million individuals infected with HIV in the United States [25]. The infection falls mainly along racial, cultural, and geographical lines and can rival rates seen in many parts of Africa (Figure 2) [26]. Incidence and rates are falling worldwide which is most likely attributed to the greater access to highly active antiretroviral therapy (HAART) medications. There were 2.6 million new infections reported in 2009 which is an almost

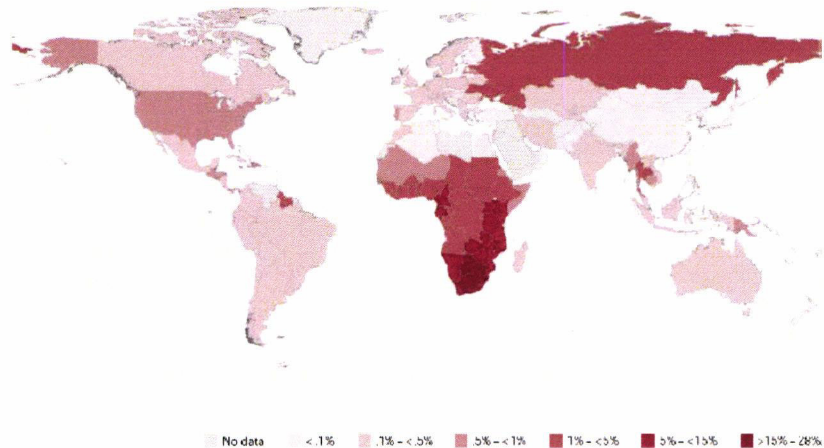


Figure 1. Global prevalence of HIV, 2009.

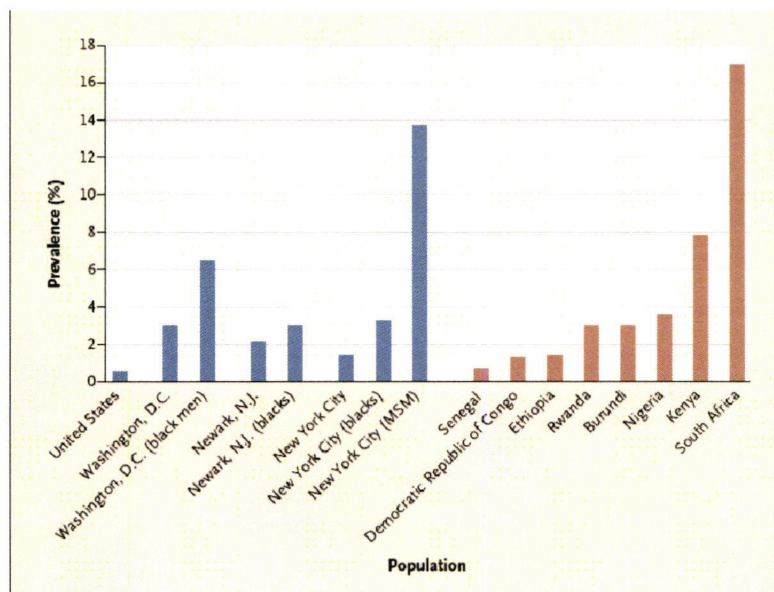


Figure 2. HIV prevalence in adults from select populations in the United States and select countries in sub-Sahara Africa. MSM stands for men who have sex with other men.

20% decrease from the previous 10 years. Other supporting evidence to this fact is that there were also 1.8 million AIDS-related deaths in 2009 as compared to 2.1 million in 2004 [24].

HAART remains the most viable option for treatment of HIV infection as this entails numerous classes of drugs in combination that can slow HIV progression. However, there are several complications that go along with HAART. HAART can be exceedingly expensive and can cause severe physiological and psychological side effects [27-29]. Another glaring concern with HAART is that HIV can eventually become resistant to the medication and eventually progress to AIDS regardless of treatment.

In response to the mounting multitudes of infected, many past and present efforts have been undertaken to develop an effective vaccine. So far, all vaccines developed have proven ineffective in preventing HIV infection and in one particular instance showed an increase in infection [30]. The most promising trial to date, known as RV144, was undertaken in Thailand from 2003 to 2006. Volunteers who received the vaccine showed a 31% lower infection rate than volunteers who received placebo injections [31]. The vaccine was a combination of a canarypox virus expressing three HIV proteins (pol, env, and gag) and a former vaccine candidate, AIDSVAX, which is a genetically engineered HIV protein, gp120. This showing of modest protection has renewed interest in gp120 as a vaccine target.

Section 2: Human Immunodeficiency Virus

HIV belongs to the virus family Retroviridae in the lentivirus genus. Viruses in this family are effectively known as retroviruses and are characterized by their enveloped nucleocapsid and their ability to reverse transcribe their diploid RNA genome into DNA, which is then inserted into the host genome [32].

There are two main types of HIV: HIV-1 and HIV-2. As noted earlier, HIV-1 is thought to have come from chimpanzees [1,2], but HIV-2 is thought to have come from sooty mangabeys, and is far less pathogenic than HIV-1. HIV-2 is mostly reserved to west Africa while HIV-1 has spread throughout the world [33]. HIV-1 can be broken down into 4 major groups: M, N, O, and P. Of these, group M is the most diverse and widespread consisting of nine subtypes, commonly known as clades, and circulating recombinant forms (CRFs) that have combined genetic information from different clades [34]. Clades tend to fall along geographical lines [35] (Figure 3), and are known to have different levels of pathogenecity [36,37]. The importance of the genetic diversity resulting in clades has important implications for drug treatment as some clades may be more apt for becoming resistant to HAART medications than others [35]. It also has significant implications for vaccine design as it is unknown if the incredible diversity of HIV would allow a vaccine designed for one clade to be effective against all other clades or CRFs [38].

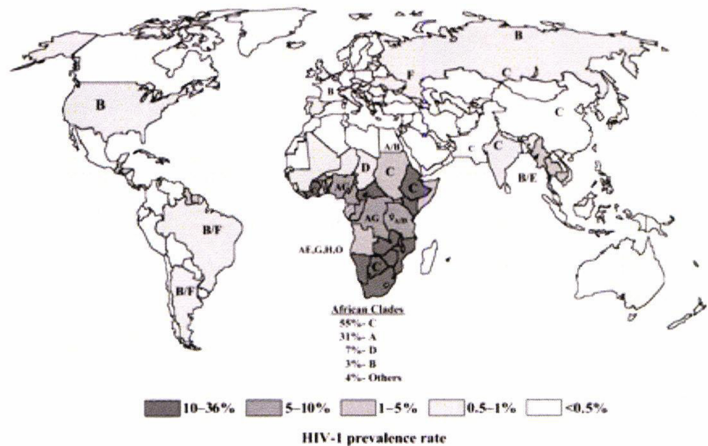


Figure 3. Geographic distribution of HIV clades

Although there are several diverse clades and strains throughout the world, the overall morphology, structure, and function of HIV remain relatively the same. The outer structure of HIV is comprised of a lipid envelope taken from the host cell interspersed with its viral receptor [39]. Inside the membrane is a viral protein matrix that stabilizes the structure [40]. Within the matrix is a conical capsid that protects and contains the viral genome and several important

enzymes [41]. Inside the capsid lies a diploid single-stranded RNA genome as well as several important enzymes and proteins such as reverse transcriptase, integrase, and proteases which ensure that the RNA is transcribed to DNA, the DNA is integrated into the host genome, and viral gene products are cleaved to make all necessary proteins, respectively [42]. A brief HIV anatomy pictorial can be found in Figure 4.

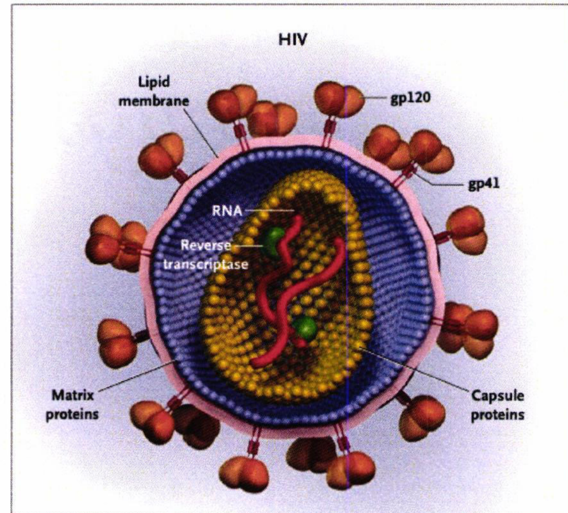


Figure 4. HIV anatomical overview.

http://allscienceconsidered.files.wordpres.com/2009/11/hiv.png

Although HIV contains 19 proteins, its genome only consists of 9 genes [43]. The gag protein is cleaved into four smaller proteins, p17, p24, p7, and p6, which are responsible for the structural integrity of the virus [44]. Pol, in conjunction with gag, produces the reverse transcriptase, viral proteases, integrase, and RNAse [45]. Env produces the viral glycoproteins gp120 and gp41 which make up the viral receptor, and are responsible for cell invasion [46]. Tat is one of two vital regulatory proteins and is a transactivator of HIV gene expression [47,48]. Rev is the second of the regulatory proteins and promotes nuclear export, stabilization, and utilization of viral mRNAs [48]. Vif sabotages a host antiviral protein APOBEC-3G which mutates viral nucleic acids [48]. Vpr regulates the importation of the viral pre-integration complex (PIC), a nucleoprotein complex containing the viral genome and associated viral and host proteins [49]. Vpu degrades host receptor CD4 production and enhances viral release [50]. Nef promotes T-cell activation and downregulates the major histocompatibility complex (MHC) proteins [51].

HIV utilizes gp120 to invade the host cell. gp120 first docks with the host via the membrane bound cellular CD4 receptor [46]. This attachment of gp120 to CD4 allows for a conformational shift of gp120 that exposes critical residues of gp120 to a chemokine coreceptor, CCR5 or CXCR4, on the host's membrane which initiates binding of gp120 to the coreceptor [52-54]. This then allows gp41 to penetrate the host membrane and subsequently break down into a hair loop structure which brings the host and viral membranes into close proximity [55,56]. This ultimately fuses the membranes together releasing the PIC inside the host [57,58].

After invasion, the PIC is transported down a system of microtubules via the host protein dynein to the nucleus [59,60]. During transport, reverse transcriptase begins to transcribe its single-stranded viral RNA into double-stranded DNA. Once at the nucleus, nuclear import signals contained within the PIC allow for infiltration into the nucleus. Subsequently, the viral protein integrase inserts the viral DNA into the host genome [61].

After integration, viral genomic RNA and mRNA are transcribed. The mRNAs are then translated into proteins, which then undergo numerous modifications (i.e. cleavage, glycosylation) and assemble with the genomic RNA at the host cell plasma membrane [62]. Once assembly has taken place, the virus buds from the cell and incorporates the host cell plasma membrane into its structure. After budding, the virion matures when the proteases cleave the polyproteins into functional proteins and enzymes. An overview of the HIV lifecycle can be found in Figure 5.

HIV's rapid mutability is due to the highly error-prone nature of reverse transcriptase. Reverse transcriptase has no proofreading ability which leads to base substitution errors at a frequency of 1 in 2000 to 1 in 4000 [63]. Mutation can also be caused by recombination of viral strains through viral superinfection [64,65]. These mutations can lead to

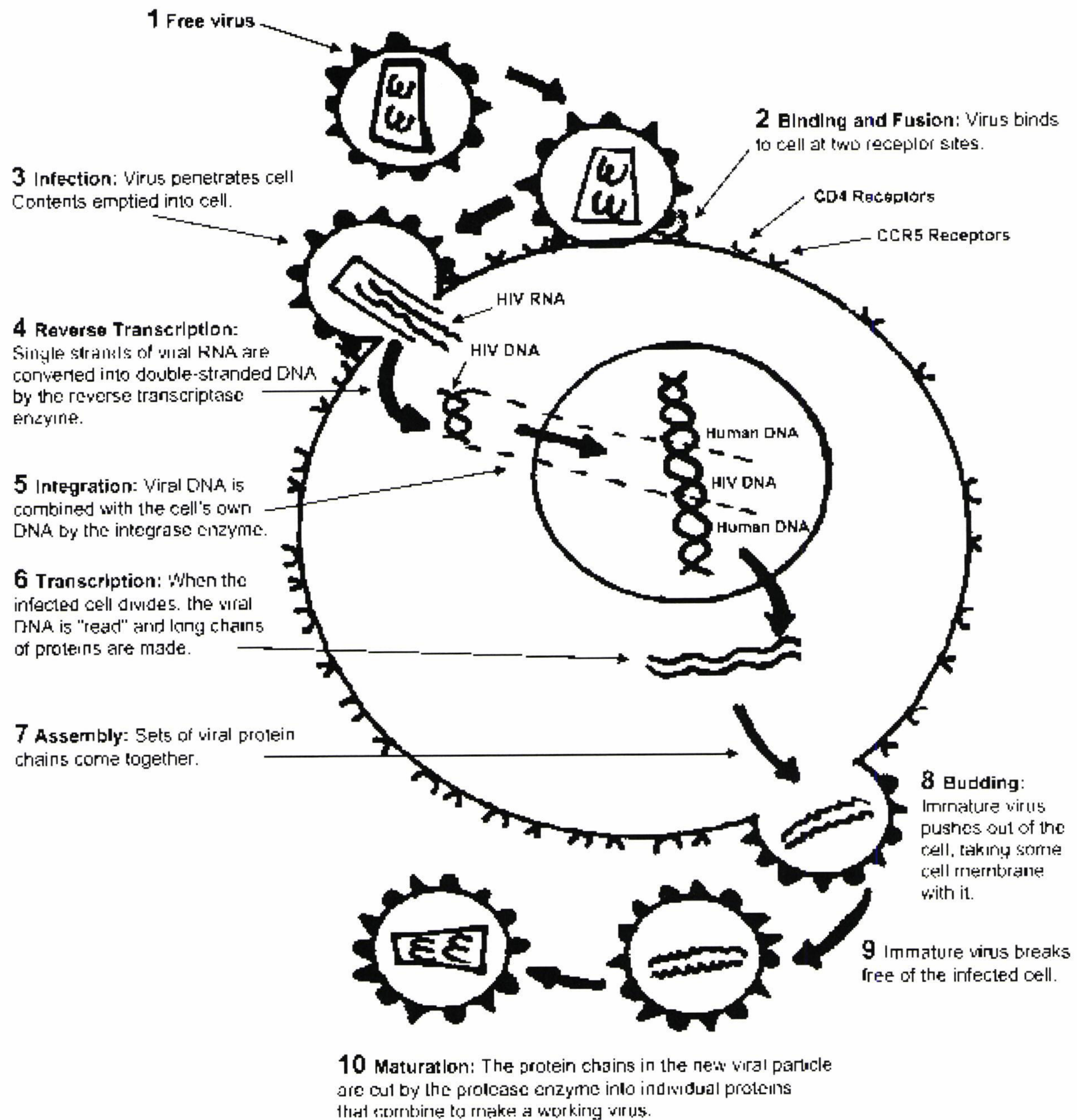


Figure 5. HIV lifecycle overview.

http://aids.about.com/blcycle.htm

drug resistance of HIV [35]. HAART medications are used to suppress viral loads, but approximately 10% of infected individuals are resistant to at least one antiretroviral medication [66], and up to half of patients report therapeutic failure after two to three years [67]. HIV cannot be eliminated completely, even though HAART can suppress HIV to incredibly low levels. This is because cell reservoirs such as peripheral-blood mononuclear cells continue to produce HIV despite antiretroviral treatment [68].

In the absence of antiretroviral treatment, the mean time to the development of AIDS is 7.7-11 years depending on age [69]. But, there is tremendous inter-individual difference in susceptibility, progression, and outcome of HIV. Several categories have arisen to describe these individuals. Rapid progressors are those individuals that acquire HIV and progress to AIDS fairly rapidly. Research has shown that these individuals progress to AIDS in approximately three years after HIV infection [70]. On the opposite end of the spectrum are long-term nonprogressors (LTNPs). Even in the absence of antiretroviral medication, these individuals remain clinically and immunologically stable and progress much more slowly to AIDS than other infected individuals [71,72]. There is also a very rare set of individuals known as HIV controllers [73]. These individuals can suppress HIV to low (<2,000 RNA copies/mL) and even undetectable (<50-75 RNA copies/mL) levels without any antiretroviral medications. Highly exposed, persistently seronegative individuals are continuously exposed to HIV, yet remain uninfected [74]. There are even highly rare individuals that are immune to HIV. These people have a 32 base pair deletion in their CCR5 co-receptor gene that codes for a defective CCR5 co-receptor and prevents HIV from initially infecting cells [75]. The identification of these different groups suggests that host genetic factors influence HIV susceptibility, progression, and outcome. Several gene variants have been associated with susceptibility, progression, and outcome

including HLA and CCR5 [76], but the variants identified thus far only account for approximately 15% of the variance observed in viral load and progression [77]. Thus, there are more genes that contribute to the variance that have yet to be identified.

Section 3: HIV and the Immune System

The immune system consists of numerous types of cells working together to fight against pathogens, but HIV is a complex Trojan horse that targets and destroys our immune system for its own propagation. HIV can target several cell types including monocytes/macrophages, microglial cells, and dendritic cells [78-80]. However, it is HIV's targeting and destruction of CD4+ T-cells that leads to the severe loss in immune function and eventually to the development of AIDS [21,22].

During the early stages of HIV infection, viral replication is already quite widespread in the lymphatic tissue [81,82]. During the initial infection, there is a large increase in viremia with several million virions per milliliter of blood [83] as well as a steep decline in the number of CD4+ T-cells [84]. This is followed by a strong HIV-1 specific activation of cytotoxic CD8 T-lymphocytes (CTL) and a suppression of viremia [84,85]. Virions are trapped in the lymphoid tissue by a follicular dendritic cell (FDC) network which allows for infection of macrophages and CD4+ T-cells and the establishment for permanent viral reservoirs [86,87]. The lymphoid tissue acts as the viral reservoir and principle site of HIV replication during the course of infection with HIV expression considerably higher in the lymphoid tissue compared to peripheral blood [87].

After viral invasion and reverse transcription in quiescent CD4+ T-cells, the viral DNA remains unintegrated into the host genome [88] and requires activation of the cell for integration

as well as for production of new virions [89]. This makes the lymphoid tissue an extremely favorable place for activation for several reasons. There is very close cell to cell contact between CD4+ T-cells and antigen-presenting cells (APC), virions on the surface of FDCs [86,87], and an abundance of proinflammatory cytokines such as IL-1, IL-6, and TNF-α which promotes activation and subsequent production of viral replication [90]. Activation and replication leads to infection of surrounding CD4+ T-cells and spreading throughout the lymphoid tissue. From this point, HIV is able to spread throughout the body from its viral reservoir. As HIV progresses, the immune system breaks down, and the host becomes more susceptible to opportunistic infections and eventually leads to the death of the host.

Although the immune system provides several layers of protection against invading pathogens, HIV is able to circumvent these mechanisms in various ways to ensure its survival. This not only has devastating effects on the CD4+ T-cell population but also has serious consequences for other immune cells. While HIV employs its own immune evasion strategies, host genetic and immunological variations have their own tactics to combat the infection and slow HIV progression.

Dendritic cells (DCs) are a major source of APCs and are primarily the first type of cell to encounter HIV [91]. DCs can capture HIV on their surfaces, and when DCs and CD4+ T-cells interact during antigen-specific stimulation, DCs can then transmit the virus from their surfaces to the CD4+ T-cell [92]. Early in infection DC numbers drop significantly and have impaired function [93].

Macrophages are another source of APCs and are a main target for HIV infection. Macrophages can serve as long lasting reservoirs for HIV replication [94]. Within these cells, viral replication can take place at the plasma membrane as well as within intracytoplasmic

vacuoles [95,96], with replication in the vacuoles being the preferred method in chronically infected macrophages. In HIV-infected individuals, macrophages display several deficiencies that include decreased chemotaxis, Fc receptor function, and oxidative burst response [97,98].

The generation of an HIV-specific immune response is dependent upon antigen presentation by the human leukocyte antigen (HLA) molecule to other immune cells. HLA class I molecules are present on all nucleated cells and present antigens to CTLs. HLA class II molecules are present APCs and present antigens to CD4+ T-cells. HLA on APCs could potentially present antigens differentially and therefore activate CTLs in a favorable or unfavorable manner and affect the progression of HIV. Several studies have shown that particular HLA alleles are predictive of slower HIV progression and HIV control [99,100] but particular alleles have also been shown to be disadvantageous to the host and associated with rapid progression of HIV [101]. Specifically, HLA-B57 variants, predominantly B5701 and B5703, are strongly associated with the control of HIV-1 infection [102,103] while B35-Px is associated with accelerated disease progression due to its decreased function in binding HIV-1 peptides and activating a CTL response [104].

CTLs are able to identify and kill HIV-infected cells [84,85]. Blood, lung, and cerebrospinal fluid have all been shown to harbor HIV-specific CTL responses [105] and these responses have been shown to be against both structural and regulatory proteins of HIV [106,107]. The CTL response is most likely responsible for the suppression of HIV in initial infection [84,85]. While the HIV-specific CTL response is constant, it does diminish over time [108]. CTLs can be activated from a memory state or from a naïve state, however activation requires CD4+ T-cells to secrete cytokines to activate CTLs from their naïve state [109]. HIV can avoid the cytotoxic effects of CTLs in several ways. In a process termed fugetaxis, HIV

gp120-CXCR4 binding actively induces the release of proteins that repel CTLs [110], and can downregulate MHC molecules in infected cells [51]. HIV can also infect CTLs if they express CD4 [111].

While the HIV-specific CTL response is thought to be a dominant factor in HIV progression and control, this remains a point of debate as the mechanism of protection remains unknown. For example, there is no known association between control and the number of HIV-specific CTLs [112,113]. CTL protection may have more to do with quality rather than quantity. HIV controllers have a much greater ability to produce numerous cytokines and perforin [114,115] that enhance the immune response and killing of HIV-infected cells. Likewise, the ability of CTLs to proliferate upon HIV antigen stimulation has been linked to HIV nonprogressors [116]. The increase in performance of HIV-specific CTLs is possibly due to optimal presentation of preferential HLA alleles that can present a larger number of HIV peptides, create a larger CTL response, and target highly conserved proteins [117-119].

B cells are a major part of the humoral arm of the immune system and produce anti-HIV antibodies in response to HIV infection. B cells start to produce anti-HIV antibodies when stimulated by CD4+ T-cells [120], and a humoral response can be usually be detected within two weeks [121] but can take up to six months [122]. During HIV infection, B cells become negatively impacted despite not being an infectable target for HIV. B cells undergo hyperactivation [123], increased spontaneous Ig secretion [124], and increased susceptibility to apoptosis [125].

Broadly acting neutralizing antibodies are quite rare among HIV-1 infected individuals and could be due to the fact that the predominant target epitopes of Env and Gag are poorly immunogenic [126]. Neutralizing antibody levels tend to peak towards the end of HIV infection

in LTNPs, whereas regular progressors lose their neutralizing antibody response over the course of their infection [127,128]. A groundbreaking study recently identified a very powerful neutralizing antibody that is able to neutralize approximately 90% of the HIV isolates tested [129].

Natural killer (NK) cells represent part of our innate immune system. Nomenclature was based upon observations of a cell population that was naturally reactive and able to lyse tumor cells without sensitization to them [130]. These cells are highly cytotoxic and can be activated through various cytokines and through their Fc receptors binding of antibodies. Soon after HIV infection, NK cell numbers increase, but then fall back to baseline levels soon after. While NK cells have the potential to kill HIV infected cells, they experience severe dysfunction even in the early stages of infection [131]. The drop in cytotoxic activity is likely due to decreased perforin expression [132].

NK cells in LTNPs tend to produce higher levels of interferon gamma than do regular progressors [133]. A polymorphic receptor on NK cells known as the killer-cell immunoglobulin-like receptor (KIR) in combination with HLA-B alleles appear to have beneficial protective effects in HIV progression [134]. In particular a KIR variant KIR3DLI in combination with HLA-B57 is thought to facilitate a strong cytotoxic response by activating developing NK cells [134].

Another imperative component to the innate immune system are neutrophils. These cells have short life spans but have very potent anti-pathogenic qualities. Neutrophils have the ability to phagocytize microbes and other particles and release powerful enzymes through a process known as degranulation, which destroys infected cells or other microorganisms. As with other immune cells, HIV has a negative impact on neutrophils. Even though neutrophils are not

infected by HIV, they still display defects in chemotaxis, bacterial killing, and phagocytosis in HIV-infected individuals [135].

CD4+ T-cells are a critical component in adaptive immunity. These cells regulate other immune cells in the fight against disease. They are the main target of HIV and their destruction is the primary cause of AIDS. HIV can cause cell death of CD4+ T-cells in several ways, both direct and indirect. CTLs can destroy HIV-infected cells [84,85], but direct infection by HIV can cause cell death through several other distinct mechanisms. After infection, continuous viral budding can disrupt the integrity of the cell membrane causing cell death [136]. An infected cell can fuse together with other non-infected CD4+ T-cells to form large multinucleated cell complexes known as syncytium that allows for HIV to deplete many cells from a single cell infection [137]. Direct infection of HIV can also induce apoptosis as it can inhibit anti-apoptotic protein Bcl-2 and activate pro-apoptotic protein caspase 8 [138,139]. Indirect killing of cells is caused by a few known mechanisms. Various free-floating HIV proteins can interact with cellular proteins and receptors which induce apoptosis [140,141]. HIV can also cause CD4+ T-cells to be indirectly depleted by activation-induced cell death [142] which is a mechanism by which CD4+ T-cells self-terminate to avoid prolonged immune activation.

CD4+ T-cells have shown conflicting results as far as their variability in relation to progression and control of HIV. For instance, a strong polyclonal Gag-specific response by CD4+ T-cells which is maintained at high levels throughout the duration of infection is correlated with some LTNPs [143]. On the other hand, some LTNPs show reduced CD4+ T-cell activation [144] which effectively reduces the number of cells that are at risk of infection to HIV thus slowing the progression of the disease. LTNPs have shown to have a high elevation in IL-7 and the IL-7 receptor on CD4+ T-cells and maintain phenotypically naïve CD4+ T-cells [145].

As illustrated, HIV has potent mechanisms to avoid and disrupt the immune system that allows for extensive replication and propagation of the virus. But, variations in host genetics have been shown to slow and control the progression of HIV. These variations are very important as they elucidate ways in which the body can fight HIV and may lead to novel therapeutics and vaccine opportunities in the future. However, the genetic variants identified to date only accounts for 15% of the variance seen in viral load and progression [77]. This means that there are other genes that play a role in progression that need to be investigated and accounted for.

Section 4: FcγRs and GM Allotypes

The main purpose of the immune system is to identify and eradicate invading pathogens. The immune system fights off these pathogens through two distinct and interconnected systems: innate and adaptive immunity. Innate immunity is the first line of defense and provides immediate protection to the host. It supplies a quick and general response to pathogens through innate immune cell activation, inflammation, and complement. Since the innate immune system largely does not retain a memory capability, this response does not provide long-term protection against particular antigens. Adaptive immunity on the other hand does have the ability to remember and recognize antigens which it then uses to generate specific attacks against the invading pathogen. The adaptive response can be activated by APCs from the innate immune response or by other infected cells presenting foreign antigens. These responses are primarily mediated through the various classes of T-cells as well as B cells. The humoral arm of adaptive immunity is composed of circulating immunoglobulins secreted by terminally differentiated B cells also known as plasma cells. These antibodies contain a variable region that are specific for

a particular antigen and are capable of binding this antigen once it is encountered. Antibodies also contain a constant region which can be a significant source of interaction with effector cells or complement. The majority of innate immune cells contain receptors that are capable of binding the Fc portion of the constant region of antibodies. Receptors that bind the Fc portion of IgG are known as Fc gamma receptors (FcγRs). Upon binding, several possible immune responses can take place including phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC). Thus, antibodies provide a bridge between the innate and adaptive immune responses.

There are six known FcγRs in humans: FcγRI, FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa, and FcγRIIIb. These are all encoded by genes on chromosome 1. These genes are thought to have come about by gene duplication and subsequent gene mutation as they contain a high degree of homology [146,147]. The cytoplasmic domains of the FcγRs show a high degree of variability whereas variability in the extracellular domain tends to be minimized with the exception of FcγRI which contains an extra immunoglobulin domain.

These variations give rise to the unique signaling and binding characteristics that are observed for each of the FcγRs. For instance, of the FcγRs, FcγRIIb is inhibitory and is used to counterbalance the activatory response of the other receptors through its immunoreceptor tyrosine-based inhibitory motif in the cytoplasmic domain [148]. In contrast, FcγRIIa and FcγRIIc activate immune response through their immunoreceptor tyrosine-based activatory motifs [148]. As for binding, FcγRI is the only high affinity receptor of the FcγRs and contains an extra immunoglobulin domain in its extracellular domain [148,149]. The other receptors contain only two immunoglobulin domains and are considered low affinity receptors [148]. While all of the FcγRs can avidly bind to complexed IgG, only FcγRI (and FcγRIIIa to a lesser

extent) can bind to monomeric IgG [150]. Individual FcγRs also show a high specificity and affinity for particular IgG subclasses [150]. There are also functional polymorphisms among particular FcγRs that give rise to differential binding affinities between alleles for the various IgG subclasses [150].

FcγRIIa is located on numerous effector cells including monocytes, macrophages, neutrophils, dendritic cells, and platelets. FcγRIIa is polymorphic at position 131 which codes for either histidine (H) or arginine (R) due to an A to G nucleotide substitution [151]. This amino acid change causes a minor differential in binding affinity for IgG1 [150], but a much larger differential for IgG2 [150-152] with histidine being the higher affinity allele. The differential in binding between the two alleles could affect the degree of the immunological response as lower affinity binding would lead to a loss in immunological activation of the effector cell while enhanced binding would lead to a rise in activation. These alleles are associated in a variety of diseases including lymphoma [153], malaria [154], and systemic lupus erythematosus [155]. FcγRIIa alleles have also been shown to be influential in the progression of HIV as those individuals who are FcγRIIa-131R/R progress faster to a low CD4+ T-cell count [156].

FcγRIIIa has been found on several effector cells but is primarily contained on NK cells. FcγRIIIa is allelic at position 158 which codes for either a valine (V) or a phenylalanine (F) due to a G to T nucleotide substitution [157]. This amino acid change causes a differential in binding affinity for all of the IgG subclasses [150] with valine being the higher affinity allele. The differential in binding between the two alleles could affect the activation and immunological response by NK cells as they there would be poorer binding and activation caused by the phenylalanine allele but higher binding and activation caused by the valine allele. These alleles

have been associated in a variety of diseases including inflammatory bowel disease [158] and rheumatoid arthritis [159]. But, FcγRIIIa alleles have shown conflicting results with regards to its influence on HIV progression [156,160].

This conflict could arise from the fact that other immunological factors have not been taken into account. FcγRs do not independently and spuriously activate an immune response in the presence of an antigen. An immunological response, such as ADCC, is induced upon FcγR binding to the Fc region of an antigen-bound IgG molecule. All of the FcγRs including their polymorphisms have shown a differential in binding to the various IgG subclasses [150]. What has not been considered is that the constant regions of the IgG subclasses are highly polymorphic and could potentially influence FcγR-Fc binding affinity and thus influence the strength of the immunological response.

IgG molecules are heterodimers that consist of four polypeptide chains: two heavy chains and two light chains which are linked by disulfide bridges. Structurally, IgG can be broken down into three domains: the Fab, the hinge, and the Fc. The Fab domain is responsible for antigen binding which takes place in the variable region. The hinge region is the amino acid sequence between the Fab and Fc domains. It contains considerable variability between IgG subclasses and determines the flexibility of the IgG molecule. The Fc region is responsible for FcγR and complement binding for the induction of an immune response.

IgG heavy and light chains can also be broken down into its variable and constant regions. The structure of the variable region determines its ability to bind specific antigens. The constant region of the heavy chain is distinctive to each subclass and exclusively comprises the hinge and Fc regions while contributing a section to the Fab domain as well. The heavy chain constant region is highly polymorphic among the IgG subclasses which gives rise to several

antigenic determinants known as GM. GM allotypes are inherited in a Mendelian fashion and are expressed as autosomal codominant genes [161]. These genes ($\gamma 1$, $\gamma 2$, and $\gamma 3$ corresponding to the heavy chains of IgG1, 2, and 3) are closely linked on chromosome 14 and have a linkage disequilibrium that is fairly absolute with recombination events happening on an evolutionary scale. These markers are transmitted in defined groups known as haplotypes and are race specific. Currently there are 18 known allotypes contained within the IgG subclasses with 4 being on IgG1, 1 on IgG2, 13 on IgG3, and none being discovered so far on IgG4.

GM allotypes have been associated with several diseases and pathogens including autoimmune diseases [162-164], multiple sclerosis [165], bacterial infections [166], and hepatitis C virus [167]. However, GM allotypes have not been investigated for their influence on HIV progression. Nearly all of the GM allotypes are contained within the Fc portion of IgG, and since attachment of the Fc receptor to the Fc region of the antibody is essential to eliciting an immune response, GM allotypes could influence Fc-FcγR binding. As it has been shown that FcγRIIa and FcγRIIIa variants have differential binding affinities for the various IgG subclasses [150], and that these variants influence HIV progression [156,160], it would be of great interest to investigate how GM allotypes, individually or epistatically with FcγRIIa and FcγRIIIa variants, influence HIV progression. Determination of common genotypes in differentially progressing groups could lead to new avenues of viewing and fighting HIV infection.

As mentioned previously, vaccine trials to date have shown a myriad of results ranging from potentially harmful [30] to modestly protective [31]. In one failed vaccine trial (AIDSVAX), participants were vaccinated with recombinant gp120 (rgp120). Even though the vaccine failed to reduce the HIV infection rate of participants, it did elicit a variety of immune responses. These responses, in particular to gp120, have gained renewed interest due to the

modest protection afforded in the Thai trial in which a combination of AIDSVAX and a recombinant canarypox vaccine (ALVAC) expressing several HIV proteins were used. One recent genome-wide study examined genetic factors that were associated with AIDSVAX-induced gp120-specific IgG2 response among the vaccine recipients. The study concluded that GM23 was significantly associated ($p = 8.5 \times 10^{-19}$) with gp120-specific IgG2 titers [168]. Efficient IgG2 responses to polysaccharides [166,169] and IgG2 levels have been associated with GM23 [170], and since HIV gp120 is highly glycosylated with carbohydrates, GM23 could influence HIV outcome and progression. HIV-1 Env and Gag specific IgG2 antibodies have been correlated with control of HIV [171,172], but the mechanism of protection remains unknown. Although IgG2 is thought to induce ADCC weakly, it has clearly been shown that anti-gp41 IgG2 antibodies can induce an ADCC response against HIV [173]. Thus, GM23 may be able to influence IgG2-mediated ADCC of HIV and at least in part explain the protection and control afforded by IgG2 antibodies.

Significance

Presently, there is no vaccine available for HIV. While the Thai trial provided a modest protection against HIV, there appears to be no vaccine on the horizon. As there are no medications approved for preventive maintenance against HIV, current therapies for mitigating HIV infection rates include education and safe sex practices. Those that do acquire HIV have little recourse in their choices against the virus. HAART, while effective, is expensive and only delays the progression of HIV while at the same time potentially causing serious side effects in some. Yet, HIV controllers and LTNPs have shown remarkable resilience against HIV even in the absence of HAART. This suggests that host genes play a role in the outcome and

progression of the disease. Thus, it is vital to explore the genes and mechanisms that are responsible for the control and slower progression of the disease. Studies have implicated FcγRIIa and FcγRIIIa genotypes in HIV progression. While these receptors' genotypic variation has been taken into consideration, the variance found in the receptors' ligand, the Fc of IgG, has not. It begs the question then that if variance found in the receptor can influence progression, why not its ligand? GM markers remain uninvestigated in control and progression of HIV. Studies into these allotypes in combination with FcγRIIa and FcγRIIIa genes could further shed light on the genetic influence of immune responses that could potentially contribute to the control and progression of HIV. Results from these studies could be used to improve potential therapeutics and vaccine designs against HIV.

Hypothesis

I hypothesize that GM alleles, individually or epistatically with particular FcγRIIa and FcγRIIIa alleles, contribute to the control and rate of progression of HIV infection, and that the underlying mechanisms include anti-gp120 mediated ADCC of HIV infected cells.

Specific Aims

Specific Aim 1: Determine if GM allotypes, individually or together with FcγRIIa or FcγRIIIa variants are associated with control and progression of HIV infection.

Specific Aim 2: Determine if anti-gp120 mediated ADCC of HIV infected cells is influenced by GM23 and FcγRIIa variants.

Chapter 2: FcγRIIa, FcγRIIIa, GM, and KM in the Control of HIV Replication

Introduction

HIV infection affects approximately 33.3 million people worldwide and is characterized by severe loss of CD4+ T-cells over several years leading AIDS. Without HAART, the mean time to the development of AIDS for an infected individual is 7.7-11 years after initial seroconversion depending on age [69]. Yet, there are remarkable differences in progression and outcome of the infection for some individuals. For instance, some highly exposed, persistently seronegative individuals remain uninfected even after repeated exposures to HIV [74], whereas rapid progressors develop AIDS within 4 years after seroconversion [70]. Other very rare individuals are able to suppress HIV to low and sometimes undetectable levels [73]. This suggests that host genetic factors influence the outcome of HIV infection. Several gene variants have been associated with susceptibility, progression, and outcome, including HLA and CCR5 [76], but they account for approximately only 15% of the variance observed in viral load and progression [77]. This suggests that there might be additional genes that contribute to this variance that have yet to be identified.

ADCC is a prominent mechanism underlying the protection provided by anti-HIV antibodies [174,175]. ADCC is triggered upon ligation of FcγR to the Fc of IgG molecules. It follows that genetic variation in FcγR and Fc—where the majority of the GM determinants are located—could contribute to the interindividual differences in ADCC, resulting in differential host control of HIV replication. Loci encoding GM and FcγR markers are located on chromosomes 14 and 1, respectively. FcγRIIa and FcγRIIIa genotypes have been shown to

influence HIV susceptibility and disease progression [156,160,176], but the role of GM determinants in HIV virologic control has not been investigated. Similarly, the role of KM allotypes (encoded by a gene on chromosome 2), which are associated with susceptibility to many infectious diseases [167,177-179], in the outcome of HIV infection has not been investigated.

Genes do not act in isolation: there is growing body of evidence that epistasis—modification of the action of a gene by one or more other genes—plays a significant role in determining the rate of progression to AIDS [180]. Determinants expressed on Fc (GM) and FcγR are probably some of the most likely ligand-receptor candidate pairs for gene-gene interactions in the human genome. Thus, the aim of this investigation was to determine whether particular GM, KM, FcγRIIa, and FcγRIIIa genotypes were individually or epistatically associated with the host control of HIV replication.

Materials and Methods

Study patients

Informed consent was obtained from study subjects enrolled in the Study of the Consequences of Protease Inhibitor Era (SCOPE) cohort at the University of California, San Francisco. The study protocol was approved by the Institutional Review Board for human research at respective institutions. Blood was collected from a total of 73 HIV-infected controllers (40 Caucasian Americans, 33 African Americans) and 100 HIV-infected non-controllers (74 Caucasian Americans, 26 African Americans). Controllers were classified as those individuals that were chronically infected for more than one year, naïve to antiretroviral therapy, and had three documented plasma HIV RNA levels < 2,000 copies/mL over at least a

12-month period (http://cfar.ucsf.edu/pdf/cores/scope-criteria-11-08-2010.pdf). Non-controllers were classified as those individuals that were chronically infected for more than one year, had no antiretroviral therapy for one year preceding sample collection, and had HIV RNA levels > 10,000 copies/mL.

GM, KM, and FcγR Genotyping

DNA was isolated from non-viable PBMC pellets using Qiagen's Blood Core Kit per manufacturer's protocol.

IgG1 allelic markers GM3 and GM17 (arginine to lysine substitution, a G→A transition in the CH1 region of the γ1 gene) were determined by direct DNA sequencing. PCR was used to amplify the CH1 region of the γ1 gene using the following primers: 5'-CCCCTGGCACCCTCCTCCAA-3' and 5'-GCCCTGGACTGGGGCTGCAT-3' [181]. The double-stranded 364 bp DNA product was then purified and sequenced on an ABI Prism 377.

IgG2 allelic marker GM23 (valine-to-methionine substitution, a G→A transition in the CH2 region of the γ2 gene) was determined using a nested PCR-RFLP method. A 915 bp fragment that includes the polymorphic site was amplified using the following primers: 5'-AAATGTTGTGTCGAGTGCCC-3' and 5'-GGCTTGCCGGCCGTGGCAC-3' [182]. A 197 bp fragment was then amplified from the 915 bp fragment using the following primers: 5'-GCACCACCTGTGGCAGGACC-3'and 5'-TTGAACTGCTCCTCCCGTGG-3'. The 197 bp product was digested by the restriction enzyme NIaIII. This resulted in the following product sizes for each genotype: GM23(+/+), 90 bp, 63 bp, and 44 bp; GM23(-/-), 134 bp and 63 bp; and GM23(+/-), 134 bp, 90 bp, 63 bp, and 44 bp.

IgG3 hetero-allelic markers GM5 (asparagine-to-serine substitution, a AT→GC transition in the CH3 region of the γ3 gene) and GM21 (proline-to-leucine substitution, a C→T transition in the CH2 region of the γ3 gene) were determined using a PCR-RFLP method. A 765 bp fragment that includes the polymorphic sites was amplified using the following primers: 5'-CTGAACTCCTGGCAGGACCGT-3' and 5'-GCTTGCCGGCTATCGCACTC-3'. A 685 bp fragment was then amplified from the 765 bp fragment using the following primers: 5'-GCACCACCTGTGGCAGGACC-3'and 5'-TTGAACTGCTCCTCCCGTGG-3' [183]. The 685 bp product was digested by the restriction enzyme MspAI1. This resulted in the following product sizes for each genotype: GM5/5, 171 bp, 158 bp, and 156 bp, 137 bp, and 63 bp; GM21/21, 327 bp, 295 bp, and 63 bp; and GM5/21, 327 bp, 295 bp, 171 bp, and 158 bp, 156 bp, 137 bp, and 63 bp.

The κ-chain is triallelic—KM1, KM1,2, and KM3 alleles. KM1 allele is rare; 98% of the individuals positive for KM1 are also positive for KM2. Thus, positivity for KM1 includes both KM1 and KM1,2 alleles. The KM alleles were determined by a previously described PCR-RFLP method [184].

A change in the nucleotide at position 497 of FcγRIIa gene from A to G results in change of amino acid histidine to arginine (H/R131). A 366 bp fragment amplified using the following primers: 5'-GGAAAATCCCAGAAATTCTCGC-3'and 5'-CAACAGCCTGACTACCTATTACGCGGG -3' [185]. The 366 bp product was digested by the restriction enzyme BstU1. This resulted in the following product sizes for each genotype: FcγRIIa H/H: 343 bp and 23 bp; FcγRIIa R/R: 322 bp, 23 bp, and 21 bp; FcγRIIa H/R: 343 bp, 322 bp, 23 bp, and 21 bp.

A change in the nucleotide at position 559 of the FcγRIIIa gene from T to G results in phenylalanine to valine substitution (F/V158) in the membrane proximal IgG like domain of FcγRIIIa. The FcγRIIIa alleles were determined by the TaqMan® SNP Genotyping Assays supplied by Applied Biosystems, following manufacturer's protocols.

Due to technical reasons, certain samples were not typed for certain genotypes causing slight variations in the sample number for each genotype.

Statistical Analysis

Fisher's exact tests were used to determine the significance of the genotype frequency differences between controllers and non-controllers. Dominant and recessive tests of the genetic effects were constructed; however, due to low minor allele frequencies some models were not explored. All interaction effects were tested in logistic regression models that also allowed for main effects of the genes. Interaction tests were constructed as 1 degree of freedom tests, assuming either recessive or dominant genetic effects of each genetic marker. All tests were two-tailed with an $\alpha = 0.05$ level. Due to varying genotype frequencies across European ancestry and African ancestry populations, tests of differences in genotype frequencies and gene-gene interactions were conducted separately for Caucasian and African American subjects in the cohort. In total main effects of six loci and eight possible interactions between the four GM/KM loci and two FcγR loci were explored. The p values for the associations were not adjusted by Bonferroni's method. Such adjustment is controversial and we believe that, instead of performing such adjustment in this work, the best approach would be to test in an independent sample.

Results

The distribution of GM, KM, and FcγR genotypes in HIV controllers and non-controllers is given in Table 1. None of the genotype frequencies by itself differed significantly between the two groups. However, certain combinations of FcγR and GM genotypes were differentially distributed between the two groups of Caucasian Americans (Table 2). Among FcγRIIa R non-carriers (i.e. H/H homozygotes), GM21 non-carriers (i.e. GM5 homozygotes) had over seven-fold greater odds of being controllers than the carriers of this allele (OR = 7.47; $p = 0.0214$). Testing epistasis between FcγRIIa and GM21 in a logistic regression model, the interaction was statistically significant ($p = 0.0255$). These GM determinants also interacted with FcγRIIIa alleles. Among the carriers of the FcγRIIIa V allele, GM21 non-carriers had over three-fold greater odds of being controllers than the carriers of this allele (OR = 3.26; $p = 0.0495$). Testing epistasis between FcγRIIIa and GM21 in a logistic regression model, the interaction was trending towards statistical significance ($p = 0.0503$). Among FcγRIIa R-carriers and FcγRIIIa V non-carriers, the GM21 genotype frequencies between controllers and non-controllers were not significantly different ($p = 0.817$, 0.560, respectively). No significant associations were observed in African American subjects.

Discussion

The results reported here show significant interactive effects of particular FcγR and GM genotypes on the host control of HIV replication. A plausible mechanism underlying this association could involve epistatic contribution of these loci to the ADCC of HIV-infected cells or to the antibody-dependent cell-mediated virus inhibition. The interacting FcγRIIa and FcγRIIIa alleles—H and V, respectively—are high affinity alleles, that is, they bind the Fc

region of IgG antibodies better than their allelic counterparts [186,187]. Alleles at both loci have been shown to be risk factors for HIV infection and progression, but no consistent pattern has emerged [156,160,188], which could be due to the fact that all studies thus far have examined the genes encoding the receptors, but not those coding for their ligand (Fc/GM). Results reported here underscore the importance of simultaneously examining the ligand (GM) and the receptor (FcγR) genes for their possible contribution to the host control of HIV replication. It is possible that GM5-expressing anti-HIV IgG antibodies have higher affinity for the FcγRIIa H and FcγRIIIa V alleles, which could enhance the magnitude of ADCC against HIV-infected cells, leading to a better control of HIV replication. This is analogous to the reported allelic interaction between particular killer cell immunoglobulin-like receptors and their HLA-C ligand in the resolution of hepatitis C virus infection [189].

We did not find an interactive effect of GM and FcγR alleles on the control of HIV replication in the African American cohort. The reasons for these racial differences are not clear. The inability to detect an association in this group could be due to its relatively small size. Alternatively, GM and FcγR alleles could interact with the allelic determinants of another as-yet-undetermined gene whose frequencies are different in the two populations. Such racial disparities in genetic associations are not unprecedented.

If GM and FcγR genes contribute to the host control of HIV replication, as suggested by the results presented here, why have they not been detected by the genome-wide association studies (GWAS) of HIV control? One likely reason is the absence of GM genes in the HapMap panel [190]. IgG gene segments harboring GM genes are highly homologous and apparently not amenable to the high throughput genotyping technology used in GWAS. Another contributing factor might be the inability of most GWAS to detect epistatic interactions. Therefore, a

candidate gene approach involving a large sample size would be necessary to confirm and extend the findings from this study.

Table 1. Distribution of GM, KM, and FcγR genotypes among HIV controllers and non-controllers

	Caucasian Americans				African Americans			
Genotypes	Controllers		Non-Controllers		Controllers		Non-Controllers	
	n	%	n	%	n	%	n	%
GM3/3	18	45.0	27	36.5	2	6.1	1	3.8
GM3/17	17	42.5	38	51.4	7	21.2	6	23.1
GM17/17	5	12.5	9	12.2	24	72.7	19	73.1
GM23(+/+)	7	17.5	14	18.9	2	6.1	1	3.8
GM23(+/-)	18	45.0	29	39.2	3	9.1	5	19.2
GM23(-/-)	15	37.5	31	41.9	28	84.8	20	76.9
GM5/5	19	47.5	28	38.9	28	84.8	23	88.5
GM5/21	16	40.0	35	48.6	5	15.2	3	11.5
GM21/21	5	12.5	9	12.5	0	0.0	0	0.0
KM1/1	1	2.5	0	0.0	4	12.1	4	15.4
KM1/3	4	10.0	18	24.3	9	27.3	12	46.2
KM3/3	35	87.5	56	75.7	20	60.6	10	38.5
FcγRIIa H/H	10	25.0	21	28.8	5	15.2	5	19.2
FcγRIIa H/R	18	45.0	37	50.7	12	36.4	12	46.2
FcγRIIa R/R	12	35.0	15	20.5	16	48.5	9	34.6
FcγRIIIa V/V	5	12.8	7	9.9	1	3.0	3	11.5
FcγRIIIa F/V	13	33.3	34	47.9	17	51.5	12	46.2
FcγRIIIa F/F	21	53.8	30	42.3	15	45.5	11	42.3

ɔle 2. Distribution of particular FcγR-GM genotype combinations in Caucasian Americans in relation to HIV control :us

'R Genotype	GM Genotype	Controllers		Non-Controllers		*p*	OR (95% CI)
		n	%	n	%		
'RIIa R non-carriers	GM21 non-carriers	7	70	5	23.8	0.0214	7.47 (1.39 - 40.2)
	GM21-carriers	3	30	16	76.2		
'RIIa R-carriers	GM21 non-carriers	12	40	23	45.1	0.817	0.811 (.325 - 2.03)
	GM21-carriers	18	60	28	54.9		
'RIIIa V non-carriers	GM21 non-carriers	7	33.3	13	44.8	0.560	0.615 (.192 – 1.97
	GM21-carriers	14	66.7	16	55.2		
RIIIa V-carriers	GM21 non-carriers	11	61.1	13	32.5	0.0495	3.26 (1.03 - 10.4)
	GM21-carriers	7	38.9	27	67.5		

Chapter 3: FcγRIIa, FcγRIIIa, and GM in the Progression of HIV Infection

Introduction

HIV progression is remarkably variable. Although the mean time to AIDS is 7.7-11 years after seroconversion [69], rapid progressors can advance to AIDS within 4 years [70] while LTNPs can survive for much longer even in the absence of HAART [71,72]. Several genetic factors have been associated with the progression rate of HIV including FcγRIIa [156]. The low affinity allele of FcγRIIa has been associated with a faster progression to a CD4+ T-cell count of < 200/mm^3 (a clinical definition of AIDS) in HIV infected individuals. FcγRIIa's ligand, Fc of IgG, is highly polymorphic. These polymorphisms, known as GM, have been shown to be influential in a number of other diseases [162-167]. Thus, variations of the Fc region may be able to contribute to the variability seen in HIV progression to a low CD4+ T-cell count. Determinants expressed on Fc (GM) and FcγR are probably some of the most likely ligand-receptor candidate pairs for gene-gene interactions in the human genome. Thus, the aim of this investigation was to determine whether particular GM allotypes individually or epistatically with FcγRIIa, are associated with the progression of HIV.

Progression of HIV among the FcγRIIIa genotypes has produced conflicting results [156, 160]. This may be due to the fact that GM allotypes and possible epistatic interaction with FcγRIIIa was not investigated or considered. Thus, GM- FcγRIIIa interactions were also analyzed for progression to a low CD4+ T-cell count.

Materials and Methods

Study patients

Informed consent was obtained from study subjects enrolled in the Multicenter AIDS Cohort Study (MACS) from Johns Hopkins University, Northwestern University, UCLA, and the University of Pittsburgh. The study protocol was approved by the Institutional Review Board for human research at respective institutions. MACS is an ongoing prospective study of the natural and treated histories of HIV-1 infection in 6972 homosexual and bisexual men that were recruited from 1984 to 2003. Genotyping results from a total of 462 infected white/non-Hispanic and 25 white/Hispanic participants were analyzed. Subjects analyzed from this study were the same subjects analyzed from a previous study [156]. Subjects include 337 individuals who seroconverted after entering the study, and 114 subjects that entered the study seropositive in 1984 with a CD4+ T-cell count > 500/mm^3 of blood.

GM Genotyping

GM typing methods were done as described in the previous chapter. FcγRIIa and FcγRIIIa genotypes were determined in a previous study [156]. Due to technical reasons, certain samples were not typed for certain genotypes causing slight variations in the sample number for each genotype.

Statistical Analysis

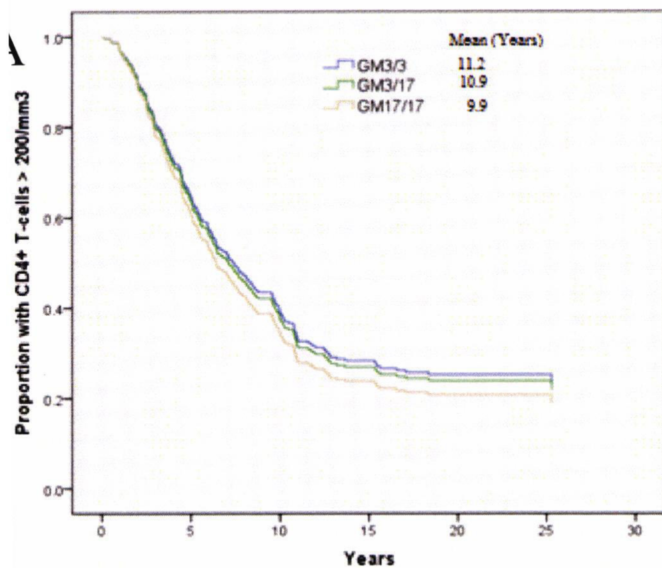
Cox regression models were used to determine whether GM genotypes alone or with FcγRIIa or FcγRIIIa predicted HIV progression to a CD4+ T-cell count < 200/mm^3. Age at time of seroconversion or entry into MACS was used as covariate in the model. Statistical software IBM SPSS Statistics 19 was used for all statistical analyses.

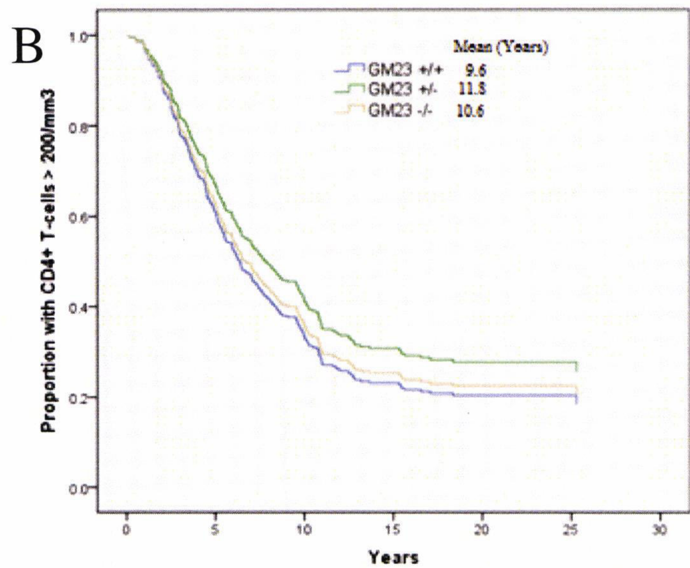
Results

Using Cox regression models, we determined that no GM genotypes contained in the GM3/17 locus (p = .855), GM23 locus (p = .286), and GM5/21 loci (p = .531) were associated with disease progression to a CD4+ T-cell count < 200/ mm^3 by themselves (Figure 6). However, a Cox regression model examining interaction with FcγRIIa H carriers vs. non-carriers (i.e. R homozygotes) and GM3 carriers vs. non-carriers (i.e. GM17 homozygotes) yielded a significant association with disease progression (p = .0424). When this model was applied to other GM loci, no significant interactions were observed.

Individual FcγRIIa genotypes were further investigated to determine if particular genotypes in combination with the GM3/17 locus influenced progression. Among FcγRIIa H non-carriers, GM3 non-carriers were associated with quicker progression to a CD4+ T-cell count < 200/mm^3 compared to carriers of this allele (p = 0.027) (Figure 7A). GM3 non-carriers had a mean progression time to CD4+ T-cell count < 200/mm^3 in 4.6 years compared to 9.4 years for GM3 carriers.

These individual FcγRIIa genotypes were also investigated with other GM loci. Although a Cox regression model examining interaction with FcγRIIa H carriers vs. non-carriers and GM23 heterozygotes vs. non-heterozygotes was non-significant (p = .215), we determined that among FcγRIIa H non-carriers, GM23 non-heterozygotes (i.e. GM23+/+ or GM23-/-) were associated with quicker progression to a CD4+ T-cell count < 200/mm^3 compared to heterozygotes (p = .027) (Figure 7B). Gm23 non-heterozygotes had a mean progression time to a CD4+ T-cell count < 200/mm^3 in 6.8 years compared to 10.0 years for GM23 heterozygotes.





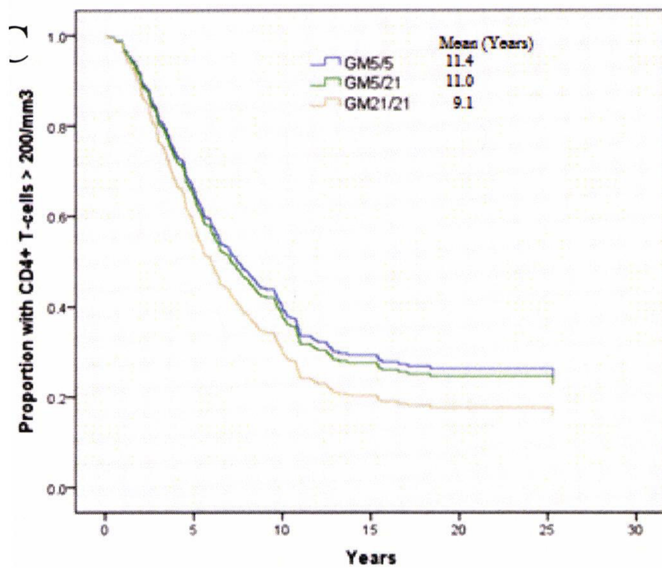


Figure 6. Cox regression estimates of progression to a CD4+ T-cell count < 200/mm^3. (A) Progression curves of the GM3/17 locus genotypes. (B) Progression curves of the GM23 locus genotypes. (C) Progression curves of the GM5/21 loci genotypes.

A Cox regression model examining interaction between FcγRIIa H carriers vs. non-carriers and GM5 carriers vs. non-carriers was also non-significant ($p = .109$). But, among FcγRIIa H non-carriers, GM5 non-carriers (i.e. GM21 homozygotes) were associated with quicker progression to a CD4+ T-cell count < 200/mm^3 compared to carriers of this allele ($p = .017$) (Figure 7C). GM5 non-carriers had a mean progression time to a CD4+ T-cell count < 200/mm^3 in 4.6 years compared to 9.2 years for GM5 carriers.

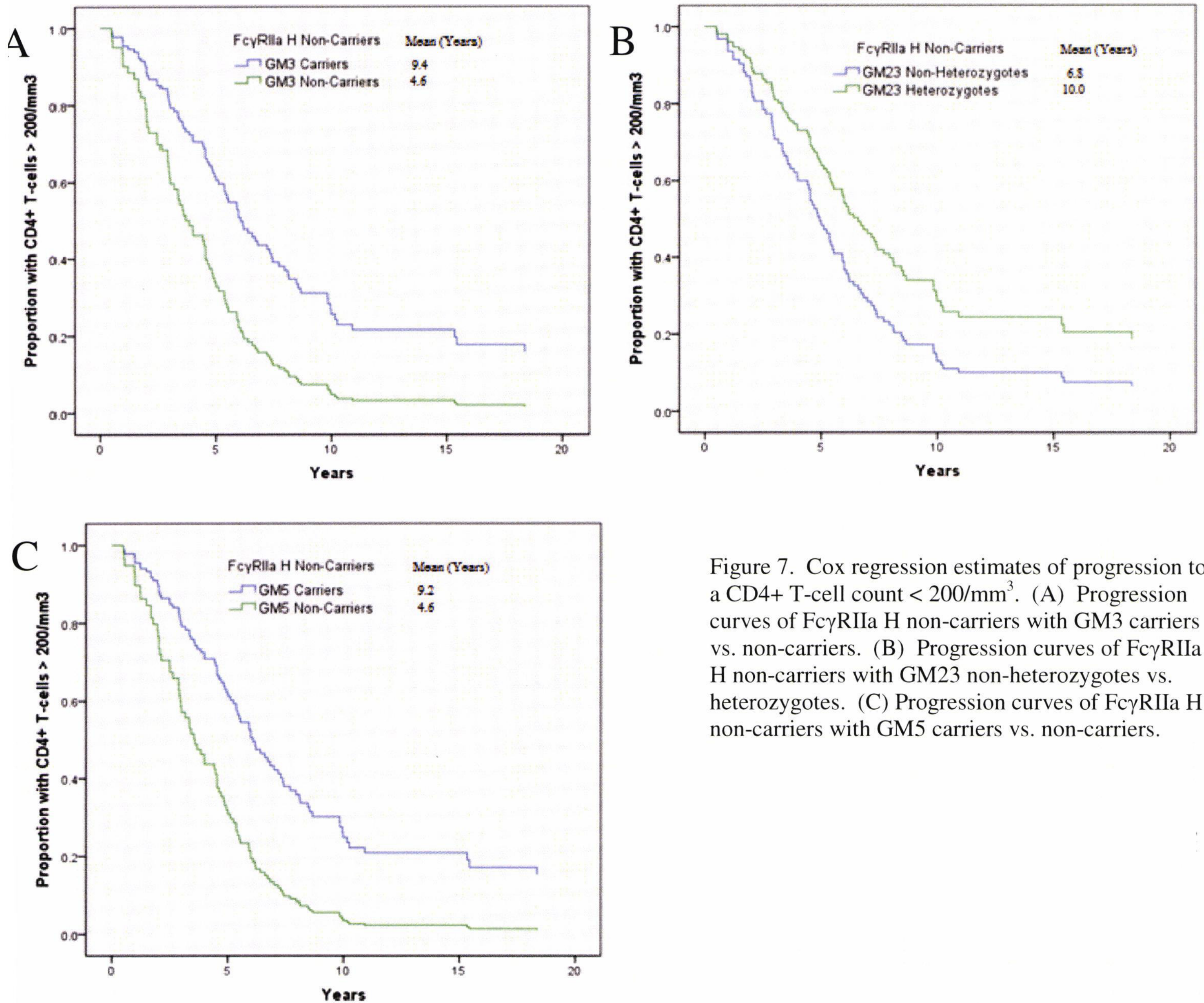


Figure 7. Cox regression estimates of progression to a CD4+ T-cell count < 200/mm^3. (A) Progression curves of FcγRIIa H non-carriers with GM3 carriers vs. non-carriers. (B) Progression curves of FcγRIIa H non-carriers with GM23 non-heterozygotes vs. heterozygotes. (C) Progression curves of FcγRIIa H non-carriers with GM5 carriers vs. non-carriers.

Cox regression models examining interaction with FcγRIIIa and GM showed no significant interactions. But a Cox regression model examining interaction FcγRIIIa F carriers vs. non-carriers (i.e. V homozygotes) and GM23 heterozygotes vs. non-heterozygotes trended towards significance (p = .067). Individual FcγRIIIa genotypes were further investigated to determine if particular genotypes in combination with the GM23 locus influenced progression. Among FcγRIIIa F non-carriers, GM23 non-heterozygotes were associated with quicker progression to a CD4+ T-cell count < 200/mm^3 compared to heterozygotes (p = 0.010) (Figure

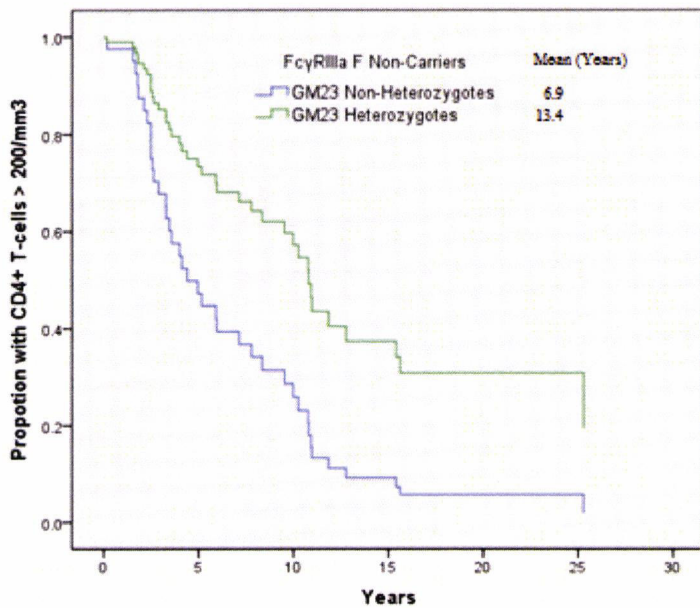


Figure 8. Cox regression estimate to a CD4+ T-cell count < 200/mm^3. Progression curves of FcγRIIIa F non-carriers with GM23 non-heterozygotes vs. heterozygotes.

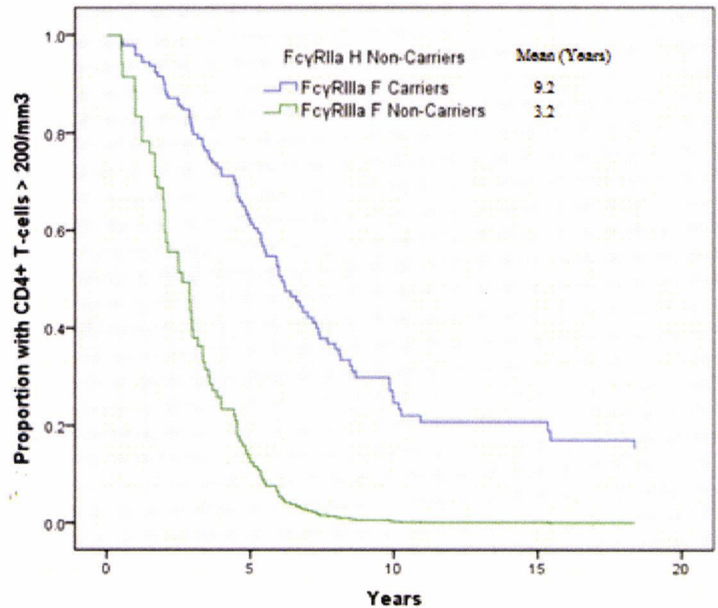


Figure 9. Cox regression estimate to a CD4+ T-cell count < 200/mm^3. Progression curves of FcγRIIa H non-carriers with FcγRIIIa F carriers vs. non-carriers.

8). GM23 non-heterozygotes had a mean progression time to a CD4+ T-cell count < 200/mm^3 in 6.9 years compared to 13.4 years for GM23 heterozygotes.

FcγRIIa and FcγRIIIa combinations were also investigated for possible associations with progression to a CD4+ T-cell count < 200/mm^3. A Cox regression model examining FcγRIIa H carriers vs. non-carriers and FcγRIIIa F carriers vs. non-carriers revealed a significant association with progression ($p = .0054$). Individual FcγRIIa genotypes were further investigated to determine if particular genotypes in combination with the FcγRIIIa genotypes influenced progression. We determined that, among FcγRIIa H non-carriers, FcγRIIIa F non-carriers were associated with quicker progression to a CD4+ T-cell count < 200/mm^3 compared to carriers of this allele ($p = 0.000396$) (Figure 9). FcγRIIIa F non-carriers had a mean progression time to a CD4+ T-cell count < 200/mm^3 in 3.2 years compared to 9.2 years for FcγRIIIa F carriers.

Discussion

The results reported here show significant interactive effects of particular FcγR and GM genotypes on the progression of HIV infection to a CD4+ T-cell count < 200/mm^3. It is possible that epistatic interaction of the various FcγR and GM alleles contribute differentially to the levels of an immunological response, such as ADCC, which could influence the progression rate of HIV to a low CD4+ T-cell count. The FcγR-GM interactions observed were among those homozygous for FcγRIIa and FcγRIIIa alleles, R and V respectively, which are risk factors for faster progression of HIV [156, 160]. In FcγRIIa H non-carriers, when combined with GM17 or GM21, progression was significantly accelerated to a low CD4+ T-cell count. Given that FcγRIIa R has decreased binding affinity for the IgG subclasses [150], this allele could have a decrease in binding affinity for GM17 or GM21 containing antibodies leading to a decrease in ADCC of HIV infected cells. It is known that the FcγRIIa R allele has decreased binding affinity for the IgG subclasses, but antibodies are also polymorphic and could potentially lead to alterations in binding and augment the progression of HIV to a low CD4+ T-cell count.

The observed effect may also be due in part to antibody-dependent enhancement (ADE), in which non-neutralizing antibodies directed against a virus actually increase the infectivity of the virus. Subneutralizing concentrations of anti-HIV antibodies have been shown to cause ADE of HIV mediated by various FcγRs including FcγRI, FcγRIIa, and FcγRIIIa [191,192, 193]. IgG1 and IgG3 concentrations are lower in individuals containing GM17 or GM21, respectively [170]. Thus, it is likely that individuals homozygous for GM17 or GM21 may produce subneutralizing concentrations of antibodies leading to an enhancement of HIV infection mediated by FcγRIIa containing the R/R genotype. As macrophages and monocytes contain FcγRIIa and are actively infected by HIV, these cells would be infected at a higher rate which

would increase the viral reservoir leading to an enhancement in viremia and potential for T-cell infection and depletion. Potentially, protective FcγR genotypes abrogate ADE which would explain why enhanced progression to a low CD4+ T-cell count was not observed in FcγRIIa H carriers in combination with GM17 homozygotes or GM21 homozygotes.

Among FcγRIIa H non-carriers or FcγRIIIa F non-carriers, when combined with GM23 non-heterozygotes, progression to a low CD4+ T-cell count was significantly accelerated. IgG2 concentrations are also influenced by GM23 [170] with those containing GM23 having higher concentrations of Ig2 while those without GM23 have lower concentrations. FcγRIIa H non-carriers or FcγRIIIa F non-carriers lacking GM23 could contain lower titers of IgG2 which could potentially have a decrease in anti-HIV neutralizing and non-neutralizing antibodies. Even though neutralizing antibodies can mediate ADCC, lower titers of antibodies could potentially decrease the size and breadth of neutralization and ADCC while possibly increasing ADE. In contrast, FcγRIIa H non-carriers or FcγRIIIa F non-carriers homozygous for GM23 could contain higher titers of IgG2 which could potentially have an increase in anti-HIV neutralization and ADCC of HIV infected cells, but also an increase in uninfected bystander CD4+ T-cells bound by free floating gp120 [194]. Thus, FcγRIIa H non-carriers or FcγRIIIa F non-carriers that are heterozygous for GM23 could fall into protective zone between possible thresholds of underactivation and overactivation.

A significant interactive effect was also observed between FcγRIIa and FcγRIIIa. The presence of particular genotypic combinations of these receptors showed a marked increase in acceleration to CD4+ T-cell count $< 200/mm^3$. While the presence of FcγRIIa H non-carriers is predictive of a quicker progression to a low CD4+ T-cell count [156], the addition FcγRIIIa V/V genotype is likely an additive effect for accelerated progression. As there is no protective allele

for progression to a low CD4+ T-cell count present in this combination, abrogation of deleterious effects previously described is not possible. Thus there is an enhancement of the acceleration of HIV progression to CD4+ T-cell count < $200/mm^3$.

These results underscore the importance of investigating not only the receptor genes in disease but also the receptor's ligand. The FcγR-GM interactions observed demonstrate how particular genotypic combination can influence the progression of HIV. From these results, particular GM allotypes can differentially influence progression rates among certain genotypes of FcγRIIa and FcγRIIIa. This highlights the necessity of investigating not just the overall interactive effect between alleles, but also the effect between particular genotypes.

Chapter 4: GM23 and FcγRIIa Influence in Anti-gp120 ADCC

Introduction

Recently, a genome-wide association study has found that the IGHG2 allele that codes for GM23 allotype is a major determinant of IgG2 antibody responses to gp120 [168]. The most successful HIV vaccine trial to date, known as RV144, was a combination of two vaccines and showed a modest protection to HIV. This trial has renewed interest in gp120 as a vaccine target, as recombinant gp120 was utilized in one of the vaccines. As mentioned previously, gp120 is highly glycosylated with polysaccharides, and efficient IgG2 responses to polysaccharides have been associated with GM23 [166,169]. Since IgG2 has been shown to mediate ADCC of HIV infected cells[173], it follows that GM23 could potentially influence an anti-gp120 ADCC response mediated by IgG2 antibodies. ADCC can be induced by antigen-complexed IgG2 when bound by FcγRIIa. FcγRIIa genotypes have been shown to be influential in the progression of HIV [156]. This receptor contains a functional polymorphism that affects its ability to bind IgG2 and could logically influence the ADCC response towards HIV.

This demonstrates that there is a genetic basis for investigating differential ADCC rates between genetically dissimilar HIV infected individuals. Yet, GM23's role in ADCC remains uninvestigated. As IgG2 is an important partner in immunity and contains its own allelic variants, it would be very important to investigate the contribution of GM23 as well as FcγRIIa variants in gp120-mediated ADCC of HIV infected cells.

Materials and Methods

FcγRIIa Typing

Informed consent was obtained from volunteer and blood was collected in EDTA-coated vacutainer tubes. The study protocol was approved by the Institutional Review Board for human research. DNA was extracted from the blood using the Qiagen Puregene Kit via manufacturer's protocols. A 366 bp fragment was amplified using the following primers: 5'-GGAAAATCCCAGAAATTCTCGC-3'and 5'-CAACAGCCTGACTACCTATTACGCGGG -3' [185]. The 366 bp product was digested by the restriction enzyme BstU1. This resulted in the following product sizes for each genotype: FcγRIIa H/H: 343 bp; FcγRIIa R/R: 322 bp; FcγRIIa H/R: 343 bp and 322 bp.

GM23 Typing

GM23 was previously typed from healthy donor plasma obtained from MUSC hospital using a standard hemagglutination inhibition assay [195].

Purification of Neutrophils

Blood from volunteers homo- and heterozygous for the H and R alleles of FcγRIIa was collected in EDTA-coated vacutainer tubes. Neutrophils were then isolated from the blood using Cedarlane Lympholyte-poly via manufacturer's protocols [196].

Purification of IgG2

Serum albumin was depleted from healthy donor plasma using ion exchange chromatography. Briefly, plasma was dialyzed against sodium phosphate buffer and then passed through an ion exchange column containing diethylaminoethyl covalently linked to Sepharose and collecting the flow through [197]. IgG2 was then be purified from the total IgG using an

affinity chromatography column using anti-IgG2 antibodies bound to beads that capture IgG2. This was then eluted and concentrated into the desired concentration.

Cell Line and Antibody

The HIV producing cell line HUT 78/HIV-1_{SF2} and anti-gp120 IgG2 human monoclonal antibody (mAb) F425-be48 were provided by the NIH AIDS Research and Reference Reagent Program. Cells were grown in culture using RPMI 1640 media supplemented with 10% FBS.

Determination of Anti-gp120 mAb Binding

A FACS analysis was performed to determine binding of F425-be48 to the HUT 78/HIV-1_{SF2} cell line. Briefly, cells were harvested, washed, and aliquoted into a desired concentration of 1,000,000 cells in 100 ul of PBS. 1 μg of antibody was added to the cells and allowed to sit for 1 hour over ice with occasional agitation every 15 minutes. Cells were then washed thoroughly with PBS twice and placed in 100 μl of PBS. A FITC-conjugated goat anti-human IgG antibody (Sigma-Aldrich) was then added and left to sit for 30 minutes over ice with occasional agitation every 15 minutes. Cells were then washed thoroughly with PBS twice and placed in 500 μl of 4% paraformaldehyde. Cells were then processed on a BD FACSAria II.

ADCC of HIV Infected Cells

HUT 78/HIV-1_{SF2} cells were harvested, washed, and aliquoted into a desired concentration of 1,000,000 cells in 100 ul of PBS into two tubes. 1 μg of F425-b4e8 was added to one set of cells. Both were allowed to sit for 1 hour over ice with occasional agitation every 15 minutes. Cells were then washed thoroughly with PBS and aliquoted into 96 well plates at

5,000 cells per well in triplicate. Neutrophils were then added to the wells in the following effector to cell ratios: 10:1, 5:1, 2.5:1, 1.25:1, .625:1, .3125:1, .15625:1, and 0:1 (Illustration 1). Plates were centrifuged and placed in a 37°C incubator for 4 hours. After this time, the plates were centrifuged again and ADCC activity measured by lactate dehydrogenase release using Promega's Cytotox 96 Non-Radioactive Cytotoxicity Assay per manufacturer's protocols.

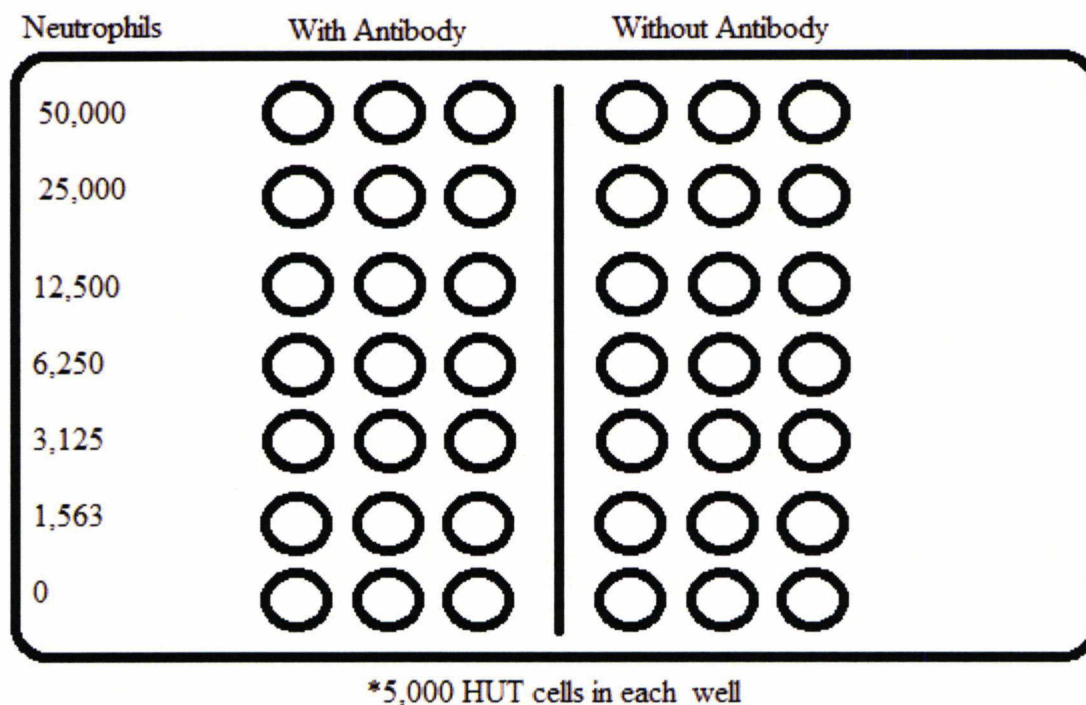


Illustration 1. ADCC plate setup

Results

Although the IgG2 mAb F425-b4e8 clearly binds to the HUT 78/HIV-1_{SF2} cell line (Figure 10), ADCC was not observed in experiments regardless of the presence of the anti-gp120 mAb or FcγRIIa genotype present on neutrophils. This negated the possibility of performing further experimentation investigating the influence GM23 in ADCC.

Discussion

Although the mAb F425-b4e8 has been shown to bind to gp120 and neutralize HIV [198,199], its non-neutralizing potential had not been investigated up to this point. This left open the possibility that F425-b4e8 was capable of mediating ADCC. The reasons behind this antibody's inability to induce ADCC are unclear.

One possibility is that F425-b4e8 a strictly neutralizing antibody. This antibody binds to the V3 loop of gp120 [200]. The vast majority of neutralizing antibodies recognize the V3 loop [198,201] and antibodies can contain neutralization or ADCC inducing capabilities. However, antibodies to HIV have been shown to induce neutralization and ADCC [198]. Antibodies that contain the capability to induce ADCC in one strain of HIV have been shown to bind another strain but lose its ADCC ability [198] meaning that even though F425-b4e8 may not be able to induce ADCC with the particular strain used in these experiments, it could potentially induce ADCC against other strains of HIV. Another possibility could be analogous to a study that a particular antibody could bind and neutralize HIV isolates effectively, but that its ADCC

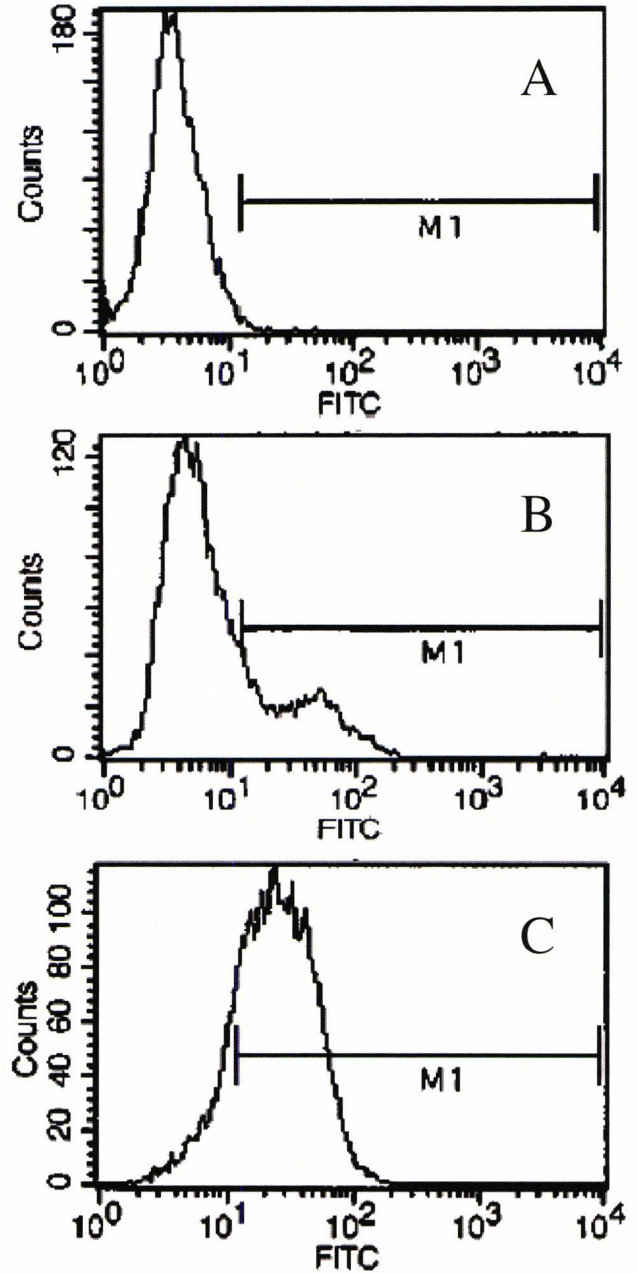


Figure 10. FACS analysis of anti-gp120 IgG2 F425-b4e8 binding HUT 78/HIV-1_{SF2}. (A) Cells alone. (B) Cells + FITC-conjugated anti-IgG antibody. (C) Cells + F425-b4e8 + FITC-conjugated anti-IgG antibody.

capabilities were completely abrogated due to amino acid substitutions in its Fc region [202]. It could be that residues have been substituted on this antibody as well leading to the inability to induce ADCC.

The cell line used in these experiments was an aggressive T-cell lymphoma infected with HIV. The HUT78 cell line has been shown to produce IL-4 when stimulated by CD3 [203]. IL-4 can induce the downregulation of FcγRIIa in neutrophils [203]. It is possible then that the HIV infection in the HUT78 cells could stimulate IL-4 production which then causes neutrophils to downregulate FcγRIIa leading to a loss of ADCC.

IgG2 contains the GM23 allotype. F425-b4e8 is negative for the GM23 allotype. Binding studies have not been done between IgG2 allotypes and FcγRIIa genotypes to determine binding efficacy. It may be that GM23 negative IgG2 antibodies have a marked reduction in binding to any of the FcγRIIa genotypes. This possibility could account for the lack of ADCC observed in these results.

We were not able to include a positive control for these experiments as we did not have access to an anti-gp120 mAb that was known to induce ADCC with this particular cell line. Thus, the inability to induce ADCC in these experiments could potentially be due to errors in the ADCC assays.

Future Directions

Results presented here provide a unique insight into the interactions between receptors and ligands and how this influences outcomes and progression of disease. The FcγR-GM interactions observed showed that in combination with particular FcγR genotypes, GM was influential not only in progression of HIV but also in the control of HIV replication. The size of our controller cohort was admittedly small. Approximately 1 in 300 HIV infected individuals is able to control HIV replication without the use of HAART. However, one future direction would be to confirm the results observed by testing in a different cohort of controllers.

Particular FcγR-GM interactions observed were associated with increased progression to a low CD4+ T-cell count. One direction would to be to confirm this data with another set of individuals. If confirmed, another important direction would be to determine the mechanism of these interactions in relation to progression. Since particular FcγR genotypes have reduced binding affinity for the various IgG subclasses, binding studies could be conducted to determine how GM allotypes influence binding affinity and how this correlates with progression.

Binding studies could also be conducted to determine how GM23 and GM23 negative IgG2 antibodies bind to the various FcγR genotypes. This may be able to elucidate how and why antibodies may or may not contain neutralizing and ADCC inducing capabilities and why the F425-b4e8 antibody was unable to induce ADCC regardless of FcγR.

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