The Immune Response to Acute HIV-1 Infection and the Effect of HAART and HLA Alleles on the Control of Viral Replication

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Molecular Genetics and Microbiology in the Graduate School of Duke University

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ABSTRACT

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Abstract

A fraction of HIV-1 patients are able to successfully control the virus and avoid developing AIDS. It has become increasingly clear that variations in the immune response during the initial days of acute infection including the period of peak viral replication determine long term differences in disease outcomes. While the precise factor(s) necessary and sufficient for protection from AIDS is as yet unidentified, a number of factors have been correlated with protection from AIDS. Among these are the presence of a strong proliferative and multifunctional T-cell response as well as the HLA allele status of a patient. Therefore the goal of this thesis project was to 1) broadly identify the major contributors to the proliferative and multifunctional T-cell response during acute infection with HIV, 2) examine the durability of these responses and 3) elucidate the gene regulation pathway(s) by which HLA allele status determines disease outcomes.

In order to identify the major contributors to the proliferative and multifunctional T-cell response to HIV we utilized PBMC samples from a cohort of acutely infected HIV patients in the Duke and University of North Carolina infectious disease clinics. These samples were stimulated in vitro with peptides representing the HIV clade B consensus sequence and the T-cells were analyzed for proliferation and multifunctionality. Through this analysis we identified CD4⁺CD8⁺ (DP) T-cells as overrepresented within the proliferative response and the primary contributor to multifunctionality. Additionally, the acute multifunctional T-cell response was highly focused on the Nef, Rev, Tat, Vpr and
Vpu sections of the HIV proteome. We also discovered similar response patterns among a cohort of HIV controllers recruited from the Duke infectious disease clinic. In fact, the frequency of multifunctional DP T-cells was inversely correlated with viral loads among the controller cohort.

Having identified DP T-cells as HIV responding cells of interest, we next examined their durability following the removal of widespread antigenic stimulation via administration of HAART. Utilizing longitudinal samples from the acute HIV cohort we again examined T-cell proliferation and multifunctionality at approximately 24 weeks and 104 weeks post infection among patients. This experiment demonstrated that among patients who initiated HAART during acute infection there was a significant reduction in the frequency of multifunctional DP T-cells at 24 and 104 weeks post infection compared to study entry. Meanwhile the proliferative DP T-cell response was maintained longitudinally. Additionally, these patients did not exhibit the previously described increase in frequency of multifunctional CD8 T-cells as infection progressed to the chronic phase. Although the majority of patients initiated HAART during the acute stage of infection, a minority delayed HAART initiation for various lengths up to and including study cessation. Among this group of patients the frequency of multifunctional DP T-cells was maintained longitudinally. Therefore, the early initiation of HAART reduces long term frequencies of multifunctional DP T-cells while delayed HAART initiation leads to a durable multifunctional DP T-cell response. Since HIV controllers with higher frequencies of multifunctional DP T-cells maintain lower viral loads, early HAART
initiation may be detrimental to the development of immune cells capable of controlling the virus.

Finally, we examined the effect HLA alleles have on gene regulation during the initial interactions between HIV and the host immune system. This work employed 2 HIV negative patient cohorts. One cohort expressed HLA-B*35 which has previously been shown to correlate with rapid progression to AIDS following infection with HIV. The second cohort expressed HLA-B*57 which has been associated with long term non-progression following infection with HIV. PBMCs from each group were infected with HIV \textit{in vitro}. Twenty-four hours after infection these cells were sorted into CD4$^+$ T-cells, CD8$^+$ T-cells and NK-cells. Following cell sorting, mRNA was isolated and interrogated for expression changes using whole genome microarrays. This analysis revealed HLA allele specific differences in the magnitude by which CD4$^+$ T-cells, CD8$^+$ T-cells and NK-cells activate the interferon response pathway following exposure to HIV.

In total, these findings provide insight into the cell types responsible for significant portions of the acute immune response to HIV and the mechanisms by which individuals protected from progression to AIDS differ from their peers.
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Chapter 1. Introduction

1.1 T-cell Development

1.1.1 Thymic Education

T-cell progenitor cells enter the thymus lacking expression of a T-cell receptor or either the CD4 or CD8 co-receptor(1, 2). Upon contact with thymic stromal cells T-cell receptor gene rearrangement occurs leading to the selection of either a γ:δ or α:β T-cell receptor(TCR)(3). Following the successful rearrangement of the α and β chains of the TCR developing thymocytes up-regulate the expression of both CD4 and CD8 becoming “double positive” thymocytes(4). Subsequent to expression of CD4 and CD8 the developing thymocytes are exposed to MHC class I and II molecules containing self-peptides. Continued survival of a thymocyte is dependent upon positive selection for successful TCR:MHC binding but interactions of too much strength are also negatively selected(5). If the successful TCR:MHC interaction utilizes MHC class I, CD4 expression is generally down-regulated resulting in CD8 single positive naïve T-cells. Conversely, if the TCR recognizes an MHC class II molecule CD8 is down-regulated resulting in CD4 single positive naïve T-cells(6, 7). Following differentiation into either CD4 or CD8 single positive naïve T-cells, the naïve cells are allowed to leave the thymus to search for foreign antigens being presented in secondary lymphoid tissues. Despite this selection step, on average 5% of circulating T-cells are positive for expression of both CD4 and CD8 (8). It is currently unknown if these cells are allowed to leave the thymus without selecting for CD4 or CD8 mono-expression or if expression of either CD4 or CD8 is reacquired in the periphery. Once in the periphery, these DP T-cells have
been shown to be at least as reactive against foreign antigens as single positive peripheral T-cells (9).

### 1.1.2 Memory T-cell Development

Naïve T-cells utilize their expression of L-selectin and LFA-1 to traffic to the secondary lymphoid tissues (10-15). Once in the secondary lymphoid tissue they undergo a tightly regulated proliferative and differentiation process into effector and memory T-cells if they recognize a MHC:foreign antigen complex and receive a co-stimulatory signal via CD28 (16). If an MHC:antigen complex is recognized without CD28 signaling, the naïve T-cell will become anergic through a process known as peripheral tolerance (17-19). On the other hand, if co-stimulation is provided the naïve T-cell becomes activated and produces IL-2 allowing it to proliferate through an autocrine pathway (20). The resulting daughter cells in turn differentiate into effector and memory T-cells which are capable of recognizing antigen outside of lymphoid tissue and a decreased need for co-stimulation (21, 22).

Following differentiation into effector CD8 T-cells, target cells are recognized via MHC class I:antigen complexes. Upon target recognition CD8 T-cells express cytokines including interferons, pro-apoptotic molecules (TNF and/or Fas) and cytotoxins such as perforin and granzyme (23). This last category of effector molecules gives activated CD8 cells their alternative name “cytotoxic” T-cells (CTLs). Simultaneous to or in the absence of CTL activity the cytokines are also able to exert suppressive activity on pathogens through a variety of mechanisms (24, 25).
Meanwhile, activated CD4 T-cells recognize MHC class II:antigen complexes and further differentiate into a number of T-helper (T<sub>h</sub>) cell types. T<sub>h</sub>1 cells occur when the responding CD4 T-cell is exposed to IL-12 resulting in the expression of the transcription factor T-bet and cytokines such as IFN-γ, IL-2, TNF-α and TNF-β (26-28). This cytokine program allows T<sub>h</sub>1 cells to activate macrophages and CTLs to become more effective pathogen killers. T<sub>h</sub>2 cells result from the responding CD4 T-cell’s exposure to IL-4 and subsequent expression of the transcription factor GATA3 and cytokines like IL-4, IL-5, IL-10, IL-13(26-29). The T<sub>h</sub>2 cytokine program stimulates the proliferation and differentiation of naïve B-cells recognizing the same antigen as the T<sub>h</sub>2 cell thereby improving the humoral immune response. T<sub>h</sub>17 cells arise from a responding CD4 T-cell being exposed to IL-6 and TGF-β thereby triggering the expression of the transcription factor RORγt and cytokines such as IL-17 and IL-21(30-33). This cytokine program stimulates epithelial cells to produce anti-microbial proteins and as a result is thought to be important in protecting mucosal surfaces from invading bacterial and fungal pathogens(34). Additionally, a small portion of CD4 T-cells are selected to express the transcription factor FOXP3 and the surface receptor CD25. Once activated in lymphoid tissue these cells develop into regulatory T-cells (T<sub>reg</sub>) which express the immune suppressive cytokines IL-10 and TGF-β (35-37).

In addition to developing effector functions following antigen recognition, some T-cells develop into memory T-cells which persist after the recognized pathogen is cleared and provide a long-term rapid defense against reinfection (38-42). Memory T-cells are identifiable by the expression of surface markers including CD45RO, CD57.
and/or the loss of CD27 expression (Figure 1) (43). In fact, these cell surface molecules can be utilized to divide memory T-cells into distinct subpopulations with varied functions. Central memory T-cells (T_{CM}) primarily reside in secondary lymphoid tissues, are capable of self-renewal and possess limited effector functions (43, 44). T_{CM} cells are identified by their expression of CD27, CD45RO and CCR7. Effector memory T-cells (T_{EM}) primarily reside in the periphery, exhibit limited self-renewal and perform effector functions (43, 45). T_{EM} cells are characterized by the expression of CD45RO without CD27 or CCR7. Terminally differentiated effector memory T-cells (T_{TDEM}) are solely devoted to performing effector functions and are incapable of self-renewal as shown by their expression of CD57 (46-48).

As mentioned previously, a small percentage of circulating T-cells express both CD4 and CD8. The development path of these cells from exiting thymocytes to memory T-cells has not been described. Nonetheless, DP T-cells have been shown to express the same memory markers as the traditional T-cell subsets (49). In addition to expressing the requisite surface markers, DP T-cells have also been shown to contain functional memory responses directed against foreign antigens (50).
Figure 1: Memory T-cell Surface Phenotype. Adapted from Sallusto F et al. 2004(43)
1.2 HIV-1 Pathogenesis

1.2.1 Origins of HIV

In 1981 clinicians identified a new disease whereby patients presented with uncommon infections and cancers as a result of an impaired immune system (51). This new disease was given the name acquired immune deficiency syndrome (AIDS) and two years later its causative agent, human immunodeficiency virus (HIV) was identified (52-55). While HIV/AIDS was first identified in the 1980s it has since been determined that simian immunodeficiency viruses (SIV) crossed from chimpanzees and sooty mangabeys to humans on at least 8 occasions throughout the 20th century to form the viral species HIV-1 and HIV-2 (56). Following these cross-species events a worldwide HIV/AIDS epidemic took hold claiming more than 25 million lives and leaving greater than 30 million people infected with HIV (57). While spreading across the globe a number of founder events occurred leading to the generation of multiple groups and subtypes of HIV (58). While all subtypes can be found in sub-Saharan Africa, there are large regional differences in the prevalence of each subtype (59). For example within North America circulating viruses are predominantly members of subtype B while patients in southern Africa and India are mainly infected with subtype C viruses (Figure 2).
Figure 2: HIV Subtype Distribution. Adapted from Taylor BS et.al. 2008 (59).
1.2.2 HIV-1 Target Cells

HIV-1 targets dendritic cells, macrophages and CD4⁺ T-lymphocytes using its primary receptor, CD4(60-62). After binding to CD4 the HIV virion uses one of many coreceptors to enter the cell. HIV’s principal coreceptors are CXCR4 and CCR5 and the ability to utilize one or the other is used to define X4 and R5 tropic viruses(63, 64). CXCR4 is primarily found on T-cells while CCR5 is found on T-cells as well as dendritic cells and macrophages leading to the alternative viral tropism names T-cell tropic and macrophage tropic. Transmitted viruses generally utilize CCR5 and evolve into CXCR4 utilizing viruses during the course of infection(65). Increased expression of IL-7 as disease progresses has been proposed to select for this transition(66).

As a result of transmitted viruses need for CCR5, individuals homozygous for a mutation in the CCR5 gene preventing CCR5 expression are highly resistant to infection (67-69). Heterozygotes display an intermediate phenotype whereby they are infected with HIV but display a slowed progression to AIDS(69). In fact, transplantation of hematopoietic stem cells lacking a functional CCR5 gene into an HIV⁺ patient has been shown to eliminate all signs of active HIV infection(70, 71).

1.2.3 Disease Course

1.2.3.1 Acute Infection

HIV-1 infection occurs via exposure to bodily fluids containing a multitude of potentially infectious virions. Even though a large number of virions are present during the exposure event, patients are usually infected by just one CCR5 utilizing virus (72,
The single infecting virus rapidly propagates itself throughout the body during acute infection while showing a particular preference for the GI tract. This preference is a result of over 70% of gut resident T-cells expressing CCR5, and preferentially exhibiting an activated memory phenotype. Additionally the integrin α4β7, a gut homing molecule which binds HIV also aids HIV in locating gut-resident CD4+ lymphocytes. This massive targeting of gut CD4+ lymphocytes leads to a severe depletion of T-cells in the gut and the translocation of microbial products from the gut into the bloodstream. Translocated microbial products along with the ongoing HIV replication throughout the body produce systemic immune activation throughout the course of infection. In addition to the depletion of gut T-cells, there is also a depletion of CD4+ T-cells throughout the body during acute HIV infection. The depletion of CD4+ T-cells following HIV infection is caused by a combination of cytolysis and apoptosis. Apoptotic cells in turn give off microparticles which are capable of suppressing antibody production, macrophages, and T-cells. Despite the immunosuppressive microparticles, the immune system manages to respond to infection initially with a cytokine storm (IFN-α, IFN-γ, IL-15, TNF-α and others) and activation of NK and NKT cells.

Shortly thereafter, HIV-1 specific CD8+ T-cells (CTLs) are able to be detected. The appearance of these cells coincides with the peak in viremia. Additionally, their appearance heralds the onset of selective immune pressure on the viral sequence at approximately 2 weeks post-infection. The initial T-cell responses are often specific for Nef while later responses recognize Gag and Pol. About 10 weeks
later, autologous neutralizing antibodies appear and also put selection pressure on the virus envelope(96-98). These factors combine to form a partially effective immune response which drastically reduces and later maintains the viral load at a chronic set point, allowing for a partial recovery within the circulating CD4 compartment and to a lesser extent the gut(99).
Figure 3: The Immune Response to HIV Infection. Adapted from McMichael AJ and Phillips RE 1997 (100).
1.2.3.2 Chronic Infection and AIDS

Following the establishment of a viral setpoint a dynamic equilibrium is established between the evolving virus and the T-cell and antibody responses directed against it. This equilibrium can last a few months for rapid progressors, years for the typical patient or decades (possibly indefinitely) for long-term non-progressors (LTNP)/viral controllers(101). Viral load at setpoint has been well correlated with the length of the chronic phase of infection(102). During the chronic phase of infection the cytopathic effects of HIV on CD4+ T-cells, impaired thymic function, CD8+ cellular cytotoxicity, antibody mediated cytotoxicity as well as induction of apoptosis from immune activation combine to cause a slow decline in CD4+ T-cell counts(103-106). In addition to its effect on CD4+ T-cell apoptosis, chronic immune activation is an excellent correlate of disease outcomes and is absent in both LTNPs and nonpathogenic SIV infections where high SIV viral loads are maintained long term(107, 108). Chronic immune activation eventually leads CD8+ T-cells to upregulate PD-1 expression and ultimately become exhausted(109-111). These exhausted CD8+ T-cells are noted for their lack of proliferation and loss of the ability to produce multiple cellular functions in response to antigenic stimulation(109, 112-114). The loss of an effective CD8 response allows for increased viral replication which in turn further harms the CD4 compartment reducing T-cell help to both the cellular and humoral arms of the immune response. This feedback loop ultimately causes the collapse of the immune system and progression to
AIDS. Following the onset of AIDS, patients are subject to any number of AIDS associated infections and cancers, one of which may ultimately cause their death.

### 1.2.3.3 Natural Control of HIV

A small portion (~5%) of HIV+ patients experience significant delays in their progression to AIDS or may never experience progression (103). Infection with HIV containing defective nef has been shown to result in improved long term prognosis (115). As mentioned previously, ΔCCR5 heterozygotes experience a slower rate of disease progression. Similarly, increased gene copy numbers of CCL3L1 gene causes a decrease in CCR5 expression and delayed disease progression (116). The presence of HLA alleles such as B57 has long been associated with LTNPs (117, 118). Recent work has shown that viral escape from B57 restricted immune pressure can lead to viral fitness costs (119). While immune pressure is applied, it does not fully explain the protective association of the HLA-B57 allele. An additional proposed explanation for B57’s protective association is a single nucleotide polymorphism (SNP) in HCP5 which displays significant linkage to HLA-B57 and is also associated with lower viral loads (120). Importantly, HCP5 is an endogenous retroviral element and shares major sequence homology with retroviral pol (121). This sequence homology may allow for important interactions between HCP5 proteins/transcripts and HIV. A SNP in HLA-C has also been shown to associate with lowered viral loads (120). This SNP has been shown to increase expression of HLA-C and therefore responses to peptides presented by HLA-C may be important in preventing progression to AIDS (122). Additional associations between viral loads and the expression of HLA alleles and their KIR binding partners on NKs have also been
observed (123-126). In total, a variety of genetic factors have been associated with this phenomenon although none fully explain some patient’s protection from AIDS.

In addition to the above genetic factors, the functions of T-cells have also been correlated with improved disease outcomes. The ability of both CD4 and CD8 T-cells to proliferate in response to HIV antigens has been associated with better HIV disease status(127, 128). While the ability of T-cells to produce individual anti-viral molecules such as IFN-γ has not been associated with viral control, the simultaneous expression of 3 or more functions at a time within HIV and SIV-specific CD4 and CD8 T-cells has been associated with improved disease outcomes(113, 129-132). The location of these polyfunctional T-cells may also be important since they are also found in higher numbers within mucosal sites (a place of high HIV-1 replication) of controllers(132). Although the presence of polyfunctional CD8 T-cells during the chronic phase of infection correlates with protection from AIDS, they do not appear in significant numbers until the acute infection is resolved therefore they may not be involved in the initial control of HIV viremia(92). Freel et. al. have shown that CD107a (a marker for cytotoxic degranulation) and MIP-1β in combination as well as independently correlate with anti-HIV activity against a variety of viral isolates spanning multiple coreceptor phenotypes(133). In addition, MIP-1β producing HIV-specific CD8 T-cells appear to apply the strongest selective pressure on the infecting virus indicating that in vivo CD8 mediated control is not limited to cytotoxic effects(92). Interestingly, CD8 T-cells have long been observed in vitro to possess a non-cytolytic antiviral response (CNAR) to HIV(134, 135). Moreover, CNAR is higher among controllers than progressing HIV
patients(136). Unlike the polyfunctional CD8 response, CNAR is observed early in the course of HIV infection and therefore may play a role in the initial control of acute infection (94, 137). Unfortunately, the identity and mechanism of the factor(s) responsible for CNAR activity are as yet unknown. Finally, DP T-cells have also been shown to possess HIV-specific responses within both acute and chronic HIV patients(50, 138). Unlike the CD4 and CD8 T-cell compartments, no systematic examination of DP T-cell capabilities or their effect on viral control has been reported.

In summary, multiple genetic factors and T-cell phenotypes have been associated with viral control but, no one correlate has been shown to explain the entirety of protection. Consequently, it is unclear if there are as yet undiscovered universal correlates of protection or if a variety of combinations of multiple factors render a subject protected from acquisition of HIV and/or AIDS.

1.2.3.4 Therapeutic Control of HIV

Currently over 25 antiretroviral drugs, targeting multiple steps in the replication cycle of HIV, have been successfully developed for use against HIV-1(139). Highly active antiretroviral therapy (HAART), the simultaneous administration of three or more antiretroviral drugs utilizing at least two methods of action was developed in 1996 and has since become the standard of care(140). HAART has been extremely effective at controlling viral loads and is responsible for saving more than 3 million years of life in the United States alone(141). Worldwide there are many competing guidelines for the timing of treatment initiation, within the United States the Department of Health and Human Services currently recommends the initiation of therapy when CD4 counts fall
below 500/µL. Early administration of HAART has been shown to reduce HIV transmission within discordant couples. Additionally, early HAART has been shown to improve the replenishment of CD4 T-cells in the gut. Therefore, earlier administration of HAART holds promise for the therapeutic containment of the pandemic. As a result, altering treatment guidelines so that HAART is initiated before CD4 counts fall below 500/µL has been suggested but remains controversial among clinicians. Importantly, the effect of early HAART initiation on the development of potentially beneficial immune responses (neutralizing antibodies, CNAR, polyfunctional T-cells, etc) directed against HIV is unclear. Given that polyfunctional CD8 T-cells have previously been positively correlated with improved disease outcomes and their frequency has been shown to correlate with days post infection the timing of HAART may be of particular importance to this correlate of protection. Consequently, further study to determine the effects of early HAART administration on the frequency of polyfunctional T-cells are necessary to guide treatment decisions of clinicians.

1.3 Focus of this Work

In the greater than 25 years since HIV was identified as the causative agent of AIDS the world has seen its prevalence explode into a global pandemic. As a result, tremendous resources have been devoted to developing therapies and vaccines to combat HIV/AIDS. With the development of HAART tremendous progress has been made in therapeutically controlling the virus once an individual is infected. Unfortunately, this treatment does not eliminate the virus from the individual and therefore must be maintained for life if AIDS is to be successfully prevented. Thus difficulties with side-
effects, treatment adherence and cost often impede long-term control of HIV. As described above, it has become evident that the earliest stages of the immune response to HIV infection are important in determining the long-term prognosis of a patient. Nonetheless, no clear correlate of protection has been identified within the acute immune response. Therefore, the overall goal of this work was to determine 1) the ability of a previously neglected cell type, DP T-cells, to respond to HIV antigens during the acute stage, 2) the durability of this response following the removal of large scale antigen stimulation and 3) how previously described genetic determinants of progression risk alter the immune response within the initial hours following infection. Specifically, Chapter 2 describes my work that identified that DP T-cells are a major contributor to the acute anti-HIV response and includes the first ever description of multifunctionality within the DP compartment(143). Chapter 3 describes my work to examine the long-term anti-HIV response in the DP compartment, as well as the CD4 and CD8 T-cell compartments, with and without the early administration of HAART. My data reveals that HAART timing differentially affects distinct T-cell functions and compartments. Finally, chapter 4 addresses the differences engendered by variable HLA status on the very earliest response to infection with HIV and reveals an important role for limited IFN responses in controlling HIV infection.
Chapter 2. Double Positive T-cell Response to HIV During Acute Infection

2.1 Introduction

Immature T cells express both CD4 and CD8 while undergoing thymic development(144). Traditionally it was believed that expression of either CD4 or CD8 was permanently lost once T-cells transitioned to naïve T-cell status and exited the thymus. Despite this, it has been observed that both healthy and diseased humans, chickens, monkeys, mice, rats and pigs exhibit a circulating pool of CD4−CD8+ double positive (DP) T-cells(8, 145-149). The DP population generally represents around 3% of circulating T-cells but shows considerable variability across individuals(8). In patients suffering from various neoplastic and infectious diseases, as well as in some ostensibly healthy individuals, circulating DPs can represent up to 43% of circulating T-cells(150, 151).

Circulating DPs have often been thought to arise from thymic leakage of immature T-cells(145, 152). Nonetheless, sporadic studies have examined the origins and functional abilities of extra-thymic DPs. DPs have been shown to localize to the sites of inflammatory processes in a variety of autoimmune disorders as well as in infectious diseases, such as HCV(151, 153, 154). It has been reported that DPs with a highly activated memory phenotype and expressing both HIV co-receptors CCR5 and CXCR4 are located in the intestine, where HIV replication preferentially occurs during acute infection(49, 155). Kitchen et. al. and others have also shown that HIV is capable of
infecting DP cells both in vitro and in vivo(156-160). Since DP cells are located at the sites of active HIV infection, express the appropriate co-receptors and are capable of being infected, DP cells may be important targets of acute HIV infection.

The ability of the immune system to quickly respond during acute HIV infection and thereby decrease viral loads to low levels is believed to be an important determinant of long term prognosis(161). Interestingly, Howe and collaborators have shown that HIV infected patients possess circulating HIV-specific DP T-cells during the acute phase(138). These HIV-specific DP cells expressed interferon-γ and were either co-expressing IL-2 or a marker for degranulation (CD107a), and patients were more likely to have bifunctional DP cells than either bifunctional CD4 or CD8 T-cells. Simultaneous expression of 3 or more functions at a time within HIV and SIV-specific CD4 and CD8 T-cells has previously been associated with improved disease outcomes(113, 132, 162-164). CD4 and CD8 T-cell proliferation in response to HIV antigens has also been associated with better HIV disease status(127, 128). Therefore, in addition to the possibility of being a target of acute HIV infection, DP cells may represent an important component of the HIV-specific cellular immune response. At present, little is known about the breadth and functional profile of the DP response to HIV. As a result, we sought to determine the ability of circulating DP cells to respond to HIV antigens with a wide range of functionalities during the acute phase of HIV infection. Finally, we went on to compare these acute responses with those observed in a group of HIV-1 controllers.
2.2 Materials and Methods

2.2.1 Subjects

Persons with acute HIV infection were identified based on clinical presentation or by screening conducted by the state of North Carolina’s Screening and Tracing Active Transmission (STAT) Program that has identified individuals with acute HIV infections (AHI) since 2002. Subjects are identified as being acutely infected through a combination of reported symptoms and serology(165). Acutely infected patients were then referred for further evaluation at either Duke University or the University of North Carolina-Chapel Hill. Following the provision of written informed consent the referred patients were enrolled in either studies of antiretroviral treatment or an untreated longitudinal study (depending on patient choice) if they were 1) EIA Negative and nucleic acid amplification test (NAT) positive 2) EIA positive, NAT positive, Western Blot negative/indeterminate or 3) EIA positive, NAT positive, Western Blot positive and documented EIA negative within 45 days. Thirty patients who had been infected for a median of 43 (range 22-105) days before entry were studied (Table 1) (165). Due to the study location, all 30 patients were presumed to have been infected with HIV-1 clade B viruses. At study entry blood samples were acquired from these patients and peripheral blood mononuclear cells (PBMCs) were isolated using standard density gradient centrifugation. Isolated PBMCs were cryopreserved in fetal calf serum supplemented with 10% DMSO and stored in vapor phase liquid nitrogen within 8 hours from collection. Viral load and CD4 counts were also obtained at study entry. This acute
patient cohort had a median viral load of 259789.5 (range 688-1,1503,872) copies/mL and a median CD4 count of 476.5 (range 6-1175) cells/mm³.

Additionally, 9 virus controllers were separately recruited from the Duke Adult Infectious Diseases Clinic with informed consent under Duke University Medical Center IRB approval. Virus controllers were required to have been diagnosed as HIV positive for greater than 1 year, be antiretroviral therapy naïve, have CD4 counts >600 cells/mm³ blood and have been controlling virus replication to less than 2,700 viral RNA copies/mL blood (Table 2) (94, 133, 166, 167).
### Table 1: Acute Patients

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Table 2: Controller Patients

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2.2.2 Peptides

Fifteen amino acid peptides overlapping by 11 amino acid residues representing the HIV-1 Clade B consensus sequences of ENV (#9480), Gag (#8117), Nef (#5189), Pol (#6208), Rev (#6445), Tat (#5138), VPR (#6447) and VPU (#6444) were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Individual peptides were resuspended in DMSO and pooled, at a final concentration of 500μg/mL, into a total of six peptide pools representing gp120, gp41, Gag, Pol peptides #5461-5585, Pol peptides #5586-5709, and a combination of Nef, Rev, Tat, VPR and VPU (VVNRT).

2.2.3 Proliferation Assays

Cryopreserved PBMC were thawed and washed twice with RPMI containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (R10) and enumerated using a Guava Count system (Millipore). Following cell counting the PBMCs were washed twice with PBS, and subsequently resuspended at 20x10^6 cells/ml. The cells were stained with Carboxyfluorescein succinimidyl ester (CFSE) for 8 minutes mixing once at 4 minutes(168). CFSE staining was quenched with 100% human serum for 2 minutes, and after washing with PBS, the cells were resuspended in R10 containing human serum. CFSE stained cells were then plated at 1x10^6 cells/mL and stimulated with peptide pools representing HIV GP120, GP41, Gag, Pol pool 1, Pol pool 2, VVNRT(1 ug/mL) or anti-CD3 (eBioscience) and -CD28 (BD Biosciences) antibodies. Stimulated cells were then incubated at 36°C with 5% CO2 for 6 days. Following 6 days of stimulation the cultures
were washed twice with PBS and stained with a violet vital dye (Invitrogen) for 20 minutes at room temperature in the dark. Cells were again washed twice with PBS and then stained with anti-CD3 APC-Cy7 (BD Biosciences; clone SK7), -CD4 PE-Cy5.5 (eBioscience; OKT4) and -CD8 Qdot605 (Invitrogen; 3B5) antibodies for 20 minutes at room temperature in the dark. Following antibody staining the cells were washed thrice with PBS and fixed with 1% paraformaldehyde. Stained and fixed cells were then refrigerated in the dark until acquisition.

2.2.4 Intracellular Cytokine Staining Assays

Cryopreserved PBMC were thawed, resuspended at 2x10^6 cells/mL in R10 and rested overnight at 36°C with 5% CO₂. After the overnight rest the PBMCS were counted and resuspended at 1x10^6 cells/mL in R10. Cells were then stimulated for 6 hours at 36°C with 5% CO₂ with peptide pools representing CMV pp65, HIV GP120, GP41, Gag, Pol pool 1, Pol pool 2 or VVNRT (1 µg/mL). Stimulations were performed in the presence of 1µg/mL each of anti-CD28 (BD Biosciences; L293) and CD49d (BD Biosciences; L25) antibodies, anti-CD107a PE-Cy5 (eBioscience; H4A3), 5µg/mL Brefeldin A (Sigma-Aldrich) and 1 µg/mL Monensin (BD Biosciences). Following the stimulation, the cells were washed with PBS containing 1%FCS and surface stained with Aqua Blue vital dye (Invitrogen), anti-CD4 PE Cy5.5 (eBioscience; OKT4), -CD8 Qdot605 (Invitrogen; 3B5), -CD27 PE-Cy7 (BD Biosciences; M-T271), -CD57 Qdot565 (AbD Serotec; TB01) and –CD45RO PE-TR (Beckman Coulter; UCHL1) for 20 minutes at room temperature in the dark. Following surface staining the cells were washed again with PBS containing 1% FCS and subsequently fixed and permeabilized with
cytofix/cytoperm and perm/wash buffer (BD Biosciences) for 20 minutes and washed twice. Following the fix/perm step cells were stained intracellularly with anti-CD3 Qdot655 (Invitrogen; S4.1), -IFN-γ Alexa700 (BD Biosciences; B27), -IL-2 APC (BD Biosciences; MQ1-17H12) -MIP-1β FITC (R&D Systems; 24006), and –perforin PE (Cell Sciences;B-D48) for 20 minutes at room temperature in the dark. Subsequent to intracellular staining cells were washed with perm/wash buffer and then fixed with 1% paraformaldehyde. Following fixation cells were refrigerated in the dark until acquisition.
2.2.5 Flow Cytometry Acquisition and Analysis.

Within 18 hours of staining, fully stained cells from the proliferation and intracellular cytokine staining (ICS) assays were acquired on a custom LSRII (BD Biosciences) using FACSDiva. Following acquisition flow data was analyzed using FlowJo software v.9.3.2 (TreeStar). For all assays gates were set to include singlet events, live CD3\(^+\) cells, lymphocytes and CD4\(^+\)/CD8\(^+\)/CD4\(^+\)/CD8\(^+\) subsets. For the proliferation assays CFSE\(^{\text{low}}\) populations were then identified from each lymphocyte subset (Figure 4). For the ICS assays the naïve population (CD27\(^-\)/CD45RO\(^-\)) was identified and excluded from each lymphocyte subset. Within the memory population, cellular function positive populations were identified individually for all cellular functions except perforin which was only defined as positive if both perforin\(^+\) and IFN-\(\gamma\)^\(^+\) (Figure 5). Using a boolean gating strategy, the 32 combinations of the 5 cellular functions were identified. Based on these frequencies we also calculated the total frequency of families of subsets expressing the same number of functions.
Figure 4: CFSE Assay Gating Strategy. Proliferation assay gating strategy. Representative flow cytometry plots are shown to display the gating strategy used in identifying proliferated cells. Briefly, geometry gates were used to identify singlets. From the singlets, vital dye $^+$CD3$^+$ cells were isolated. Within the vital dye $^+$CD3$^+$ cell a second geometry gate was used to select for lymphocytes. CD4$^+CD8^-$, CD4$^+CD8^+$ and CD4$^-$CD8$^+$ T-cell subsets were then identified from within the lymphocyte geometry gate. Within each T-cell subset a CFSE$^{\text{low/}}$ gate was drawn to identify cells which had undergone proliferation.
Figure 5: ICS Assay Gating Strategy. Representative flow cytometry plots are shown to display the gating strategy used in identifying cells expressing any of 5 functions. Briefly, geometry gates were used to identify singlets. From the singlets, vital dye-CD3+ cells were isolated. Within the vital dye-CD3+ cell a second geometry gate was used to select for lymphocytes. CD4+CD8-, CD4+CD8+ and CD4-CD8+ T-cell subsets were then identified from within the lymphocyte geometry gate. Within each T-cell subset a CD27-CD45RO- gate was drawn to exclude naïve T-cells. Expression of CD57, CD107a, IFN-γ, IL-2, MIP-1β and perforin was then determined from within each memory population.
2.2.6 Statistical Analysis.

For the proliferation assays relative proliferation values were obtained by subtracting the average CFSE\textsuperscript{low} population frequency of a patient samples’ two unstimulated wells from the CFSE\textsuperscript{low} population frequency following stimulation and then dividing the resulting value by the average CFSE\textsuperscript{low} population frequency of a patient samples’ two unstimulated wells. For the intracellular cytokine staining assays Pestle was used for background subtractions and for frequency analysis Prism (Graphpad) and SPICE were used. PESTLE and SPICE were kindly provided by Dr. M. Roederer Vaccine Research Center, NIH, Bethesda, MD.

DP response ratios were calculated by first multiplying each patients’ frequency of HIV-specific DP, CD4 and CD8 T-cells by the mean cell count in the DP, CD4 and CD8 compartments for each stimulation condition to obtain a normalized HIV-specific DP, CD4 and CD8 cell count. The normalized HIV-specific DP cell count was then divided by the sum of the normalized HIV-specific DP, CD4 and CD8 cell counts to give the DP response ratio.

Comparisons of responses within patient groups were performed using a Wilcoxon Matched Pairs test (Prism). Comparison across patient groups used a Mann-Whitney U test (Prism). Correlations were obtained using linear regression (Prism). No adjustments for multiple comparisons were performed and P values should be interpreted with this in mind.
2.3 Results.

2.3.1 HIV-specific Proliferation of DP cells.

Previous studies reported a correlation between the magnitude of HIV-specific proliferation of T-cells and improved disease outcomes (127, 128). Nonetheless, HIV-specific proliferation of DP cells has not been previously examined. Therefore, PBMCs from HIV+ patients were analyzed for their proliferative ability in response to stimulation with peptides representing the HIV clade B consensus peptide sequence. Following 6 days of stimulation CD4+CD8− (CD4), CD4+CD8+ (DP) and CD4−CD8+ (CD8) populations were identified by flow cytometry (Figure 4). The loss of CFSE staining was used to indicate cells within these populations which had undergone proliferation during the stimulation (168). As expected, we observed proliferative responses to the HIV proteome within both the CD4 and CD8 compartments of the acutely infected subjects. The relative proliferation within the CD8 compartment was greater than that seen in the CD4 compartment for both the acute (median, range 1.78, -4.93-25.5 vs. 0.93, -3.86-31.22) and controller patients (2.65, -0.19-29.69 vs. 0.40, -0.32-20.89), although this difference was only significant in the controller cohort (Figure 6A). In both acutely infected (3.78, -3.42-36.36) and controller patients (6.31, -0.13-98.13), we observed significantly greater relative proliferation within the DP compartment following HIV antigen stimulation than the CD4 compartment. DP proliferative responses were also higher than those observed in the CD8 compartments of both cohorts, but reach statistical difference only in the acute cohort (p= 0.01). Next, we determined the portion of the total T-cell proliferative response to HIV which was attributable to the DP compartment.
in the acute and controller cohorts, a calculation we call the DP response ratio. In both cohorts, the median proliferative DP response ratio was approximately 16% (range 0-78; 0-39) (Figure 6B). In 5 of 30 acute patients the DP response ratio was greater than 40%. We also sought to determine if there was a relationship between the time since infection and the magnitude of the proliferative response. There was no correlation between the CD4 proliferative response and time following infection (Figure 6C). However, the HIV specific proliferative response within the DP and CD8 compartments increases with time since infection (p = 0.0198, 0.0406), suggesting that both the DP and CD8 proliferative responses gain strength as the immune system partially controls viral replication (Figure 6D and E). Meanwhile, the magnitude of the HIV-specific proliferative response within the controller cohort did not correlate with viral loads within these patients (p = 0.6312).
Figure 6: Total HIV-Specific Proliferation. A, Cells were stained with CFSE and stimulated for 6 days with peptide pools representing the HIV proteome. The HIV-specific relative proliferation was calculated for each T-cell subtype and plotted for each patient in the acute and chronic cohorts. B, The percentage of the total anti-HIV proliferative response coming from the DP compartment was calculated for each patient in the acute and chronic cohorts. C, The days post infection at which an acute blood sample was drawn was plotted against the HIV-specific relative proliferation in the CD4 compartment D, DP compartment E, CD8 compartment.
2.3.2 Polyfunctionality of HIV-specific DP cells.

The polyfunctionality of HIV-specific cells was analyzed by staining for the expression of CD107a, IFN-γ, IL-2, MIP-1β and perforin after a six hour stimulation. HIV-specific functionality was determined according to the gating strategy displayed in Figure 5. Following the identification of each individual functional population, Boolean gating was applied to identify cells expressing all possible combinations of the 5 functions. We observed a significantly higher frequency of CD8 T-cells expressing 3 or more functions in the acutely infected and controller groups than was observed within the CD4 compartment (Figure 7A). HIV-specific multifunctional cells within the DP compartment (20, -0.19-100) were significantly more frequent than in the CD8 compartment (0.34, 0.01-24.84) in the acute infection group (p = 0.0002). Of interest, 100% of patient Z68’s DP cells were specific for HIV and multifunctional at study entry.

Using the multifunctional response we again compared the DP response ratios across cohorts to determine the fraction of all HIV-specific multifunctional T-cells which reside within the DP compartment (Figure 7B). The acute cohort had a median multifunctional DP response ratio of 73% (0-92%) while the controllers exhibited a median of 1% (0-100%). It is notable that the controllers exhibited an extremely variable DP response ratio with the majority well below the acute cohorts’ median and two patients presenting a DP ratio of 100%. Due to this high variability the response ratios of the two groups only trended towards a significant difference (p = 0.0828).
In order to understand the differentiation stage of the whole and HIV-1-specific multifunctional DP cell subsets, we also examined their CD27, CD45RO, and CD57 expression. Within the general memory population of the CD4, DP and CD8 memory compartments (Figure 8A-F), we observed that the CD4 memory compartment was predominantly CD27+CD45RO+CD57−; the CD8 memory compartment was evenly distributed between CD27 and CD45RO expression status with the frequency of cells lacking CD57 expression being approximately two-fold higher than those displaying CD57 expression. The DP compartment was often intermediate to the frequencies displayed in either the CD4 or CD8 compartments but more closely resembled the CD8 compartment. When the HIV-1-specific subsets were analyzed, we found that in both the acute and controller cohorts, a median of 92% (33-99%; 50-100%) of multifunctional DP cells lacked CD57 expression and therefore were not terminally differentiated (Figure 7C). Finally, within the timespan covered by our acute cohort there were no significant correlations between the frequencies of CD4, DP or CD8 multifunctional cells and time post-infection (Figure 9A, B and C). Conversely, the frequency of HIV-specific multifunctional DP cells within the controller cohort correlated with the viral loads within these patients (p = 0.0282).
Figure 7: Total HIV-Specific Multifunctionality. A, Cells were stimulated for 6 hours with peptide pools representing the HIV proteome and then expression of CD107a, IFN-γ, IL-2, MIP-1β and perforin. Using Boolean gating expression of all possible combinations of these functions was determined. The HIV-specific frequency of cells expressing 3, 4 or 5 of these functions within each T-cell subtype was plotted for each patient. B, The percentage of the total 3, 4 or 5 function response coming from the DP compartment was calculated for each patient in the acute and chronic cohorts. C, The percentage of multifunctional DP cells lacking expression of CD57.
Figure 8: Memory Phenotypes. The CD4, DP and CD8 memory T-cell populations were analyzed for their expression of CD27, CD45RO and CD57. The frequency of A, CD27+CD45RO+CD57+ B, CD27-CD45RO+CD57+ C, CD27-CD45RO-CD57+ D, CD27+CD45RO+CD57- E, CD27-CD45RO+CD57- and F, CD27-CD45RO-CD57- cells within the memory population are shown for each T-cell compartment.
Figure 9: Multifunctionality vs. Time. The days post infection at which an acute blood sample was drawn was plotted against the HIV-specific 3, 4 or 5 function response in the A, CD4 compartment B, DP compartment C, CD8 compartment.
2.3.3 Antigen-Specific Contribution to HIV-specific responses.

Next, we sought to identify the antigenic regions responsible for evoking the proliferation and multifunctionality observed within the DP compartment. Within the acute HIV infection cohort patients we observed no significant antigen-specific differences in proliferative capacity of the DP cells (Figure 10A). On the other hand, the controllers’ DP cells focused on all non-Env peptide pools (Figure 10B). In fact, the DP proliferative response to Gag was significantly higher within the controller cohort than within the acute cohort (p = 0.0439). All other antigen-specific responses were not significantly different between the patient cohorts (p-values summarized in Table 3).

In contrast to their proliferative response, the acute patients’ multifunctional response was most pronounced to VVNRT, though responses were robust to most of the peptide pools (Figure 10C). Meanwhile, the controllers’ multifunctional response mirrored their proliferative response as it was again focused on the recognition of Gag and VVNRT antigens (Figure 10D). We compared the multifunctional responses to each antigen between patient cohorts and observed that they were significantly higher among the acute patients than the controllers for all peptide pools except Pol pool 1 which displayed a trend towards significance (p-values summarized in Table 3).

We also compared the proliferative and functional responses across assays for the two peptide pools (Gag and VVNRT) that generated strong functional and proliferative responses. This analysis revealed no correlation between the level of proliferating DP cells and the frequency of multifunctional DP cells (Figure 11A and B). This was
observed in both the acute and controller cohorts.
Figure 10: HIV Antigen Specific Responses. A, The relative proliferation of the DP compartment against each HIV antigen pool for patients in the acute cohort. B, within the controller cohort. C, The frequency of antigen specific 3, 4 or 5 function memory T cells responding to each HIV antigen pool in the acute cohort. D, within the controller cohort.
Figure 11: Relationship between Multifunctionality and Proliferation. A, The frequency of antigen specific 3, 4 or 5 function DP cells is plotted against the relative proliferation of DP cells in response to gag stimulation. B, in response to VVNRT stimulation.
**Table 3: p-values From Comparing Acute DP Functions to Controller DP Functions**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>GP120</th>
<th>GP41</th>
<th>Gag</th>
<th>Pol 1</th>
<th>Pol 2</th>
<th>VVNRT</th>
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<tbody>
<tr>
<td>Relative Proliferation</td>
<td>0.6074</td>
<td>0.4121</td>
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<td>0.2659</td>
<td>0.1427</td>
<td>0.8849</td>
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<td>Frequency of 3,4 &amp; 5 Function Cells</td>
<td>&lt; 0.0001</td>
<td>0.0294</td>
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<td>0.0909</td>
<td>0.001</td>
<td>0.0041</td>
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2.3.4 Relationship between responses within the DP subset and the CD4+ and CD8+ T-cell subsets.

It is not clear if DP cells leave the thymus as DP cells or if they are derived in the periphery from the CD4 and/or CD8 compartments. In order to gain some insight into the origins of the DP response we also determined the degree to which the magnitude of HIV-1-specific DP responses resembled that found within either the CD4 or CD8 compartments. Within the acute cohort, the level of proliferated DP cells was directly related to the proliferation observed within the CD4 compartment following stimulation with each HIV antigen (Figure 12A and not shown). Furthermore, the magnitude of DP proliferation following stimulations with Gag, both Pol pools and VVNRT also correlated with the proliferation observed in the CD8 compartment (Figure 12A and not shown).

Similarly, the controller DP proliferative response correlated with the CD4 response following stimulations with Gag and both Pol pools (Figure 12B and not shown). While the DP anti-Gag and VVNRT proliferative response correlated with the observed CD8 proliferation. Conversely, there were no significant correlations between the acute DP multifunctional response and the CD4 response. The magnitude of the acute anti-gp120 multifunctional DP response did correlate with the multifunctional frequencies observed in the CD8 compartment (Figure 12C and not shown). Meanwhile the controller DP multifunctional responses did not correlate with any CD4 or CD8 multifunctional responses (Figure 12D and not shown). The p-values obtained from each of these comparisons is summarized in Tables 4 and 5.
Figure 12: CD4 or CD8 Responses vs. DP Responses. A, The antigen specific relative proliferation in the CD4 or CD8 compartment for each patient is plotted against that patient’s antigen specific relative proliferation in the DP compartment for patients in the acute cohort. B, for patients in the controller cohort. C, The antigen specific frequency of 3, 4 or 5 function CD4 or CD8 cells is plotted against that patient’s antigen specific frequency of 3, 4 or 5 function DP cells for patients in the acute cohort. D, for patients in the controller cohort.
Table 4: p-values From Linear Regression Analysis of DP Proliferation vs. CD4 or CD8 Proliferation

<table>
<thead>
<tr>
<th>Proliferation</th>
<th>GP120</th>
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<th>Gag</th>
<th>Pol 1</th>
<th>Pol 2</th>
<th>VVNRT</th>
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</thead>
<tbody>
<tr>
<td>Acute DP vs. CD4</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.0111</td>
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<tr>
<td>Acute DP vs. CD8</td>
<td>0.9695</td>
<td>0.1022</td>
<td>0.0128</td>
<td>0.0006</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Controller DP vs. CD4</td>
<td>0.0814</td>
<td>0.7922</td>
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<td>&lt; 0.0001</td>
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<td>Controller DP vs. CD8</td>
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Table 5: p-values From Linear Regression Analysis of DP Multifunctionality vs. CD4 or CD8 Multifunctionality

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<th>Antigen</th>
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<th>Gag</th>
<th>Pol 1</th>
<th>Pol 2</th>
<th>VVNRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute DP vs. CD4</td>
<td>0.8183</td>
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<td>Acute DP vs. CD8</td>
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<td>Controller DP vs. CD4</td>
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<td>0.9583</td>
<td>0.2861</td>
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<tr>
<td>Controller DP vs. CD8</td>
<td>0.5481</td>
<td>0.7554</td>
<td>0.504</td>
<td>0.7157</td>
<td>0.694</td>
<td>0.5631</td>
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</table>

*a Significant p-values are driven by a single outlier patient response*
2.4 Discussion

Relative to the extensive analysis of the CD4+ and CD8+ T-cell compartments, the role of circulating CD4+CD8+ T-cells (DP cells) in immune responses to pathogens and cancer has rarely been studied. As a result, their immune function is poorly understood. In this study, we present the first ever evidence of antigen-specific DP cells simultaneously exhibiting 3 or more functions.

Previously, a cohort of patients recently infected with HIV-1 was shown to contain DP cells which produced IFN-γ alone or in combination with either IL-2 or expression of CD107a in response to HIV(138). This prior work was reported as the response to the HIV proteome as a whole, leaving the antigen-specific breadth of DP cells unknown. Additionally, we present the first ever examination of HIV-specific DP cells within patients who naturally control HIV-1 infection. In this study, we sought to determine the antigen specificity of DP cells and if DP cells were capable of mounting a wide variety of response modalities (CD107a, IFN-γ, IL-2, MIP-1β, and/or perforin as well as proliferation) or were restricted to a narrow range of response types. This was done using peptide pools representing the majority of the HIV proteome to stimulate cells isolated from acutely infected HIV patients. This study has shown that within these patients, DP cells represent an immune subset which is capable of proliferation and mounting a multifunctional response. We show for the first time that antigen-specific DP cells are capable of expressing 3 or more functions at a time. In fact, within the acute cohort studied here, anti-HIV T-cells expressing all 5 functions were almost exclusively DP cells (data not shown). Moreover, the multifunctional cells from the acute cohort
were primarily focused on the VVNRT peptide pool, while the controller DP cells were focused on non-Env antigens.

The presence of multifunctional cells within the CD8 compartment during chronic infection has previously been shown to correlate with improved disease outcomes(113). Therefore, it will be important to track the DP response in treatment naïve patients to determine if multifunctional DP cells are as protective as multifunctional CD8 cells. Interestingly, the presence of multifunctionality in the DP population did not correlate with multifunctionality in the CD8 compartment. Our previous findings have shown that it may take as long as 55 weeks to develop significant multifunctional CD8 responses(92). As a result, it is unclear if there is truly a lack of a relationship between DP and CD8 multifunctionality or if multifunctionality within the DP compartment may precede development of CD8 multifunctionality. Nonetheless, within our limited cohort of controllers there was a correlation between the frequency of multifunctional DP cells and lower viral loads at the time of sample acquisition. This suggests that multifunctional DP cells may have an impact on the successful control of virus replication. Importantly, not all controllers exhibit high levels of multifunctional DP cells. Therefore, DP cells are not the sole mechanism by which control can be achieved. Instead, they may act in combination with other important immune response modalities such as ADCC, CNAR and/or neutralizing antibodies.

We have shown that HIV-specific DP cells display both proliferative and multifunctional capabilities. While both types of immune response were present within the DP compartment, their magnitudes were not correlated with each other. This
indicates that these are distinct response modes. Although since the assays were performed in parallel rather than simultaneously or in series this could not be demonstrated conclusively. These two qualitative aspects of the anti-HIV immune response have previously been correlated with improved disease outcomes when examined within other T-cell compartments. It is important to note that the samples in this study were obtained while the patients’ immune response would be expected to establish a partial control of viral replication toward the viral setpoint. Therefore, the DP responses described here are temporally associated with an expected decline in viral load and consequently may be important mediators of the temporary viral control experienced late in acute infection. For that reason it will be important to isolate HIV-specific DP cells in order to test their ability to inhibit HIV in vitro either through direct inhibition of viral replication, by cytotoxic killing of HIV infected cells and/or stimulation of antibody dependent cellular cytotoxicity. Additionally, recent improvements in humanized mouse models may allow the selective addition and subtraction of DP cells in order to determine the role DP cells may play in the initial decline in viral loads following acute infection (169-172). If DP cells are able to not just respond to HIV antigens but in fact inhibit HIV they will become a therapeutic target. It is important to note that a therapeutic vaccine trial has previously shown the ability to significantly increase HIV-specific IFN-γ production by DP cells in chronically infected patients with a single intramuscular injection of REMUNE (a gp120 depleted immunogen) (50). While unsuccessful in improving disease outcomes, this vaccine trial was able to demonstrate that not only are HIV-specific DP cells present in chronic infection but that memory T-cell responses
result in the expansion of HIV-specific DP cells in addition to traditional CD4 and CD8 expansion. The combination of this vaccine trial and our finding of a correlation between DP multifunctionality and improved viral control indicate the potential of DP cells within the context of therapeutic HIV vaccine development. Additionally, Pahar and colleagues have shown that DP cells are highly enriched in the gut which is an important target site for protective HIV vaccine development (49). Our study revealed that the circulating HIV-specific multifunctional DP population generally lacks expression of CD57 and is therefore not terminally differentiated. Thus, it may be possible to generate a long lasting protective DP memory pool. Unfortunately, Brenchley et.al. have shown that CD57 memory T-cells preferentially harbor HIV (156). Therefore, the DP cells we may want to elicit for protective immunity may also be the subset of DP cells HIV prefers to infect. As a result, extensive examination of dynamics between DP mediated anti-HIV responses and infectability may be necessary.

Given previous work showing that DP cells are generally highly differentiated, our observation that they show evidence of proliferation following 6 days raises important questions about DP cell origins, at least in the HIV-1 infection model. Since a portion of DP cells resemble central memory T-cells or even naïve T-cells it is possible that the DP population is a self-sustaining population (138). Alternatively, a portion of single positive CD4 and/or CD8 T-cells could transition to DP status following antigenic activation and differentiation. In fact, previous work has shown that heavy stimulation (CD3/28, SEB, etc.) of CD8 T-cells sustained over multiple days causes dim expression of CD4 (159, 173, 174). We observed CD4^{bright} in addition to CD4^{dim} DP cells within
both stimulated and unstimulated conditions therefore sustained strong stimulation of CD8 cells is unlikely to explain the origin of the DP cells described in this study.

Additionally, DP cells were also present and highly active in the ICS assay for which stimulations only lasted 6 hours rather than multiple days as in the above studies. If the HIV-specific DP cells we observe are originating from a single positive population the strong correlation between proliferated CD4 and DP cells means that it is likely that the CD4 compartment would be the primary source. Similarly, Colombatti and colleagues’ work showing that DP cells show greater clonal similarity with CD4 cells than CD8 cells further supports this hypothesis(175).

In summary, we have demonstrated that DP cells are capable of mounting a robust and highly diversified response to HIV antigens. Additionally, the DP response is maintained in viral controllers and similar responses within the CD4 and CD8 compartments have previously been correlated with improved disease outcomes(113, 127, 128, 164). Therefore, it is important that this study form the basis of further work delineating the effect this response has on viral replication and long term disease outcomes. Furthermore, these DP cells may be an important vaccine target which means we must work to more fully understand DP cell origins and development. In total, we have established DP cells as a major responding population to acute HIV infection and established a basis for extensive exploration of their origins and role in successfully combating HIV and other pathogens.
Chapter 3. Longitudinal Analysis of the Anti-HIV T-cell Response in the Presence of HAART

3.1 Introduction

Many aspects of both the innate and the adaptive immune response to HIV take shape only after exposure to HIV for multiple weeks or months. There is a well described cytokine storm which is initiated immediately upon infection with HIV(87). This storm begins with the production of IL-15, IFN-α, and TNF-α. Serum IL-15 and IFN-α quickly return to basal levels, while TNF-α remains elevated through peak viremia. Subsequent to the initial cytokine increases IL-10, IL-18, and IFN-γ are elevated within patients’ serum. Additional cytokine serum levels wax and wane as acute infection progresses. In total, these secretions of the innate and adaptive immune system create an ever evolving cytokine milieu under which the humoral and cellular immune response to HIV develops.

The humoral response to HIV also develops along a well described pathway(176). Initially, antibodies are directed against non-neutralizing gp41 epitopes. Followed only months later by antibodies against neutralizing gp120 epitopes of autologous virions(177). Even then only a small subset of patients will ultimately develop broadly neutralizing antibodies(178). Despite the lack of broadly neutralizing antibodies, the anti-HIV antibody response develops autologous neutralizing antibodies as well as other antiviral functions such as antibody dependent cellular cytotoxicity (179-181). Within the cellular immune response, polyfunctional CD8 responses have been shown to develop over the course of the initial year following infection (92). The frequency of these
polyfunctional T-cells during the chronic stage of infection has been correlated with improved disease outcomes (113). Therefore, continued exposure to HIV antigens may be necessary for the long-term development of these and other critical anti-HIV immune responses. Conversely, CD4 T-cell proliferative and cytolytic responses are detectable early in infection and have been correlated with delayed disease progression (127, 182, 183). Similar to the CD4 correlates and unlike the important antibody and CD8 T-cell responses, multifunctional DP T-cells are present during the acute stage of HIV infection (143). Consequently, long term exposure to HIV antigens is not necessary for their initial development, although it is unclear if consistent antigenic stimulation is necessary for their maintenance.

The administration of HAART has been shown to rapidly decrease circulating viral loads to undetectable levels. Unfortunately, the cost, frequency/duration and side-effects of HAART lead to a variety of adherence issues. In turn, poor adherence to HAART regimens can lead to the evolution of HIV strains resistant to HAART. Therefore, historically many clinicians have delayed HAART initiation until late in the chronic phase of infection and/or the onset of AIDS when patients are most at risk of opportunistic infections. Over time reductions in cost, side-effects and burdensomeness of treatment as well as the recognition of improved immunologic restoration following early-HAART have led to earlier recommended HAART initiation time points (78, 184-187). Recently, the early administration of HAART has also been shown to reduce transmission rates (142). Therefore, the initiation of HAART upon diagnosis with HIV is becoming increasingly common (185, 188). It is unclear how this change in treatment
philosophy may alter the long-term immune response to HIV. We hypothesized that while the early removal of HIV antigens improves the restoration of the wider immune system it could stunt the development of a robust CD8 response. In addition, the rapid removal of HIV antigens could deprive multifunctional DP T-cells of the antigenic stimulation necessary for their long-term survival. Therefore we sought to describe the longitudinal T-cell response within a cohort of patients initiating therapy during the acute phase of HIV infection. Additionally, we analyzed a small group of patients who heterogeneously delayed HAART initiation, as well as a group of viral controllers.

3.2 Materials and Methods

3.2.1 Subjects

Persons with acute HIV infection were identified based on clinical presentation or by screening conducted by the state of North Carolina’s Screening and Tracing Active Transmission (STAT) Program has identified individuals with acute HIV infections (AHI) since 2002. Subjects are identified as being acutely infected through a combination of reported symptoms and serology(165). Acutely infected patients were then referred for further evaluation at either Duke University or the University of North Carolina-Chapel Hill. Following the provision of written informed consent the referred patients were enrolled in either studies of antiretroviral treatment or an untreated longitudinal study (depending on patient choice) if they were 1) EIA Negative and nucleic acid amplification test (NAT) positive 2) EIA positive, NAT positive, Western Blot negative/indeterminate or 3) EIA positive, NAT positive, Western Blot positive and documented EIA negative within 45 days. Thirty-five patients who had been infected a
median of 43 days (range 22-105) before study entry were enrolled (Tables 6 and 7) (165). Due to the study location all patients were presumed to have been infected with HIV-1 clade B viruses. At study entry blood samples were acquired from these patients and peripheral blood mononuclear cells (PBMCs) were isolated using standard density gradient centrifugation. Isolated PBMCs were cryopreserved in fetal calf serum supplemented with 10% DMSO and stored in vapor phase liquid nitrogen within 8 hours from collection. Viral load and CD4 counts were also obtained at all time points for which patient with samples were available. At study entry, this patient cohort had a median viral load of 210,521 (range 688-11,503,872) copies/mL and median CD4 count of 448 (range 6-1,912) cells/mm$^3$. Within 8 weeks of study entry 21 patients initiated HAART therapy (early therapy) while the remainder ($n = 14$) delayed the initiation of therapy a median of 397.5 days (range 81- >721) days.

Additionally, 9 virus controllers were separately recruited from the Duke Adult Infectious Diseases Clinic with informed consent under Duke University Medical Center IRB approval. Virus controllers were required to have been diagnosed as HIV positive for greater than 1 year, be antiretroviral therapy naïve, have CD4 counts >600 cells/mm$^3$ blood and have been controlling virus replication to less than 2,700 viral RNA copies/mL blood (Table 2) (133, 166, 167).
**Table 6: Early HAART Group**

<table>
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<tr>
<th>Patient</th>
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<th>CD4 Count</th>
</tr>
</thead>
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Table 7: Delayed HAART Group

<table>
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<tr>
<th>Patient</th>
<th>Days post infection</th>
<th>Viral Load</th>
<th>CD4 Count</th>
<th>Days to Rx</th>
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<td>54</td>
<td>6281</td>
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<tr>
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3.2.2 Peptides

Fifteen amino acid peptides overlapping by 11 amino acid residues representing the HIV-1 Clade B consensus sequences of ENV (#9480), Gag (#8117), Nef (#5189), Pol (#6208), Rev (#6445), Tat (#5138), VPR (#6447) and VPU (#6444) were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Individual peptides were resuspended in DMSO and pooled, at a final concentration of 500μg/mL, into a total of six peptide pools representing gp120, gp41, Gag, Pol peptides #5461-5585, Pol peptides #5586-5709, and a combination of Nef, Rev, Tat, VPR and VPU (VVNRT).

3.2.3 Proliferation Assays

Cryopreserved PBMC were thawed and washed twice with RPMI containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (R10) and enumerated using a Guava Count system (Millipore). Following cell counting the PBMCs were washed twice with PBS, and subsequently resuspended at 20x10^6 cells/ml. The cells were stained with Carboxyfluorescein succinimidyl ester (CFSE) for 8 minutes mixing once at 4 minutes(168). CFSE staining was quenched with 100% human serum for 2 minutes, and after washing with PBS, the cells were resuspended in R10 containing human serum. CFSE stained cells were then plated at 1x10^6 cells/mL and stimulated with peptide pools representing HIV GP120, GP41, Gag, Pol pool 1, Pol pool 2, VVNRT(1 ug/mL) or anti-CD3 (eBioscience) and -CD28 (BD Biosciences) antibodies. Stimulated cells were then incubated at 36°C with 5% CO₂ for 6 days. Following 6 days of stimulation the cultures
were washed twice with PBS and stained with a violet vital dye (Invitrogen) for 20 minutes at room temperature in the dark. Cells were again washed twice with PBS and then stained with anti-CD3 APC-Cy7 (BD Biosciences; clone SK7), -CD4 PE-Cy5.5 (eBioscience; OKT4) and -CD8 Qdot605 (Invitrogen; 3B5) antibodies for 20 minutes at room temperature in the dark. Following antibody staining the cells were washed thrice with PBS and fixed with 1% paraformaldehyde. Stained and fixed cells were then refrigerated in the dark until acquisition.

3.2.4 Intracellular Cytokine Staining Assays

Cryopreserved PBMC were thawed, resuspended at 2x10^6 cells/mL in R10 and rested overnight at 36°C with 5% CO₂. After the overnight rest the PBMCS were counted and resuspended at 1x10^6 cells/mL in R10. Cells were then stimulated for 6 hours at 36°C with 5% CO₂ with peptide pools representing CMV pp65, HIV GP120, GP41, Gag, Pol pool 1, Pol pool 2 or VVNRT (1 ug/mL). Stimulations were performed in the presence of 1ug/mL each of anti-CD28 (BD Biosciences; L293) and CD49d (BD Biosciences; L25) antibodies, anti-CD107a PE-Cy5(eBioscience; H4A3), 5ug/mL Brefeldin A (Sigma-Aldrich) and 1 ug/mL Monensin (BD Biosciences). Following the stimulation, the cells were washed with PBS containing 1%FCS and surface stained with Aqua Blue vital dye (Invitrogen), anti-CD4 PE Cy5.5 (eBioscience; OKT4), -CD8 Qdot605 (Invitrogen; 3B5), -CD27 PE-Cy7 (BD Biosciences; M-T271), -CD57 Qdot565 (AbD Serotec; TB01) and –CD45RO PE-TR (Beckman Coulter; UCHL1) for 20 minutes at room temperature in the dark. After surface staining, the cells were washed again with
PBS containing 1% FCS and subsequently fixed and permeabilized with cytofix/cytoperm and perm/wash buffer (BD Biosciences) for 20 minutes and washed twice. Following the fix/perm step cells were stained intracellularly with anti-CD3 Qdot655 (Invitrogen; S4.1), -IFN-γ Alexa700 (BD Biosciences; B27), -IL-2 APC (BD Biosciences; MQ1-17H12) -MIP-1β FITC (R&D Systems; 24006), and –perforin PE (Cell Sciences;B-D48) for 20 minutes at room temperature in the dark. Subsequent to intracellular staining cells were washed with perm/wash buffer and then fixed with 1% paraformaldehyde. Following fixation cells were refrigerated in the dark until acquisition.

3.2.5 Flow Cytometry Acquisition and Analysis.

Within 18 hours of staining, fully stained cells from the proliferation and intracellular cytokine staining (ICS) assays were acquired on a custom LSRII (BD Biosciences) using FACSDiva. Following acquisition flow data was analyzed using FlowJo software v.9.3.2 (TreeStar). For all assays gates were set to include singlet events, live CD3+ cells, lymphocytes and CD4+/CD8+/CD4+/CD8+ subsets. For the proliferation assays CFSElow populations were then identified from each lymphocyte subset (Figure 4). For the ICS assays the naïve population (CD27+CD45RO−) was identified and excluded from each lymphocyte subset. Within the memory population, cellular function positive populations were identified individually for all cellular functions except perforin which was only defined as positive if both perforin+ and IFN-γ+ (Figure 5). Using a Boolean gating strategy, the 32 combinations of the 5 cellular
functions were identified. Based on these frequencies we also calculated the total frequency of families of subsets expressing the same number of functions.

3.2.6 Statistical Analysis.

For the proliferation assays relative proliferation values were obtained by subtracting the average CFSE\text{low} population frequency of a patient samples’ two unstimulated wells from the CFSE\text{low} population frequency following stimulation and then dividing the resulting value by the average CFSE\text{low} population frequency of a patient samples’ two unstimulated wells. For the intracellular cytokine staining assays Pestle was used for background subtractions and for frequency analysis Prism (Graphpad) and SPICE were used. PESTLE and SPICE were kindly provided by Dr. M. Roederer, Vaccine Research Center, NIH, Bethesda, MD.

DP response ratios were calculated by first multiplying each patients’ frequency of HIV-specific DP, CD4 and CD8 T-cells by the mean cell count in the DP, CD4 and CD8 compartments across all stimulation conditions to obtain a normalized HIV-specific DP, CD4 and CD8 cell count. The normalized HIV-specific DP cell count was then divided by the sum of the normalized HIV-specific DP, CD4 and CD8 cell counts to give the DP response ratio.

Comparisons of responses within patient groups were performed using a Wilcoxon Matched Pairs test (Prism). Comparison across patient groups used a Mann-Whitney U test (Prism). Correlations were obtained using linear regression (Prism). No adjustments for multiple comparisons were performed and P values should be interpreted with this in mind.
3.3 Results

3.3.1 Longitudinal Analysis of T-cell Proliferation

Previous work showed that CD4, DP and CD8 cells within our acute patient cohort exhibited similar total HIV proliferative responses at study entry when compared to viral controllers. In order to examine the evolution and longevity of this response we stimulated PBMCs obtained approximately 24 and 104 weeks following study entry. Follow up patients were divided into two groups according to the timing of HAART initiation. One group received HAART within 2 weeks of study entry (“treated group”, n = 21) while the second group delayed HAART initiation beyond the acute phase of HIV infection (“delayed group”, n = 14).

Within the CD4 and DP T-cell compartments total HIV-specific proliferation did not significantly differ between all time points for both the treated and delayed groups (Figure 13A, B). With the exception of the treated week 104 time point’s proliferative response being significantly lower than the delayed week 24 time point, the CD8 compartment also showed no significant alterations to longitudinal HIV-specific proliferation (Figure 13C). This is likely a result of mild but not significant decreases over time in the proliferative capacity of the early treatment group’s CD8 compartment paired with a mild but not significant increase in proliferative capacity from entry to week 24 in the delayed therapy group. Similarly, all time points across both treatment groups showed similar proliferative responses to the HIV controllers.
Figure 13: Longitudinal HIV-specific Proliferation. Cells were stained with CFSE and stimulated for 6 d with peptide pools representing the HIV proteome. The HIV-specific relative proliferation was calculated for each T cell subtype and plotted for each patient in each treatment cohort (T = treatment initiated early, D = delayed treatment initiation) A, CD4 compartment. B, DP compartment. C, CD8 compartment. * = p < 0.05
3.3.2 Cross-Compartment Analysis of Proliferative Response

Viral controllers exhibit a significantly higher level of HIV-specific relative proliferation within the DP compartment than within the CD4 compartment (Figure 14A). At study entry the DP T-cell proliferative response to HIV is significantly higher than either the CD4 or CD8 response (Figure 14A). At week 24, patients who were treated immediately exhibit a similar relative response profile as at entry (DP response > CD4/CD8 response) while the delayed treatment group resembles the viral controllers (DP response > CD4 response) (Figure 14B). Similarly, at week 104 the early treatment group still maintains the entry response profile. Meanwhile the delayed treatment group exhibits no significant difference between CD4, DP or CD8 proliferative responses and therefore has lost the controller response profile (Figure 14C). While the significant differences between DP and CD4 or CD8 relative proliferation levels was lost over time in the delayed therapy group these changes do not manifest themselves in significant changes to the DP response ratio (Figure 15).
Figure 14: Cross-Compartment Comparison of HIV-specific Proliferation. Cells were stained with CFSE and stimulated for 6 d with peptide pools representing the HIV proteome. The HIV-specific relative proliferation was calculated for each T cell subtype and plotted for each patient A, Entry and Controller samples. B, Week 24 samples. C, Week 104 Samples. * = p < 0.05, ** = p < 0.01, *** = p < 0.001
Figure 15: Proliferative Response Ratio. Cells were stained with CFSE and stimulated for 6 d with peptide pools representing the HIV proteome. The percentage of the total anti-HIV proliferative response coming from the DP compartment was calculated as described in the methods for each patient and plotted (T = treatment initiated early, D = delayed treatment initiation).
3.3.3 Longitudinal Analysis of T-cell Multifunctionality

The ability of T-cells to simultaneously perform multiple functions in response to HIV has been associated with improved disease outcomes. Therefore, we also analyzed the ability of the patients’ CD4, DP and CD8 T-cells to produce IFN-γ, IL-2, MIP-1β, and Perforin as well as express CD107a. The CD4, DP and CD8 compartments exhibit higher frequencies of HIV-specific multifunctional (≥3 functions) T-cells at entry than within the controller cohort (Figure 16A-C). Within the CD4 and CD8 compartments, this differential is rapidly lost whether HAART is initiated immediately or delayed (Figure 16A, C). The DP compartment exhibits a similar pattern when therapy is initiated early (Figure 16B). However, when therapy is delayed multifunctional DP cells are maintained at frequencies similar to study entry and higher than the controller cohort (Figure 16B).
Figure 16: Longitudinal HIV-specific Multifunctionality. Cells were stimulated for 6 h with peptide pools representing the HIV proteome and then stained for expression of CD107a, IFN-γ, IL-2, MIP-1β, and perforin. Using Boolean gating expression of all possible combinations of these functions was determined. The HIV-specific frequency of cells expression three, four, or five of these functions within each T cell subtype was plotted for each patient. (T = treatment initiated early, D = delayed treatment initiation) A, CD4 compartment. B, DP compartment (Median D-Wk24 = 4.81, D-Wk104 = 5.69). C, CD8 compartment. * = p < 0.05, ** = p < 0.01, *** = p < 0.001
3.3.4 Cross-Compartment Analysis of Multifunctionality

The frequency of multifunctional T-cells was also compared across T-cell compartments. Similar to the proliferation response, controllers exhibited a higher frequency of multifunctionality within the DP compartment than the CD4 compartment (Figure 17A). At study entry, the DP compartment of acute patients exhibited significantly higher multifunctional frequency than either the CD4 or CD8 compartment (Figure 17A). Irrespective of when therapy was initiated this relationship between the DP compartment and both the CD4 and CD8 compartment was maintained at both weeks 24 and 104 (Figures 17B and C). Multifunctional DP response ratios did not exhibit significant differences across all patient groups and time points, although this may be due to the high subject to subject variability observed in this measurement (Figure 18). To assess the degree to which multifunctional DP cells were differentiating as acute disease gave way to chronic infection we also assessed CD57 expression (a marker for terminal differentiation). Across all patient groups and at all time points greater than 90% of multifunctional DP cells were found to lack CD57 expression meaning that these cells were capable of being long-lived and/or further expansion (Figure 19).
Figure 17: Cross-Compartment Comparison of HIV-specific Multifunctionality. Cells were stimulated for 6 h with peptide pools representing the HIV proteome and then stained for expression of CD107a, IFN-γ, IL-2, MIP-1β, and perforin. Using Boolean gating expression of all possible combinations of these functions was determined. The HIV-specific frequency of cells expression three, four, or five of these functions within each T cell subtype was plotted for each patient. (T = treatment initiated early, D = delayed treatment initiation) A, Entry and Controller samples. B, Week 24 samples. C, Week 104 Samples. * = p < 0.05, ** = p < 0.01, *** = p < 0.001
Figure 18: Proliferative Response Ratio. Cells were stimulated for 6 h with peptide pools representing the HIV proteome and then stained for expression of CD107a, IFN-γ, IL-2, MIP-1β, and perforin. Using Boolean gating expression of all possible combinations of these functions was determined. The percentage of the total anti-HIV multifunctional response coming from the DP compartment was calculated for each patient and plotted (T = treatment initiated early, D = delayed treatment initiation).
**Figure 19: CD57 expression.** The percentage of multifunctional DP cells lacking expression of CD57 was determined for each patient and plotted. (T = treatment initiated early, D = delayed treatment initiation).
3.4 Discussion

The early initiation of HAART has been shown to generate superior outcomes for both controlling the spread of HIV and limiting immune damage. As a result, it has been suggested that therapeutic intervention begin before some portions of the normal immune response to HIV begin. In this study, we present a longitudinal analysis of the HIV-specific immune response following HAART initiation during acute infection. In addition, we present the first analysis of the longevity of antigen-specific DP T-cells following the removal of large-scale antigenic stimulation.

Previous work showed that the frequency of multifunctional cells within the CD8 compartment increased throughout the first 84 weeks of infection (92). The aforementioned work was performed in the absence of therapy and focused on autologous Gag-specific responses. In the present study, we sought to examine the ability of not only multifunctional CD8 T-cells but also CD4 and DP T-cells to develop and/or be maintained in the presence of HAART. Furthermore, we expanded the breadth of analysis to include stimulation with nearly all of the HIV proteome and the analysis of HIV-specific proliferative responses in addition to multifunctionality.

We report that following the early initiation of HAART there is no increase in HIV-specific proliferation or multifunctionality within the CD4 or CD8 compartment. In the cohort for whom the initiation of HAART was delayed, the patients did not show a durable increase in either CD4 or CD8 proliferation or multifunctionality. In fact, the removal of antigenic stimulation appeared to result in a small decrease in the frequency of multifunctional CD8 cells. This is consistent with the waning of many vaccine
induced immune responses following long periods without antigenic stimulation in the form of either infection or the administration of booster vaccine doses (189). At first glance, the absence of an increase in CD8 multifunctionality within the delayed therapy cohort is contradictory to previous findings(92). Therefore, it is important to note that the functions analyzed in the two studies differ in that the prior work examined TNF-α while this study examined perforin expression instead. In fact, the prior work also revealed that the majority of TNF-α⁺ cells were also IFN-γ⁺. As a result, the lack of TNF-α may have reduced the reported CD8 multifunctionality. In addition, the prior study utilized autologous peptide sequences compared to the consensus sequences presented here. Minor differences in sequence recognition may result in differential responses to the antigenic stimulation. Consequently, a lack of homology between a patient’s circulating virus and the consensus sequence may have resulted in reduced detection of CD8 multifunctionality. Finally, in the previous work CD8 multifunctionality was largely absent in samples obtained prior to 55 weeks post onset of symptoms. Only the third and final timepoint (week 104) of the present study was after 55 weeks of infection. By week 104, 8 of the delayed therapy cohort had already initiated therapy. As a result, during the timeframe when CD8 multifunctionality would be expected to appear, the majority of delayed therapy patients lacked large scale in vivo antigenic stimulation.

The DP compartment was shown to experience an even more marked decline in multifunctional frequency following early HAART initiation despite the maintenance of HIV-specific proliferation. Significantly, patients who delayed the initiation of HAART did not exhibit significantly lower multifunctional DP cell frequency compared to study
entry. Conversely, this group also did not show a consistently higher multifunctional frequency than the early treatment group. It is possible that the heterogeneous mixture of therapy initiation times within the delayed therapy group is responsible for these seemingly conflicting results. Supporting this is the fact that Howe and colleagues observed an increase in bifunctional HIV-specific DP T-cells from early to chronic infection in the absence of therapy(138). It is unclear if the administration of a vaccine or structured treatment interruptions would cause these multifunctional cells to return and/or expand rapidly. Given the lowered frequency of HIV-specific multifunctional DP and CD8 T-cells following the administration of therapy it may be difficult to specifically target them within the context of a therapeutic vaccine. Therefore, if multifunctional DP and/or CD8 T-cells prove important to a successful therapeutic vaccine strategy it may be vital to delay therapy initiation until these cells develop a more durable response. While our analysis revealed that the frequency of both DP and CD8 T-cells with multifunctional HIV-specific responses declined following early HAART administration, DP multifunctionality was nonetheless maintained at a higher frequency than CD8 multifunctionality. This may be due to higher levels of memory cells being established before therapy initiation and/or the DP compartment being more sensitive to low levels of viral replication. Consequently, the need to delay therapy to ensure significant multifunctional frequency may be less pronounced within the DP compartment.

When considering the possibility of targeting HIV-specific cells for therapeutic expansion it is important to know their differentiation status. Previous work showed that the DP compartment is generally highly differentiated (49). However our findings in
Chapter 2 revealed that within the subset of DP-cells which are specific for HIV and multifunctional, terminal differentiation is rare during the acute phase. Consistent with the DP compartment’s maintained proliferative capability, we show that this lack of terminal differentiation is maintained regardless of the timing of HAART initiation. Accordingly, allowing the multifunctional population to expand and/or be maintained at a high level will not render them unresponsive to therapeutic attempts at expansion.

In summary, we have demonstrated that DP cells consistently respond to HIV antigens at similar or greater frequencies compared to CD8 T-cells and that the timing of HAART initiation effects long term HIV-specific multifunctional T-cell but not proliferative frequencies. As a result, this study reveals the need for further studies elucidating if multifunctional frequency is causative of improved disease outcomes or merely correlated with those outcomes. If multifunctional frequency is in fact altering disease outcomes this study also reveals that minimum frequencies for the desired effect must be established. In turn, these minimum frequencies can then be used to better inform physicians and public health officials on the proper timing of HAART initiation and/or evaluate the effectiveness of therapeutic interventions targeting the expansion of multifunctional populations.
Chapter 4. Effect of HLA-type on the Earliest Anti-HIV Response within Peripheral Blood

4.1 Introduction

The role of adaptive immunity against HIV-1 infection has been widely discussed in regard to both humoral and cellular mediated immunity. While many correlates of protection have been identified neither antibodies acting via neutralization and/or ADCC nor CTL responses have been identified as surrogates of protection in long-term non-progressor (LTNP) individuals (190-194). As a result, we are left without an unequivocal correlate of protection that may be related to the innate or adaptive components of the immune system. Despite this lack of a clear surrogate of immune protection within the innate and/or adaptive immune responses, it has been shown that expression of particular HLA alleles has a strong correlation with progression to overt disease (118, 120).

Therefore a question remains: what host factors are linked with HLA allele expression and responsible for the epidemiological heterogeneity in infection susceptibility and progression rates? In fact, following exposure to HIV-1 gene expression and activation, of cellular subsets involved in the immune response, may occur at different levels in selected individuals (i.e. HLA-B57* +). These different pathways of gene activation/expression may ultimately have an impact on disease progression.

The SIV model comparing Rhesus Macaques (RMs) and Sooty Mangabeys (SMs) can provide important clues to the genetic factors determining disease progression. SIV
infection in RMs leads to rapid progression to simian AIDS, characterized by chronic
immune activation and increased T-cell apoptosis. In contrast, SIV infection of SMs is
essentially nonpathogenic and is characterized by minimal T-cell apoptosis and activation
despite the maintenance of detectable virus load. This stark contrast in disease
progression is likely the result of host-virus adaptations leading to differing levels and
persistence of immune activation particularly in interferon signaling pathways in RMs
compared to SMs (195, 196).

In humans, clinical studies have identified patient populations displaying a normal
(NP), rapid (RP), or long-term non-progression (LTNP) pattern to full-blown AIDS
(197). Subsequently, a number of studies have drawn associations between expression of
HLA class I alleles and progression to AIDS (198, 199). Specifically, HLA-B*35 is
significantly over-represented in RP patients, whereas LTNP patient populations are
significantly correlated with expression of HLA-B*27 and B*57 alleles. Interestingly,
among the LTNP individuals, control of virus replication may occur at different clinical
stages. Recently, it has been reported that HLA-B*57+ individuals seem to better control
the early phase of infection (defined as time to CD4 count =200cells/µl), whereas HLA-
B*27+ individuals control virus replication better after this point (200). The importance
of HLA-B*57 has been further confirmed by the work of Fellay and colleagues who have
shown a strong correlation between a single-nucleotide polymorphism (SNP) associated
with the HLA-B*5701 allele and viral setpoint (201). It is possible that in LTNP, the
secretion of cytokines and/or chemokines controls virus replication either directly, by
affecting the ability of the virus to infect and replicate into the target cells (202), or
indirectly, by influencing the level of T cell activation (203). It has also been demonstrated that host gene products belonging to the APOBEC3 family can interact with the virus Vif gene product resulting in inhibition of HIV-1 replication (204). In total these studies indicate that specific gene expression profiles could be involved in the control of early virus replication.

Studies conducted by Brenchley and collaborators have highlighted the rapid depletion of the CD4 T lymphocyte compartment that takes place during the first two weeks of acute HIV-1 infection (205, 206). Despite the recovery occurring in the following weeks it is unclear to what extent certain immune subpopulations are selectively lost. Therefore, it may be difficult to identify pathways of gene expression that could have a significant impact on controlling HIV replication based on the analysis of the surviving/replenished T and NK-cell subsets at the time of HIV diagnosis or establishment of the viral set point. To overcome the uncertainty related to these losses, it is important to examine gene expression profiles before and immediately after infection has occurred. Therefore, in this study we utilized PBMCs from HIV negative individuals expressing HLA-B*35 and HLA-B*57 to evaluate the gene expression profiles of NK, CD4+ T, and CD8+ T cellular subsets before and after in vitro infection with HIV-1.

4.2 Materials and Methods

4.2.1 Subjects

Anonymous HIV negative blood donors expressing HLA-B*35 or HLA-B*57 were identified via the North Carolina Red Cross and Duke University Medical Center. Blood samples were acquired from these patients and peripheral blood mononuclear cells
(PBMCs) were isolated using standard density gradient centrifugation. Isolated PBMCs were cryopreserved in fetal calf serum supplemented with 10% DMSO and stored in vapor phase liquid nitrogen.

4.2.2 Infections

Cryopreserved PBMC were thawed, resuspended at 2x10⁶ cells/mL in R10 and rested overnight at 36°C with 5% CO₂. After the overnight rest the PBMCs were counted and resuspended at 1x10⁶ cells/mL in R10. 2x10⁷ cells were infected/mock inoculated with an CXCR4-tropic HIV-1 virus (SC24-060298), (provided by Drs. Freel, Tomaras, Greenberg) at an MOI of 1 (207). The infected cultures were then incubated for 24 hours at 36°C with 5% CO₂.

4.2.3 Cell Sorting and RNA Isolation

Following 24 hours of infection, cells were washed twice with PBS and stained with a violet vital dye (Invitrogen) for 20 minutes at room temperature in the dark. Cells were again washed twice with PBS and then stained with anti-CD3 APC, anti-CD4 FITC, anti-CD8 PerCP-Cy5.5, anti-CD16 PE-Cy7 and anti-CD56 PE-Cy7 antibodies (BD Biosciences) for 20 minutes at room temperature in the dark. Following antibody staining the cells were washed thrice with PBS and refrigerated in the dark until acquisition.

Fully stained samples were sorted on a BD FACSaria II. Live cells were identified by gating for the lack of violet vital dye staining and SSC. Following the determination of viability singlets were identified using FSC-H and FSC-A. Within the singlet gate lymphocytes were identified using SSC-A and FSC-A. The live singlet
lymphocyte population was further refined using SSC-W and SSC-A followed by FSC-W and FSC-A. The refined live singlet population was then interrogated for CD16/56 and CD3 expression. The CD16/56$^-$CD3$^-$ NK cell population was sorted into a 2mL test tube containing PBS at 4°C. Additionally the CD3$^+$CD16/56$^-$ “Non-T” population was sorted into a 2mL test tube containing PBS at 4°C. The CD3$^+$ population was further interrogated for CD4 and CD8 expression. The CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T-cell populations were sorted into separate 2mL test tubes containing PBS at 4°C. Total RNA was then isolated from each sorted population using an RNeasy kit (Qiagen) according to manufacturer’s instructions. Isolated RNA was eluted into 30µL of RNase free water and stored at -80°C.

4.2.4 Microarrays

Two hundred ng of total RNA was amplified and labeled using the Illumina TotalPrep RNA Amplification kit (Ambion). cRNA quality was assessed by capillary electrophoresis on an Agilent 2100 Bioanalyzer. Expression levels mRNA transcripts were then assessed using an HT12-V3 bead array (Illumina). Hybridization was carried out according to the manufacturer’s instructions.

4.2.5 Statistical Analysis

The arrays were scanned using Illumina’s iSCAN and quantified using Genome Studio (Illumina). Analysis of the GenomeStudio output data was conducted using R (R Development Core Team) and Bioconductor software packages. Random sequence probes are included in the bead arrays to obtain system background. Genes having intensities below system background were removed from the analysis. Quantile
normalization was applied to the remaining genes, followed by a log₂ transformation. The “Linear models for microarray analysis” (LIMMA) package was then used to fit a linear model to each probe and to perform (moderated) t-tests or F-tests on the compared groups (208-210). To control the expected proportions of false positives, the false discovery rate (FDR) for each unadjusted P value was calculated using the Benjamini and Hochberg method implemented in LIMMA. Principle component analysis was performed via multidimensional scaling in R to generate plots for evaluation of similarities or dissimilarities between data sets. Ingenuity Pathway Analysis software (IPA, Ingenuity Systems) was used to annotate genes and rank canonical pathways via gene set enrichment analysis (GSEA). For both the identification of individually significant genes and GSEA a standard of |FC|>1.3, p<0.01 and FDR<25% was used.

4.3 Results

4.3.1 Differential Gene Expression in CD4⁺ T-cells

Transmitted viruses often utilize CCR5 as their coreceptor (72). Effective in vitro infection of CD4 T-cells with these viruses requires the use of either dendritic cell intermediaries or exogenous activation of the T-cells. Both of these transmission methods would introduce potentially confounding variables to the experimental system. Therefore, we performed all infections with a CXCR4 utilizing virus that was capable of infecting resting CD4 T-cells. To ensure that our experimental system was working properly we first analyzed the CD4 RNA samples for HIV LTR expression via a PCR assay. LTR expression was observed at similar levels within both the B*35 and B*57 groups (data not shown). Second we performed a principal component analysis to
determine if the microarray data for each cell type was grouping as expected. This analysis successfully divided the samples into three subgroups consisting solely of NK cells, CD4 T-cells and CD8 T-cells (data not shown). Having shown that the gene expression signatures for the samples were properly clustering by cell type we went on to examine the effect of infection with HIV on CD4\(^+\) T-cells’ gene expression. First each HLA group’s “infection effect” was determined by finding the relative change in each gene’s expression following infection compared to the mock infected samples. Next these “infection effects” were compared across HLA groups to determine the impact B*35 and B*57 expression had on how CD4\(^+\) T-cells respond to HIV-1 exposure. No individual genes rose to significance within the CD4\(^+\) T-cell subset. However, pathways analysis revealed that the IFN-signaling, Activation of IRF by Cytosolic Pattern Recognition Receptors and Role of Pattern Recognition Receptors in Recognizing Bacteria and Viruses pathways were all significantly altered by HLA status (Figure 20A). These pathways contain a number of overlapping genes including DDX58, IFIH1 and IRF7 (Figure 20B-D). In general interferon response genes in these pathways are upregulated following infection in B*35 subjects while B*57 subjects experience limited to no upregulation. These pathways combine to highlight significant differences in the ways pattern recognition receptors and the resulting interferon signaling generate mRNA changes in response to HIV in HLA-B*57 individuals.
Figure 20: CD4 T-cell Pathways Analysis. Gene expression profiles were analyzed to identify canonical pathways with differential infection effects based upon HLA allele status within CD4 T-cells A) Heatmap showing the top 10 differentially expressed canonical pathways following infection. Shades of blue indicate the Benjamini-Hochberg corrected p-values, on a \(-\log_{10}\) scale, for each pathway either within an HLA group or between HLA groups. B) Heatmap of the interferon signaling pathway C) Heatmap of the activation of IRF by cytosolic pattern recognition receptors pathway D) Heatmap of the role of pattern recognition receptors in recognition of bacteria and viruses pathway. Bars below the dendrograms indicate the cell type analyzed and the HLA group of individual subjects. Heatmaps display the infection effect for an individual gene within each subject. An increase in gene expression following infection with HIV is indicated with shades of red, while decreases in gene expression following infection are indicated with shades of blue. Results are presented using a log2 scale.
4.3.2 Differential Gene Expression in CD8+ T-cells

Next we analyzed the differential in “infection responses” within the CD8+ T-cell compartment of B*35 and B*57 subjects. As in the CD4+ T-cell compartment, no individual genes were significantly differentially regulated as a result of HLA status. Pathways analysis revealed significantly elevated levels of the IFN-signaling, Activation of IRF by Cytosolic Pattern Recognition Receptors and Role of Pattern Recognition Receptors in Recognizing Bacteria and Viruses pathways within the B*35 group but not the B*57 group (Figure 21A and B). IFIH1 and IRF7 expression were again implicated as important differences between the HLA groups within the context of these pathways. Finally, the magnitude of the interferon response was again larger within the B*35 group than within the B*57 group.
Figure 21: CD8 T-cell Pathways Analysis. Gene expression profiles were analyzed to identify canonical pathways with significant infection effects based upon HLA allele status within CD8 T-cells A) heatmap showing the top 11 differentially expressed canonical pathways following infection. Shades of blue indicate the Benjamini-Hochberg corrected p-values, on a $-\log_{10}$ scale, for each pathway either within an HLA group or between HLA groups. B) Heatmap of the genes shared among the interferon signaling, activation of IRF by cytosolic pattern recognition receptors and, role of pattern recognition receptors in recognition of bacteria and viruses pathways. Bars below the dendrograms indicate the cell type analyzed and the HLA group of individual subjects. The Heatmap displays the infection effect for an individual gene
within each subject. An increase in gene expression following infection with HIV is indicated with shades of red, while decreases in gene expression following infection are indicated with shades of blue. Results are presented using a log$_2$ scale.
4.3.3 Differential Gene Expression in NK Cells

Finally we analyzed the infection responses of the subjects’ NK cells. This again revealed no individual gene products as being significantly differentially regulated. Similar to the CD4 and CD8 compartments strong interferon pathway responses were observed within the B*35 group but not within the B*57 group (Figure 22A). Again IRF7 was a strong driver of the observed infection response within B*35 subjects (Figure 22B). Additionally the B*57 group exhibited signatures of LXR/RXR activation, PPAR signaling while the B*35 group did not (Figure 22C and D). As with the previous interferon responses these pathways also share genes including NCOR2 and NGFR.
**Figure 22: NK Cell Pathways Analysis.** Gene expression profiles were analyzed to identify canonical pathways with differential infection effects based upon HLA allele status within NK cells. A) Heatmap showing the top 14 differentially expressed canonical pathways following infection. Shades of blue indicate the Benjamini-Hochberg corrected p-values, on a -log_{10} scale, for each pathway either within an HLA group or between HLA groups. B) Heatmap of the role of pattern recognition receptors in recognition of bacteria and viruses pathway for B*35 subjects. C) Heatmap of the PPAR signaling pathway. D) Heatmap of the LXR/RXR activation pathway. Bars below the dendrograms indicate the cell type analyzed and the HLA group of individual subjects. Heatmaps display the infection effect for an individual gene within each subject. An increase in gene expression following infection with HIV is indicated with shades of red, while decreases in gene expression following infection are indicated with shades of blue. Results are presented using a log_{2} scale.
4.4 Discussion

It has long been known that expression of different HLA alleles resulted in highly variable relative risks of infection with HIV and progression to AIDS once one was infected. In particular, HLA B*57 expression is statistically over represented among long-term non progressors while HLA B*35 is over represented among rapid progressors. The mechanisms by which this level of protection/risk is achieved are unknown. Therefore in this study we analyzed how gene expression changes following exposure of a cell culture to HIV differed according to the expression of the B*35 or B*57 HLA alleles. By sorting cells into pure populations of NK cells as well as CD4 and CD8 T-cells subsequent to HIV exposure but before RNA isolation we are able to observe the contribution varied HLA alleles have on each compartments response to HIV while maintaining the intra-compartment interactions which may be vital to a successful immune response to any pathogen.

Previous work in the SIV model has implicated differential TLR7 and TLR9 signaling as being responsible for the differential disease outcomes in monkeys following SIV infection(211). In particular, IRF7 was shown to be expressed at significantly lower levels within Sooty Mangabeys than within Rhesus Macaques. This is consistent with our pathway findings which reflect increased pattern recognition receptor and interferon signaling within the CD4, CD8 and NK cells of B*35 subjects (which may be similar to the Rhesus Macaques) relative to the B*57 subjects (which may be similar to the Sooty Mangabeys). Therefore it appears that inhibition of IRF7 and its associated pathways is a
conserved method for controlling HIV/SIV infections. Although in vivo transmission events generally occur with CCR5 utilizing viruses, in this study, we utilized a CXCR4 virus to directly infect resting CD4 T cells. Both co-receptors involved in HIV-1 entry mediate signal events when bound by HIV(212). As a result, it is possible that important HLA associated differences in gene expression could be HIV strain specific. Additionally, coreceptor usage is determined by differences in the structure of the viral envelope. It is also possible that these differences could result in differential recognition by pattern recognition receptors. Mandl and colleagues showed that SIV mediated activation of IRF7 is triggered by the pattern recognition receptors TLR7/9. These TLRs recognize pathogens via nucleic acids not patterns within their envelopes(213, 214). Therefore, we believe the pathways identified in this study are generalizable to CCR5, dual tropic, and/or alternative coreceptor utilizing viruses.

IRF7 is a strong inducer of IFN-α expression(215). Increased interferon-α production is a hallmark of the cytokine storm immediately following HIV infection and has also been associated with progressing disease (87, 216). In fact, IFN-α production has been implicated in bystander killing of uninfected CD4+ T-cells and the depletion of CD4+ T-cells as disease progresses towards AIDS(217, 218). Therefore, IFN-α mediated immune damage may be a primary driver of progression to AIDS. Interestingly, whereas human controllers keep HIV viremia at low to undetectable levels, Sooty Mangabeys remain asymptomatic while maintaining high levels of SIV viremia. As a result, the most important factor in preventing the onset of AIDS may not be controlling HIV viremia
directly but instead controlling the amount of IFN-α being produced as a result of viral replication.

Using a mouse model IFN-α has also been shown to be a powerful inhibitor of HIV infection(219). Additionally, Martinelli and colleagues have shown that HIV gp120 effectively inhibits TLR9 mediated activation of IFN-α secretion(220). Therefore evolution has led HIV to moderate IFN-α secretion, this could be because of its deleterious effects on the viruses survival. Consequently, simply blocking IFN-α signaling to avoid its bystander effects may not be sufficient. Instead, it may be necessary to fine tune the IFN-α response so that it is strong enough to combat HIV replication but weak enough that the majority of bystander effects are avoided.

In summary, we have shown that the association between improved disease outcomes and reduced IRF7 mediated pattern recognition receptor/interferon signaling previously observed in SIV is conserved in humans’ response to HIV. How this finding relates to other correlates of immune protection including multifunctional T-cells, broadly neutralizing antibodies and immunoglobulin class selection could be addressed in further studies. Therefore, it is important that the links between the various cell types producing these correlates are further explored. Further and larger studies are needed to better understand these connections and thereby inform vaccine antigen and adjuvant design decisions to ensure that they effectively target only the desired immune responses.
Chapter 5: Overall Conclusions and Future Directions

Through more than 25 years of HIV research much progress has been made. Numerous effective therapies have been developed and recently a vaccine showed modest efficacy (221). While this vaccine achieved a 31% efficacy rate at preventing infection it has not been shown to have an effect on the course of disease once someone becomes infected. Separate work on long term non-progressors and elite controllers has revealed that these patients are often clinically separable from normal progressors very early in infection. Differences in peak viremia, viral set-point and immune activation are detected following infection with HIV or SIV in protected humans and primates (222-224). As a result, it has become clear that how a patient’s immune system responds to HIV in the very earliest stages following exposure ultimately defines how much or little the disease will progress in a patient. To that end this work has focused on describing, 1) the differences in lymphocyte gene expression that occur within 24 hours of infection of samples genetically predisposed to either rapid or long-term non-progression, 2) a previously unexplored T-cell subtype’s role in the acute response to HIV infection and 3) the effect of HAART on the longitudinal maintenance of this response. As a result, we identified IFN responsiveness as a key differentiator between protected and at-risk subjects, a correlation between multifunctional DP T-cell frequency and lower HIV viral loads among controllers, the DP T-cell compartment as the primary source of multifunctional T-cells during the acute immune response, and that early administration of HAART results in lower long term multifunctional DP T-cell frequencies.
The above work examining the effect of HLA type on the immune response within 24 hours of infection revealed that genetically protected subjects exhibited reduced IFN responsiveness compared to genetically at risk subjects. This pattern was identified by our work in CD4 T-cells, CD8 T-cells and NK-cells. This finding is corroborated by Mindl and colleagues’ primate studies using SIV which have identified type one interferon and IRF7 mediated signaling as a key differentiator between pathogenic and non-pathogenic SIV infection(211). Therefore, therapeutic modulation of the type 1 interferon response could lead to improvement in disease outcomes. Type 1 interferons are important elements of the initial immune response to many pathogens and are capable of affecting nearly every cell type in the body(225). Importantly, the combination of Mindl and our studies demonstrate that this differential response to interferons is conserved across diverse immune cell types ranging from lymphocytes such as CD4 T-cells, CD8 T-cells and NK-cells to myeloid cells such as pDCs. Therefore, the potentially beneficial effects of limiting interferon response in one cell type are unlikely to be counteracted by negative effects in other immune cells. Unfortunately, there is still the possibility that long-term therapeutic reductions in interferon signaling will adversely affect the ability of a patient to respond to non-HIV pathogens. Consequently, it is necessary to identify the precise timeframe(s) following infection when reduced is IFN signaling is most beneficial. As a result, future studies are necessary in both humans and primates to elucidate the temporal requirements for the beneficial effect of reduced IFN signaling. Using anti-IFN-α antibodies one could induce a sooty mangabey like
activation state within rhesus macaques during the acute and/or chronic phases of SIV infection followed by long term monitoring for disease progression.

The presence of multifunctional CD8 T-cells during chronic infection has also been implicated in improved outcomes following HIV infection(113). Our studies determined that have also correlated the frequency of multifunctional DP T-cells with lower HIV viremia among a cohort of viral controllers. T-cells exhibiting higher orders of functionality have also been correlated with improved outcomes in numerous other diseases to include chronic infections such as HCV to TB (226, 227). Sadly, the processes underlying their successful generation are still largely unknown. However, it has been shown that the treatment of acute HCV patients with 12 to 16 weeks of pegylated IFN-α allowed the maintenance of multifunctional CD8 T-cells beyond the acute phase and into chronic infection(226). Recent work by Romain and colleagues has also shown that a mixed population of cynomolgus macaque dendritic cells (DC) transfected with HIV-Gag were able to stimulate in vivo production of multifunctional CD4 and CD8 cells(228). Furthermore, in most conditions the largest producers of IFN-α are pDCs(225). Therefore, attempts to modulate IFN signaling within the context of acute HIV infection may result in impaired multifunctionality during the chronic phase. Importantly, Romain’s study utilized a mixture of DC subtypes primarily consisting of mDCs with a small contingent of pDCs. As a result, further studies should be pursued to 1) determine the antigen presenting cell (APC) type responsible for generating multifunctional T-cells and 2) the primary source of the IFN-α produced following HIV infection. When performing these studies it is important to note that our work revealed
very little correlation between the frequency of multifunctional cells in the DP compartment and either the CD4 or CD8 compartments. Therefore, it is possible that different mechanisms are responsible for the generation of each T-cell compartments’ multifunctional cells.

Just as the mechanism behind the generation of multifunctional T-cells is unknown, the origin of peripheral DP T-cells is similarly unidentified. Historically, peripheral DP T-cells were nonfunctional cells believed to result from accidental leakage of under-developed CD4/CD8 single positive T-cells from the thymus (145, 152). Recent work by us and other groups has revealed that DP cells in fact possess functional capabilities (138, 143, 229, 230). In fact, we have shown not only the presence of these functional capabilities but also that their frequency correlates with improved control of HIV. These findings increase the importance of elucidating the origin of this T-cell compartment. We hypothesize three potential sources of peripheral DP T-cells 1) fully educated CD4^+CD8^+ thymic emigrants, 2) peripheral CD4^+CD8^- T-cells gaining CD8 expression and 3) peripheral CD4^+CD8^+ T-cells gaining CD4 expression. Experiments involving the adoptive transfer of lymphoid progenitor cells, peripheral CD4^+CD8^- T-cells or peripheral CD4^+CD8^+ T-cells into humanized mice followed by longitudinal analysis of the peripheral blood, lymph nodes and mucosal surfaces for the presence of double positive T-cells should reveal the required elements for filling of the DP T-cell compartment. Previous reports have shown that in vitro stimulation of CD4^-CD8^- or CD4^-CD8^+ positive T-cells with mitogens generates limited expression of the previously unexpressed CD4 or CD8 molecule (151, 173). Therefore, the previously described
experiments may require the addition of T-cell stimulants following adoptive transfer to fully populate the DP T-cell compartment. It is also possible, that the 3 potential sources of the DP compartment are not mutually exclusive. In addition, if CD4⁺CD8⁻ and/or CD4⁻CD8⁺ T-cells are capable of gaining expression of the previously unexpressed molecule it may also be possible for CD4⁺CD8⁺ T-cells to transition to either single positive compartment. This would lead to a dynamic equilibrium between the various T-cell compartments where a cell’s surface marker identity is as transient as its production of a given cytokine or degranulation state. This would fit with the breakdown of the classical categorization of CD4 cells as purely helper cells and CD8 cells as purely cytotoxic cells. In fact, studies have shown that the CD4 compartment contains cytotoxic capabilities and CD8 cells are capable of a range of functions beyond cytotoxic killing including noncytolytic suppression and stimulation of additional immune cell types (i.e. “helper” functionality) (183, 231). As a result, a more complete understanding of the helper and cytotoxic functionality may be that they exist on a continuum. In this continuum CD4⁺CD8⁻ T-cells possess the greatest probability of performing helper functions along with a low but important probability of performing cytotoxic functions. Meanwhile, CD4⁻CD8⁺ T-cells possess the greatest probability of performing cytotoxic functions along with a low but important probability of helper functions. Where DP T-cells fit along this continuum is as yet unclear.

We and others have shown that DP T-cells express markers for degranulation following antigenic stimulation (138, 143). Nonetheless, actual cytotoxic effects have as yet never been demonstrated within the DP compartment. An experiment whereby sorted
pure populations of CD4, DP and CD8 T-cells are entered into a cytotoxic killing assay would reveal 1) if DP T-cells are capable of direct killing and 2) the relative magnitude of killing between each T-cell compartment. Initial experiments may utilize classic immunological systems such as OVA specific TCRs in mice. The functional divisions between T-cell subtypes are often much more distinct in mice than humans, therefore experiments should also be performed utilizing human cells and human disease antigens (232, 233). As mentioned previously, T-cells can also directly inhibit the propagation of pathogens without killing the infected cells. The presence of CNAR in the context of HIV is well described although the mechanism of action is not (234, 235). Substitution of CD4CD8⁺ T-cells with DP T-cells in both contact and non-contact mediated HIV suppression assays will reveal if HIV-specific DP T-cells are also capable of this function. If the DP compartment contains CNAR these cells may provide an important point of comparison for studies directed at identifying the molecule(s) responsible for CNAR.

Given the correlation between the frequency of multifunctional DP T-cells and improved control of HIV, this immune cell population may be an attractive target for vaccine development. Previous work by Suni and colleagues has shown that a therapeutic HIV vaccine can expand the HIV-specific DP T-cell population(50). Nevertheless, much work remains to be done to determine how to best stimulate this T-cell compartment. The previously outlined studies to determine the origins of this cell population and its antiviral capabilities will inform the direction of possible vaccine protocols targeting DP T-cells. For example, if the primary source of DP T-cells is from
peripheral CD4+ T-cells gaining CD8 expression then vaccine antigens may need to be focused on known CD4 epitopes. If stimulated peripheral CD8+ T-cells generate the majority of DP T-cells then the opposite would be true and vaccines targeting DP T-cells should focus on known CD8 epitopes. Alternatively, if DP T-cells are primarily fully-educated thymic emigrants they may respond to unique T-cell epitopes, meaning extensive epitope mapping experiments may be necessary to direct the development of successful DP T-cell targeting vaccines. Follow up experiments to the DP T-cell origin experiments are likely to determine the cytokines and intracellular signaling mechanisms necessary for DP T-cell development. These results will be vital in determining the optimal adjuvant(s) to include in DP T-cell targeting vaccines. Meanwhile, the experiments determining the antiviral capabilities of DP T-cells will determine the proper metrics for evaluating candidate DP T-cell vaccines. If DP T-cells are shown to have potent cytotoxic or CNAR capabilities initial experiments evaluating vaccines should be designed to measure the strength and breadth of these functions following vaccine administration. If on the other hand DP T-cells are shown to primarily act as helpers cells, then vaccine metrics should be directed at evaluating DP T-cell cytokine production and the resulting changes in CD8+ T-cell cytotoxicity, CD8+ T-cell CNAR, B-cell antibody production, antibody affinity, etc. The experiments determining the capabilities of DP T-cells will also determine which diseases present the most attractive targets for DP T-cell vaccines. If DP T-cells are determined to primarily act as direct effector cells then bacterial pathogens and viruses such as influenza for which antibody responses are generally considered to be most effective would be less attractive for DP T-cells
Conversely, pathogens such as HSV-2 and rotavirus that are primarily contained by the T-cell compartment would be particularly attractive targets for a potential DP T-cell vaccine (238-240).

Early administration of HAART greatly reduces the risk of HIV transmission. Meanwhile, many aspects of the immune response require extended periods of antigen exposure in order to fully develop. Therefore the initiation of HAART before the immune response is fully established has been hypothesized to present a risk to the long-term immune response of a patient. This work did not find significant changes in proliferative capacity within any T-cell compartment following the initiation of therapy. Therefore, the proliferative T-cell response to HIV appears to be established very early following infection. Additionally, this response is also either highly durable in the absence of strong antigenic stimulation or the low level of viral replication which occurs despite HAART is sufficient to continually boost this aspect of the immune response.

Conversely, the multifunctional T-cell response significantly declined in all compartments following the early initiation of HAART. This effect was also observed within the CD4 and CD8 compartments of patients who delayed HAART initiation. Interestingly, patients who delayed HAART initiation exhibited an intermediate multifunctional DP T-cell response. Therefore, the timing of HAART may be of particular importance in maintaining a strong multifunctional DP T-cell response. It is also possible that rather than timing itself being important, instead it is the cumulative duration of antigenic stimulation. Consequently, it is possible that continued antigenic stimulation following the initiation of HAART could improve the durability of the DP T-
cell response. This could be achieved by therapeutic vaccination with HIV antigens and/or structured treatment interruptions allowing for the temporary return of antigenic stimulation. These approaches could be used alone or in combination to generate the cumulative antigenic stimulation required for a more durable multifunctional DP cell response. In order to determine if this is necessary, additional studies will be required to determine if the differences in longitudinal multifunctional DP T-cell responses result in long term clinical effects. It is known that low levels of HIV replication occur within treated patients despite HAART’s ability to reduce HIV viremia below the limits of detection for standard assays (241). Within the cohort of controllers the frequency of multifunctional DP T-cells is correlated with lower viral loads. Consequently, it is possible that the delayed HAART patients, who have higher frequencies of multifunctional DP T-cells, experience even lower levels of viral replication than the early HAART patients. Over the course of treatment this could manifest itself in several clinically relevant ways. First viral replication activates the immune system and chronic activation leads to immune exhaustion that is a hallmark of long term HIV infection (242-245). As a result, improved control of low level viral replication could delay the onset of immune exhaustion and improve long term patient outcomes. Additionally, viral replication allows the virus to evolve around the environmental pressures of both the immune system and the HAART regimen being administered(246). Therefore, the potential link between multifunctional DP T-cell frequency and low level viral replication could result in a decrease in HIV’s ability to successfully identify escape mutants which could have major impacts on long term disease outcomes. Importantly, structured
treatment interruptions have been shown to generate escape mutants so this would be a less than ideal manner of generating increased DP multifunctionality. If these hypotheses are true public health officials must weigh the competing interests of generating higher frequencies of multifunctional DP T-cells and reducing the potential for HIV transmission when formulating recommended treatment protocols.

In order to properly inform treatment recommendations the link between the timing of treatment initiation and multifunctional DP T-cell frequency must be well described. The delayed therapy patient group in this work is made up of 14 patients and represents a heterogeneous mixture of treatment initiation times. In order to fully understand the relationship between HAART timing and multifunctional frequency larger follow-up studies are necessary. These studies will require the development of either patient cohorts initiating therapy at regular intervals spread across the first two years following infection or a large cohort with highly diverse treatment initiation times allowing for statistical modeling of multifunctional DP T-cell frequency’s dependence on HAART initiation time. Immune responses do not increase forever in the presence of a continuous antigenic stimulation. Instead they usually plateau and/or begin to decrease following sustained antigenic stimulation. As a result, the above described studies will likely reveal a time after which further delay in treatment initiation fails to result in additional increases in multifunctional DP T-cell frequencies. When balancing the need to reduce transmission frequencies with the development of a patient’s immune response, this time-point would represent the furthest from infection one should initiate therapy. Follow-up experiments demonstrating a link between post-HAART replication levels and
multifunctional DP T-cell frequency may also reveal a plateau above which multifunctional frequency no longer alters replication levels. This may further reduce the need to delay therapy initiation for sufficient multifunctional DP T-cell frequencies to have been developed. Additionally, treatment recommendations may need to be dynamic and consider the many demographic factors which effect transmission probabilities. Therefore, patients who are more likely to transmit their infection, such as an IV drug user, may be encouraged to initiate HAART earlier than patients with lower transmission risks, such as a member of a monogamous couple where both partners are HIV positive.

Collectively, this thesis has identified a new significant contributor to the acute anti-HIV response, demonstrated that the timing of HAART initiation alters the long-term durability of this response and revealed a conserved pathway determining pathogenic versus non-pathogenic lentiviral infection. This work has the potential to direct the development of new HIV treatment methodologies, the optimal timing of HAART and the composition of candidate therapeutic and preventative vaccines. Thus, this work’s impact ranges from the basic science of immune cell differentiation to the clinics where doctors and patients are determining the optimal treatment plan for HIV patients.
Appendix A

Work performed in collaboration with Dr. Jason Stout and the Duke Infectious Diseases Clinic that is distinct from the dissertation research topic.

Introduction

Tuberculosis (TB) is one of the oldest and most successful human pathogens and has infected approximately one third of the world’s population (250). After an individual is infected with TB, the infection may have no clinical manifestation (latent TB infection), but 5-10 percent of infected individuals will progress to active TB disease months to years later (251-253). Early detection and treatment of active TB as well as preventive treatment of individuals with latent TB infection are therefore considered two of the cornerstones of TB control.

Current TB diagnostics, however, are in need of improvement. TB infection has been traditionally diagnosed using the Mantoux tuberculin skin test (TST). TST effectiveness is limited by the need for a return visit, inter-reader variability, cross-reactivity with non-tuberculosis mycobacteria as well as the Bacille Calmette-Guerin (BCG) vaccine, and poor sensitivity in immunocompromised patients (254, 255). Importantly, the TST only identifies TB infection, but does not provide any information that can distinguish latent TB infection from active TB disease. Microbiologic methods (acid-fast smear and mycobacterial culture) must be used to discriminate latent from active TB. Unfortunately, acid-fast smears are relatively insensitive for TB diagnosis, and mycobacterial culture requires clinicians to wait up to several weeks to obtain a result.
as well as significant investments in equipment. Recently, Interferon-gamma Release Assays (IGRAs) have been developed which deal with some of the TST’s limitations (256). IGRAs are performed in vitro with a single blood draw (obviating the need for return diagnostic visits), include standards to reduce inter-reader variability, and utilize TB specific antigens to reduce cross-reactivity with other mycobacteria, including BCG. The increased specificity of IGRAs has significant potential to reduce false-positive tests, particularly among BCG-vaccinated, otherwise low-risk populations, permitting public health programs to focus on high-risk persons (257). Much like TST’s, IGRAs suffer from suboptimal sensitivity in immunocompromised individuals and young children. IGRAs are also unable to differentiate latent from active TB (258).

We sought to determine whether other biomarker responses or combinations thereof, after whole-blood stimulation by TB-specific antigens, could improve upon the performance of Interferon-gamma (IFN-γ) by IGRAs. We hypothesized that combinations of multiple biomarkers could a) be more sensitive for TB infection than a single immune marker, and b) potentially distinguish individuals with active TB disease from persons with latent TB infection.
**Materials and Methods**

**Subjects**

Our cohort included persons participating in two different ongoing studies: 1) the “TB Epitopes Study” (Immunogenicity of *Mycobacterium tuberculosis* T Cell Epitopes), which collected a single blood specimen from persons with either active or latent TB and examined immune responses to a number of TB-specific epitopes, or 2) the “GIS-THIS Study” (Geographic Information Systems-based screening for TB, HIV, and syphilis), which screened participants for TB, human immunodeficiency virus, and syphilis in high-incidence neighborhoods. Both studies involved drawing blood for a Quantiferon® Gold In-Tube test, and the leftover supernatant from the Quantiferon tubes was frozen at -80ºC and subsequently used in this study. Patients involved in this study were recruited between October 1, 2008 and May 1, 2009. The present study as well as the parent protocols required written informed consent and were approved by the Duke University Institutional Review Board.

Consenting subjects were divided into three experimental groups according to TB status: active TB, latent TB and TB negative. The active TB group included subjects who either grew *Mycobacterium tuberculosis* from a clinical specimen or were diagnosed clinically per Centers for Disease Control and Prevention criteria (259). Individuals in the active TB group were at variable stages of treatment, including some who had completed treatment. The latent TB group included subjects with either a positive TST, positive Quantiferon® Gold In-Tube assay, or both, but with no signs or symptoms of active TB disease and no positive cultures for *M. tuberculosis*. Finally, the TB negative
group included subjects who tested negative with the TST and/or Quantiferon® Gold In-
Tube assays and had no clinical evidence of active TB disease.

**Quantiferon Tests**

Quantiferon® Gold In-Tube (QFT-GIT) (Cellestis Inc, Valencia CA) tests were
performed according to the manufacturer’s instructions by a single ASCP certified
medical technologist trained to perform the QFT-GIT assay by Cellestis. Briefly, whole
blood was collected by venipuncture from each subject and incubated for 16-24 hours in
three separate conditions: 1) a mixture of 3 TB antigens from RD1 and RD11 (ESAT-6,
CFP-10 and TB7.7); 2) a mitogen for a positive control; and 3) a mock stimulation for a
negative control (Nil). Following the stimulations, 150µl of supernatant was harvested
from each tube. 50µL of each supernatant was used to determine its IFN-γ concentration
by ELISA (Cellestis Inc, Valencia, CA). In addition, 100µL of each supernatant was
frozen at -80°C and analysis was performed within 16 weeks from collection.

**Multiplexed Bead Arrays**

Supernatants from each stimulation were thawed and analyzed undiluted and at a
1:10 dilution to determine the concentration of 25 potential biomarkers (IL-1β, IL-1RA,
IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 p40/70, IL-13, IL-15, IL-17, TNF-
α, IFN-α, IFN-γ, GM-CSF, MIP-1α, MIP-1β, IP-10, MIG, Eotaxin, RANTES, MCP-1) 
by using a Human Cytokine 25-plex (Biosource, Camarillo, CA). The assay procedure
was performed by a single blinded researcher for all assays and followed manufacturer’s
instructions. In summary, sample and standard dilutions were performed with the
included assay diluent. Beads coated with antibodies against the 25 potential biomarkers
were added to each well of a 96-well filter plate and washed with wash buffer. Beads were resuspended in 100µL of incubation buffer. 100µL of standards that contained a mixture of each analyte with known concentrations were added to the appropriate wells and run in duplicate. The remaining wells received 50µL assay diluents and 50 µL of either Quantiferon® supernatant or 10-fold diluted Quantiferon® supernatant. Plates were at room temperature while shaking for 2 hours to allow sample binding to the appropriate beads. Following two recommended washes with wash buffer, biotinylated antibodies against each biomarker were added to the bead: sample conjugates and incubated at room temperature while shaking for 1 hour. At the end of the incubation time, the plate was washed twice with wash buffer. Streptavidin-RPE was added to each well and the plate was incubated at room temperature while shaking for 30 minutes. After the plate was washed 3 times with wash buffer, the beads were resuspended in wash buffer. The plate was then analyzed on a Luminex 100™ instrument (Luminex, Austin, TX) using Bio-Plex Manager Software (Bio-Rad, Hercules, CA). Analyte concentrations obtained from the undiluted Quantiferon® supernatant were used unless they were above the linear range of the assay, in which case the corresponding 10-fold diluted Quantiferon® sample supernatant was used to determine the analyte concentration. In one case the analyte (MCP-1) concentration in the 10-fold diluted specimen was above the linear range; this response was deleted for analytic purposes. Analyte concentrations reported as below the limit of detection were assigned values of 0 pg/mL for analytic purposes.
**Statistical Analysis**

The biomarker response was defined as the concentration of the biomarker in the Nil tube supernatant subtracted from the biomarker concentration in the TB antigen tube supernatant (TB-Nil) as detected by the multiplexed bead array. The Wilcoxon rank-sum test was used to test the marginal association between each biomarker response and experimental group (260). Based on the biomarker profiles, we built classification models for the three infection outcomes: negative (uninfected), latent TB, and active TB. To this end conditional inference trees were used (261). For each model, the family-wise error rate was controlled at the two-sided 0.05 level. Receiver operating characteristic curves were generated based on combinations of cytokines. The Spearman rank coefficient was used to assess the relationship between interferon gamma responses in the Quantiferon® vs. the Luminex systems. The R statistical environment along with the party extension package were used to conduct the statistical analyses (261, 262) as well as SASv9.3 (SAS Systems, Cary, NC).
Results

Study Subjects

Specimens from 70 subjects were examined; demographic and clinical data are summarized in Table 8. Among the 32 persons with latent TB infection, one was currently taking isoniazid for latent TB, and 8 had received at least some prior treatment for latent TB (range 8-52 weeks of isoniazid, median 38.5 weeks), of whom 5 had completed at least 6 months of isoniazid, and one additional person was currently receiving latent TB treatment (7 weeks received). Three of the persons with latent TB had HIV; two were taking antiretrovirals at the time of blood sampling (CD4+ T-cell count 157/mm³ and 352/mm³), and one was not (CD4+ T-cell count 449 cells/mm³). Among the 12 persons with current or prior active TB disease, 10 were culture-proven and 2 were clinical cases. Nine of the active TB patients had pulmonary disease and 3 had extrapulmonary disease. Four of the active TB patients were currently on TB treatment (3-25 weeks into treatment), and 8 had completed treatment a median 8.5 months previously (range 0.5-17 months). Two of the patients with active TB had HIV, and both were on antiretroviral therapy at the time of blood sampling for the study, with CD4+ T-cell counts of 153 and 358 cells/mm³.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>TB Negative</th>
<th>Latent TB</th>
<th>Active TB</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>26</td>
<td>32</td>
<td>12</td>
</tr>
<tr>
<td>Median Age (range)</td>
<td>46.5 (26-62)</td>
<td>50 (2-66)</td>
<td>43.5 (4-93)</td>
</tr>
<tr>
<td>Female Gender</td>
<td>7 (27%)</td>
<td>15 (47%)</td>
<td>4 (33%)</td>
</tr>
<tr>
<td>Race/Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Hispanic Asian</td>
<td>0 (0%)</td>
<td>3 (9%)</td>
<td>1 (8%)</td>
</tr>
<tr>
<td>Non-Hispanic Black</td>
<td>22 (85%)</td>
<td>16 (50%)</td>
<td>8 (67%)</td>
</tr>
<tr>
<td>Non-Hispanic White</td>
<td>2 (8%)</td>
<td>9 (28%)</td>
<td>1 (8%)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>2 (8%)</td>
<td>4 (13%)</td>
<td>2 (17%)</td>
</tr>
<tr>
<td>US Born</td>
<td>24 (92%)</td>
<td>21 (64%)</td>
<td>7 (58%)</td>
</tr>
<tr>
<td>HIV Status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>25 (96%)</td>
<td>27 (84%)</td>
<td>10 (83%)</td>
</tr>
<tr>
<td>Positive</td>
<td>1 (4%)</td>
<td>3 (9%)</td>
<td>2 (17%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>0 (0%)</td>
<td>2 (6%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>3 (12%)</td>
<td>2 (6%)</td>
<td>3 (25%)</td>
</tr>
<tr>
<td>BCG Status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unvaccinated</td>
<td>25 (96%)</td>
<td>21 (66%)</td>
<td>7 (58%)</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>0 (0%)</td>
<td>10 (31%)</td>
<td>4 (33%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (4%)</td>
<td>1 (3%)</td>
<td>1 (8%)</td>
</tr>
<tr>
<td>Quantiferon result</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>26 (100%)</td>
<td>13 (41%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Positive</td>
<td>0 (0%)</td>
<td>19 (59%)</td>
<td>12 (100%)</td>
</tr>
<tr>
<td>Tuberculin skin testing</td>
<td>Not done</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5 not done
4 history of prior positive, unknown size
All positive (median 15.5 mm, range 11-25 mm)
Biomarker Analysis After Antigen Stimulation

In order to verify the performance of our Luminex assay as a diagnostic we initially compared Luminex obtained IFN-γ production values across patient groups. As expected, IFN-γ expression, as detected by Luminex, was significantly higher in the infected subjects (latent TB and active TB combined) than the TB negative group as shown in Figure 23A (p<0.001). Because most subjects’ results were at the upper or lower end of the linear range of the Quantiferon® assay, it was difficult to assess the linearity of the relationship between IFN-γ responses as measured by the Quantiferon® kit vs. Luminex. The Spearman rank correlation between responses in the two kits was 0.47 (p<0.0001).

We next analyzed each of the remaining 24 biomarkers’ ability to differentiate between uninfected and infected subjects (latent TB and active TB combined). The median levels of seven biomarkers (IP-10, MIG, IL-2, MCP-1, IL-15, and IL-1 receptor antagonist) were significantly (p<0.001) higher in the TB-infected group compared to the uninfected group (Figure 23B-F and Figure 24A). Of those, IP-10, MCP-1, and IL-15 displayed the least amount of overlap between the uninfected subjects and the TB-infected groups. IL-10 was also identified as being differentially secreted between TB infected and uninfected subjects, but did not meet Bonferroni-corrected significance (corrected for 50 comparisons, so significant p<0.001) (Figure 24B). Log-transformed p-values obtained as a result of this analysis are displayed in Figure 1G (blue bars). The
complete list of biomarkers and comparison of median values between TB-infected and uninfected persons is in Table 9.
Figure 23: Comparison of biomarker expression between TB negative, latent TB, and active TB subjects. Secretion of IFN-γ (A), IP-10 (B), MIG (C), and IL-2 (D) are significantly higher in TB infected subjects (●) than non-infected controls (●) but do not significantly differ when active TB subjects are compared to latent TB subjects (●) following TB antigen stimulation of whole blood. MCP-1 (E) and IL-15 (F) secretion are significantly increased following TB antigen stimulation of whole blood in TB infected subjects compared with non-infected controls. These biomarkers are also more highly secreted in active TB subjects than latent TB subjects. Each data point represents the concentration observed following stimulation minus the concentration observed in the negative control condition. HIV positive subjects are denoted by squares. Log p-values from Wilcoxon-rank sum tests comparing biomarker secretion in TB infected subjects to non-infected controls (blue bars) as well as active TB subjects to latent TB subjects (red bars) are also plotted (G). The line (log P=3) represents the Bonferroni-corrected significance threshold (50 comparisons).
Figure 24: Comparison of biomarker expression between TB negative and TB infected individuals. IL-1R Antagonist (A) and IL-10 (B) secretion significantly increases following TB antigen stimulation of whole blood from TB infected subjects compared to TB negative subjects (●). IFN-α (C) and IL-4 (D) were significantly increased in active TB subjects (●) compared to latent TB subjects (○). Each data point represents the concentration observed following stimulation minus the concentration observed in the negative control. HIV positive subjects are denoted squares.
### Table 9: Biomarker Expression and P-values

<table>
<thead>
<tr>
<th>Analyte</th>
<th>TB Negative</th>
<th>Latent TB</th>
<th>Active TB</th>
<th>TB negative vs. Infected</th>
<th>TB negative vs. Latent TB</th>
<th>TB Negative vs. Active TB</th>
<th>Latent TB vs. Active TB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (LQ - UQ)</td>
<td>Median (LQ - UQ)</td>
<td>Median (LQ - UQ)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1b</td>
<td>3 (-6 - 122)</td>
<td>38 (-45 - 161)</td>
<td>18 (24 - 324)</td>
<td>0.4550</td>
<td>0.8579</td>
<td>0.1210</td>
<td>0.1260</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>-321 (-827 - 641)</td>
<td>1404 (-34 - 5394)</td>
<td>4609 (2470 - 14638)</td>
<td>0.0003</td>
<td>0.0066</td>
<td>&lt;0.0001</td>
<td>0.0113</td>
</tr>
<tr>
<td>IL-2</td>
<td>0 (-3 - 0)</td>
<td>46 (0 - 267)</td>
<td>194 (68 - 287)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.1090</td>
</tr>
<tr>
<td>IL-2R</td>
<td>-12 (-41 - 24)</td>
<td>14 (-32 - 51)</td>
<td>54 (-9 - 122)</td>
<td>0.2330</td>
<td>0.4800</td>
<td>0.0997</td>
<td>0.2040</td>
</tr>
<tr>
<td>IL-4</td>
<td>1 (-6 - 6)</td>
<td>0 (-8 - 6)</td>
<td>18 (6 - 25)</td>
<td>0.4573</td>
<td>0.8457</td>
<td>0.0178</td>
<td>0.0075</td>
</tr>
<tr>
<td>IL-5</td>
<td>0 (0 - 0)</td>
<td>0 (0 - 0)</td>
<td>0 (0 - 0)</td>
<td>0.5330</td>
<td>0.6340</td>
<td>0.5470</td>
<td>0.7850</td>
</tr>
<tr>
<td>IL-6</td>
<td>316 (6 - 2302)</td>
<td>512 (3 - 2068)</td>
<td>700 (257 - 2468)</td>
<td>0.6330</td>
<td>0.7740</td>
<td>0.5250</td>
<td>0.6870</td>
</tr>
<tr>
<td>IL-7</td>
<td>0 (-8 - 7)</td>
<td>0 (-14 - 13)</td>
<td>29 (3 - 42)</td>
<td>0.3680</td>
<td>0.9300</td>
<td>0.0115</td>
<td>0.0146</td>
</tr>
<tr>
<td>IL-8</td>
<td>11234 (-1758 - 21194)</td>
<td>7353 (-912 - 26664)</td>
<td>31232 (21588 - 44526)</td>
<td>0.2280</td>
<td>0.7740</td>
<td>0.0100</td>
<td>0.0333</td>
</tr>
<tr>
<td>IL-10</td>
<td>0 (-3 - 4)</td>
<td>6 (0 - 40)</td>
<td>42 (3 - 89)</td>
<td>0.0009</td>
<td>0.0060</td>
<td>0.0027</td>
<td>0.1850</td>
</tr>
<tr>
<td>IL-12</td>
<td>0 (-20 - 19)</td>
<td>13 (-32 - 50)</td>
<td>34 (17 - 70)</td>
<td>0.0303</td>
<td>0.2100</td>
<td>0.0017</td>
<td>0.1530</td>
</tr>
<tr>
<td>IL-13</td>
<td>0 (0 - 0)</td>
<td>0 (0 - 0)</td>
<td>0 (0 - 11)</td>
<td>0.1990</td>
<td>0.4830</td>
<td>0.0598</td>
<td>0.1330</td>
</tr>
<tr>
<td>IL-15</td>
<td>0 (-16 - 16)</td>
<td>36 (13 - 75)</td>
<td>152 (105 - 203)</td>
<td>&lt;0.0001</td>
<td>0.0025</td>
<td>&lt;0.0001</td>
<td>0.0006</td>
</tr>
<tr>
<td>IL-17</td>
<td>0 (0 - 0)</td>
<td>0 (0 - 13)</td>
<td>13 (4 - 20)</td>
<td>0.0479</td>
<td>0.3460</td>
<td>0.0007</td>
<td>0.0504</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0 (-16 - 17)</td>
<td>13 (-13 - 102)</td>
<td>13 (16 - 160)</td>
<td>0.0584</td>
<td>0.1780</td>
<td>0.0300</td>
<td>0.2550</td>
</tr>
<tr>
<td>IFN-α</td>
<td>-3 (-9 - 4)</td>
<td>-4 (-10 - 10)</td>
<td>10 (7 - 15)</td>
<td>0.1690</td>
<td>0.8550</td>
<td>0.0009</td>
<td>0.0081</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>-2 (-23 - 16)</td>
<td>38 (0 - 167)</td>
<td>116 (56 - 225)</td>
<td>&lt;0.0001</td>
<td>0.0009</td>
<td>&lt;0.0001</td>
<td>0.0787</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>(-20 - 0)</td>
<td>0 (0 - 12)</td>
<td>0 (0 - 0)</td>
<td>0.2130</td>
<td>0.1970</td>
<td>0.5970</td>
<td>0.6060</td>
</tr>
<tr>
<td>MIP-1a</td>
<td>-37 (-487 - 652)</td>
<td>358 (-161 - 2260)</td>
<td>1178 (-248 - 3219)</td>
<td>0.0090</td>
<td>0.1490</td>
<td>0.1460</td>
<td>0.5940</td>
</tr>
<tr>
<td>MIP-1b</td>
<td>-28 (-1066 - 207)</td>
<td>1405 (-366 - 4971)</td>
<td>2179 (-135 - 9615)</td>
<td>0.0162</td>
<td>0.05110</td>
<td>0.0232</td>
<td>0.4270</td>
</tr>
<tr>
<td>IP-10</td>
<td>22 (0 - 52)</td>
<td>1709 (260 - 6609)</td>
<td>1886 (1169 - 3234)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.4950</td>
</tr>
<tr>
<td>MIG</td>
<td>0 (-4 - 8)</td>
<td>143 (9 - 1240)</td>
<td>698 (325 - 1206)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.1510</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>-2 (-6 - 3)</td>
<td>-7 (-27 - -1)</td>
<td>-1 (-7 - 2)</td>
<td>0.2190</td>
<td>0.0899</td>
<td>0.8410</td>
<td>0.1950</td>
</tr>
<tr>
<td>RANTES</td>
<td>1156 (652 - 2177)</td>
<td>2355 (255 - 7392)</td>
<td>10700 (1685 - 26931)</td>
<td>0.0478</td>
<td>0.2530</td>
<td>0.0043</td>
<td>0.0283</td>
</tr>
<tr>
<td>MCP-1</td>
<td>-511 (-9460 - 2444)</td>
<td>13862 (-579 - 29332)</td>
<td>30024 (25925 - 46561)</td>
<td>&lt;0.0001</td>
<td>0.0016</td>
<td>&lt;0.0001</td>
<td>0.0030</td>
</tr>
</tbody>
</table>
**Active Versus Latent TB**

Next we sought to determine if any biomarkers were differentially expressed between active and latent TB patients with a Wilcoxon rank-sum test. As expected, IFN-γ was not significantly differentially expressed between infection statuses (Figure 23A). Similarly, IP-10, MIG, IL-2 and IL-10 were also not differentially expressed between the active and latent TB groups (Figure 23B-D and Figure 24B). Using a Bonferroni-corrected significance threshold for significance (p<0.001), we observed that IL-15 was the only biomarker able to segregate subjects with latent and active TB (Figure 23F).

Using a less strict significance cutoff (p<0.01) four biomarkers were identified as having differential secretion in response to TB antigen stimulation in subjects with latent TB compared to those with active TB: MCP-1, IL-1Ra, IFN-α, and IL-4 (Figure 1E and Supplementary Figures 1A, C, and D). We report the p-values for comparisons of all 25 biomarkers’ median production in latently infected patients to that in actively infected patients in Figure 23G (red bars) and Table 9.

Although IL-1Ra, IL-4, IL-15, IFN-α, and MCP-1 responses were quantitatively different between the latent and active TB groups, there was still considerable overlap in biomarker responses, as shown in Figures 23A through F. Conditional inference trees were therefore used to explore whether a combination of responses could improve discrimination between the uninfected, latent TB, and active TB groups. A model utilizing MCP-1 followed by IP-10 (Figure 25) successfully identified all of the active TB subjects, but also misidentified 23 latent TB subjects and 2 uninfected subjects as
having an active infection. In addition, this model misclassified 2 TB uninfected subjects as having a latent TB infection and 3 latent TB subjects as being uninfected.

Since current diagnostic methods perform well at identifying the TB negative individuals we hypothesized that removing this group from consideration for the inference trees would generate a clinically more relevant model. When only the responses of the latent and active TB groups were analyzed, IL-15 response alone was the best discriminator between latent and active TB (Figure 26). This model outperformed the previous inference model, reducing the misclassified latent TB subjects from 23 to 7 but it also raised the misclassification of active TB subjects from 0 to 1.

To verify our original hypothesis that combinations of multiple biomarkers could correctly segregate individuals with active TB disease from persons with latent TB infection, we examined the performance of all pairs of biomarker responses that significantly differed between the active and latent TB groups (IL-1Ra, IL-4, IL-15, IFN-α, and MCP-1). This method determined that the combination of an MCP-1 response greater than or equal to 19,696 pg/mL and an IL-15 response greater than or equal to 82 pg/mL achieved the greatest overall accuracy in identifying patients with latent versus active TB (Figure 27). In fact, by using this combination we correctly assigned 38/44 (86.4%) of subjects with either latent or active TB to the correct disease states. The sensitivity and specificity of this two-biomarker combination for active TB (vs. latent TB) were 83% and 88%, respectively. Receiver operating characteristic curves for this combination are shown in Figure 28.
Of note, there was no significant correlation in the active tuberculosis group between time elapsed since the start of anti-tuberculosis treatment and biomarker response for interferon gamma or any of the biomarkers in the models above. The Spearman correlations and p-values between time since start of treatment and biomarker response were $r=0.44$ (p=0.15) for interferon gamma, $r=0.13$ (p=0.69) for IP-10, $r=-0.02$ (p=0.95) for IL-15, and $r=-0.36$ (p=0.25) for MCP-1.
Figure 25: Conditional inference tree for diagnosing TB uninfected, latent TB and active TB infections. Conditional inference trees were used to classify all patients into three infection/disease states. For this analysis the family-wise two-sided error rate was not allowed to exceed 0.05.
Figure 26: Conditional inference tree for diagnosing latent versus active TB infections. After excluding TB negative patients based upon current diagnostic methods conditional inference trees were used to classify all infected patients into active or latent infection statuses. For this analysis the family-wise two-sided error rate was not allowed to exceed 0.05.
Figure 27: Differentiation of active and latent TB subjects. Simultaneously analyzing IL-15 and MCP-1 concentrations allows the effective separation of active and latent TB disease states. Subjects with active TB are represented by red shapes, and those with latent TB as orange shapes. Using cutoffs of 82pg/mL and 19696pg/mL for IL-15 and MCP-1 respectively, 10/12 (83%) active TB subjects (●) and 28/32 (88%) latent TB subjects (●) were correctly identified. Incorrectly identified subjects are shown as half-shaded shapes. HIV positive subjects are denoted by squares.
Figure 28: Receiver operating characteristic curve for a combination of IL-15 and MCP-1 in discriminating active from latent tuberculosis.
Discussion

This study suggests that several biomarker responses to TB antigens may be useful for the detection of TB infection as well as for differentiating active and latent TB disease. Of particular promise in detecting TB infection was IP-10. This is consistent with previous studies which found locally high levels of IP-10 in pleural effusions of TB patients as well as within indurations resulting from a positive TST reaction (263, 264). In addition, our results confirm recent observations by Ruhwald and collaborators of IP-10 as a marker for TB infection (265, 266). Of note, the optimal cutoff for IP-10 response found by Ruhwald et al. (455 pg/ml) did not perform quite as well in our subject population (sensitivity 70.5%, specificity 96.2% in discriminating infected vs. uninfected subjects) as a lower cutoff (200 pg/ml) (sensitivity 84.1%, specificity 96.2%), illustrating that optimal cutoffs will need to be determined from more large, prospective studies. Our results are also in agreement with a smaller study that compared biomarker responses after stimulation in 8 TB patients to 7 healthy controls. The Quantiferon® Gold In-Tube system was also used for that study, which found increased MCP-1, MCP-3, IL-1Ra, and IP-10 responses to TB antigen stimulation among TB patients compared with controls (267). While immunosuppressed individuals often do not mount a detectable IFN-γ response to TB antigen stimulation, the biomarkers identified in our study may be produced at detectable levels in such patients (255). In fact, the four HIV+ TB+ patients in our study mounted IP-10 responses greater than all but one TB-uninfected patient.
These limited data support further study of IP-10 response to TB antigens as a potential diagnostic in immunosuppressed populations.

More interestingly, we also identified a biomarker combination which shows promise in distinguishing latent from active TB. Specifically, the combined analysis of IL-15 and MCP-1 responses accurately identified 86% of active and latent TB patients. This combined IL-15 and MCP-1 response pattern could either be a marker for the presence of active TB or instead could represent a response associated with greater susceptibility to development of active TB after infection. Most of our subjects with active TB were sampled after treatment completion, so it is impossible to determine from our data whether these responses might be dynamically influenced by the treatment.

Our study suffers from a number of limitations, including relatively small sample size and a heterogeneous subject population. In particular, subjects in both the latent and the active tuberculosis group were at different time points after the start of treatment. Biomarker responses to the antigens used could potentially vary during and after treatment, but this effect has not been consistent in the literature (268-270).

Additionally, there is no gold standard test for latent tuberculosis; all of the available tests have limitations, and discordant results from the same patient are the norm (258, 271). We used standard definitions of latent tuberculosis, but there was heterogeneity in this group (some had positive tuberculin skin tests with negative Quantiferon® tests, others did not have skin testing performed and only had positive Quantiferon® tests) as well. Such heterogeneity is most likely to bias our results toward the null due to increased
measurement variability, but we cannot dismiss the potential for spurious associations between biomarker responses and disease group based on misclassification or heterogeneity. Furthermore, the receiver operating characteristic curves are based on a training dataset alone, which likely overestimates model performance. We did not examine a number of potential biomarkers such as EGF or soluble CD40 ligand that have demonstrated potential to discriminate latent from active tuberculosis in other studies; at least one biomarker that seemed promising for this purpose in another study (MIP-1β) did not perform well in our study (272).

The particular biomarker responses associated with active TB disease in this study were not surprising given previous investigators’ findings. *In vitro* studies have shown that IL-15 upregulates the antimicrobial protein cathelicidin, leading to decreased *M. tuberculosis* survival (273). Furthermore, Rausch and colleagues have shown that IL-15 is required for proper CD8+ T-cell accumulation in the lungs and therefore increased mortality in knockout mice following *M. tuberculosis* infection (274). In addition to IL-15, our study identified increased MCP-1 production as an indicator of active TB. Multiple studies indicate high MCP-1 production may be detrimental to the host immune response (275). Included in these findings was discovery of a single nucleotide polymorphism (SNP) in the MCP-1 promoter which correlated with increased MCP-1 expression and increased susceptibility to active TB disease (276). While this finding held true in Mexican, Korean and Peruvian patient populations the SNP had no effect in a Russian population and even correlated with protection in Ghanaian patients (277, 278). Additional genetic and/or environmental factors are probably important for determining
the role of MCP-1 in fighting TB disease. In light of these contradictory findings it is also important that the present study be extended to diverse TB populations around the world. One possible interpretation is that MCP-1 production in response to \textit{M. tuberculosis} predisposes certain patient populations to develop active TB, and subsequent to the onset of active TB a strong IL-15 response is mounted. A proper longitudinal study would be needed to determine if IL-15 and MCP-1 production appears concurrently, one precedes the other, or if the “active” response pattern heralds active disease or follows its onset. Additional information might be gained by examining biomarker profiles after prolonged incubation, as opposed to the 16-24 hour incubation period used in our study. A recently published study used the same stimulation platform but a longer incubation (72 hours) time, and found that IL-2 supernatant concentrations at 72 hours (but not 18 hours) discriminated latent from active TB patients (279). This suggests that the simultaneous monitoring of these significant biomarkers may also provide insights into the spectrum of immune responses across the population. Distinct portions of this spectrum (e.g. strong IP-10 response combined with a weak IFN-\(\gamma\) response) may have prognostic significance for pathogenic outcomes such as progression from latent to active disease, extrapulmonary spread of TB, probability of reinfection and/or treatment efficacy. Therefore, it is important that our findings form the basis for a larger study featuring diverse well-defined patient populations who are monitored longitudinally.
Appendix B

Peer-Reviewed Publications


References


123. Fadda L & Alter G (2012) KIR/HLA: Genetic Clues for a Role of NK Cells in the Control of HIV


140. Adults HPoAGf & Adolescents a (2011) Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents. eds Research OoA & Council A.


179. Ferrari G, et al. (2011) An HIV-1 gp120 Envelope Human Monoclonal Antibody That Recognizes a C1 Conformational Epitope Mediates Potent Antibody-


Biography

I was born Marc Alan Frahm in Washington, D.C. on October 4, 1983. I lived in Darnestown and Potomac MD until graduating from Thomas S. Wootton High School. Following high school I moved to Madison, WI where I attended the University of Wisconsin-Madison for my undergraduate education. While at the University of Wisconsin I joined the Laboratory of Dr. Hector DeLuca under the guidance of Dr. Kevin Healy. Under their tutelage, I studied the developmental regulation of the intestinal calcium transport machinery as well as the regulation of Vitamin D Receptor expression. During this time, my contributions were recognized as a second author on the paper titled, \textit{1,25-Dihydroxyvitamin D$_3$ Up-regulates the Renal Vitamin D Receptor Through Indirect Gene Activation and Receptor Stabilization} which was published in the \textit{Archives of Biochemistry and Biophysics}. After graduating from the University of Wisconsin in 2005 with a Bachelor's of Science in Biochemistry I moved south to Durham, NC to attend Duke University. At Duke I pursued my Ph.D. in the Department of Molecular Genetics and Microbiology under the advisement of Drs. Guido Ferrari and Georgia Tomaras. During this time I published two first author peer-reviewed publications. The first paper is titled, \textit{Discriminating between Latent and Active Tuberculosis with Multiple Biomarker Responses} and was published in \textit{Tuberculosis}. A second paper has been published under the title, \textit{CD4$^+$CD8$^+$ T Cells Represent a Significant Portion of the Anti-HIV T Cell Response to Acute HIV Infection} and was accepted for publication in the \textit{Journal of Immunology} where it was selected as a featured article in the May 1, 2012 issue. Outside of the laboratory I have been an active member of the department serving
as class representative to the MGM student board for two years and helping plan the Jim McGinnis Memorial Lecture in 2010.