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Determination of Parameters that Constitute an HIV-Specific Immune Response Associated with Disease Progression in a Perinatally HIV-Infected Pediatric Population



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Determination of Parameters that Constitute an HIV-Specific Immune Response Associated with Disease Progression in a Perinatally HIV-Infected Pediatric Population

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

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in the

[redacted]

# Dedication

This dissertation is dedicated to my wonderful parents, Herminia and Dennis Sharp, the most supportive and loving parents a kid could ever hope for. They've always told me that they'll be proud of me no matter what I do, as long as I'm happy doing it. While I've yet to really test them on this promise, I've always believed that they'd be right behind me if I decided to chuck it all and follow my side dream of being a landscaper. I wouldn't be half the person I am today without their loving attention and constant support. Thank you so much guys for the carne asada, multitudes of USC and Lakers games, refritos, shoulders to cry on, almond champagne, camping trips, barista duties, happy times with the cats, wine-tasting trips, Happy Nail gifts, home-made flour tortillas, car repairs, sunny afternoons in the backyard, and all the countless other things that made my grad school years that much easier.

Love you tons.

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Chapter 1 is adapted from the previously published article: Sharp ER, Barbour JD, Karlsson RK, Jordan K, Sandberg JK, Wiznia A, Rosenberg MG, Nixon DF. Higher frequency of HIV-1 Specific T cell Immune Responses Observed Among African American Children Vertically Infected with HIV-1. J Infect Dis. 2005 Nov 15;192(10):1772-80.

Chapter 2 is a reprint of the previously published article: Lee AW, Sharp ER, O'Mahony A, Rosenberg MG, Israelski DM, Nolan GP, Nixon DF. Single-cell, phosphoepitope-specific analysis demonstrates cell type- and pathway-specific dysregulation of Jak/STAT and MAPK signaling associated with in vivo human immunodeficiency virus type 1 infection. J Virol. 2008 Apr;82(7):3702-12

**Determination of Parameters that Constitute an HIV-Specific Immune Response Associated with Progression in a Perinatally HIV-Infected Pediatric Population**

Elizabeth Ramona Sharp

## Abstract

Pediatric HIV infection is a major worldwide public-health challenge, with an estimated rate of 1,200 children being infected daily in sub-Saharan Africa alone. In spite of this, the HIV-specific immune response in perinatally infected children has been relatively understudied. These studies were designed to probe specific aspects of the immune response to HIV in a group of vertically infected children/adolescents, treated at the Jacobi Medical Center in the Bronx, NY. The first study investigated the association between ethnicity and HIV-specific CD8+ T cell responses. Using a cytokine-enhanced ELISpot assay to measure IFN-γ secretion in response to stimulation by HIV proteins Gag, Nef, and Tat, we show that African-American subjects displayed an immune response of higher magnitude than age and disease-matched Hispanic subjects. With the second study, we focused on defects in monocytes, specifically the signaling within, resulting from HIV infection. We utilized a recently developed intracellular staining technique, phospho-flow, to investigate the Jak/Stat signaling pathway in the monocytes of HIV-infected children. We found significantly lower levels of Jak/Stat signaling in response to stimulation in HIV-infected individuals compared to non-infected individuals. The last study focused on immunodominance patterns and differentiation profiles of Gag-specific CD8+ T cell responses in two progression groups within the same cohort of perinatally infected children. Using CDC guidelines, we classified patients into those with no immunosuppression or with severe immunosuppression. Using

the IFN-γ ELISpot assay, we then identified all epitope-specific CD8+ T cell responses to the Gag protein and compiled the immunodominance hierarchy of each patient. Utilizing multi-parameter cytokine flow cytometry, we then identified the differentiation profile and cytokine secretion profile of each epitope-specific response. We found significant differences between the progression groups with respect to immunodominance hierarchies of Gag-specific CD8+ T cell responses as well as striking differences in the differentiation profiles of CD4+ T cells, specifically an accumulation of the CCR7-CD45RA+ subset. Taken together, these studies add to the field of pediatric HIV immune responses and identify several factors, namely race, monocyte defects, and immunodominance patterns, which can play an important role in the role of the immune response in progression of HIV disease in perinatally infected children.

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**Chapter 1**



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# Introduction

*I. The Global AIDS Epidemic*

Over 25 years after the human immunodeficiency virus (HIV) was discovered, it remains a global health problem of unprecedented scope. It is estimated that 25 million individuals have already died worldwide as a result of infection with HIV and that approximately 33 million more are living with HIV/AIDS. [1]

Sub-Saharan Africa bears an enormously disproportionate burden of HIV infections worldwide. It is estimated that 67% of all people currently living with HIV/AIDS are in this region of the world. In some areas, up to 24% of the entire population is infected with HIV. The impact of this disease on Africa is difficult to fathom, in many countries it has decimated an entire generation and brought down life expectancy rates to levels not seen since the 1950's. [1]

The United States, like most highly developed countries, has significantly lower rates of HIV prevalence than sub-Saharan Africa; between .5 and 1 percent of the population are thought to be infected with the virus. [1] In spite of the lower level of HIV prevalence in the US, the epidemic in this country is unbalanced in terms of the racial groups infected. Most strikingly, African Americans comprised almost 50% of reported HIV/AIDS cases in 2006 although they only make up 13% of the general population. The Hispanic population in the US also bears a disproportionate burden of HIV infection, accounting for almost 20% of reported cases in 2006, while making up only 15% of the general population. [2]

*II. Pediatric HIV Infection*

Pediatric infection remains a major problem in the worldwide AIDS pandemic. The number of children under 15 living with HIV/AIDS has been steadily increasing since monitoring practices were first begun. UNAIDS estimates that in 2007, between 1.9 and 2.3 million children worldwide are living with HIV/AIDS, the vast majority residing in sub-Saharan Africa. [1] A mother can transmit the virus to her infant during pregnancy, during birth, or through breast-feeding. All routes can almost uniformly be prevented in the presence of antiretroviral therapy. Fortunately, as a result of increased access to treatment for pregnant women and young children, both the number of new HIV infections and the number of deaths from AIDS in children has dropped nearly 20% in the past few years [1].

*III. Natural History of HIV Infection in Adults*

Early studies in adults established the natural history of adult HIV infection in the absence of therapeutic intervention, summarized in Figure I.

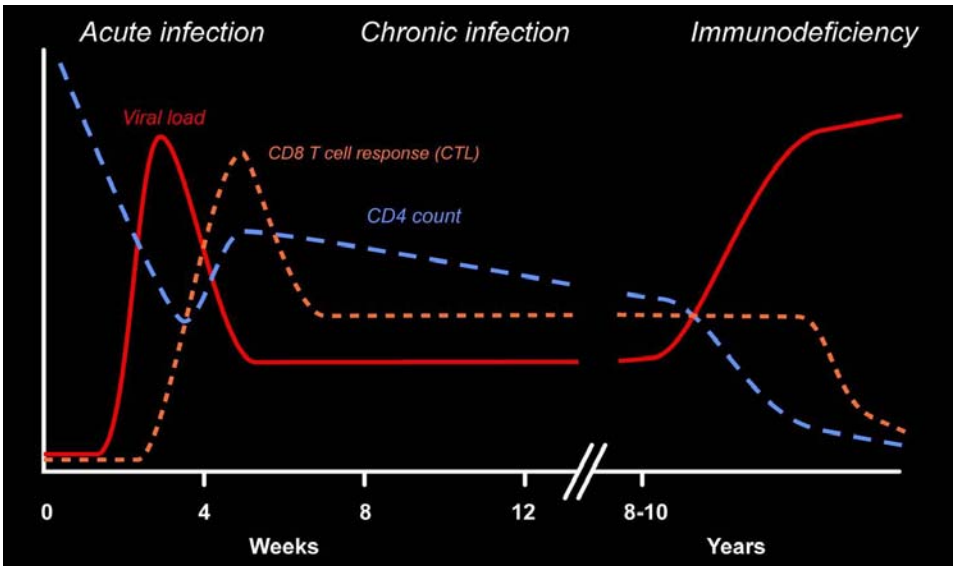


**Figure I. Natural Progression of HIV Disease in Adults**

The first stage of infection is acute infection, which occurs in the first days and weeks after transmission of the virus to an individual. During acute infection, the virus replicates almost unchecked and can reach levels of several million virion RNA copies per mL of peripheral blood. This high level of viral replication is accompanied by a marked drop in the numbers of circulating $CD4^+$ T cells. There is an innate immune response to HIV during these earliest stages of infection, involving dendritic (myeloid and plasmacytoid) cells, natural killer cells, natural killer T cells, and phagocytic cells (neutrophils, monocytes, macrophages) among other components. This early, non-specific response limits viral replication and spread, allowing the adaptive immune system sufficient time to mount a strong, effective response.

Within 2-4 weeks in most individuals, the adaptive immune system develops a response to the viral infection with an increase in HIV-specific CD8+ T-cells as well as the emergence of HIV-specific antibodies. It is widely believed that the development of this adaptive immune response is responsible for the drastic drop in viral load and accompanying rise in CD4+ T cell levels. The nadir of the viral load in an individual after resolution of primary viremia is known as the viral load set point, and has shown to be a strong indicator of future disease progression. [3, 4]

The early stages of chronic infection are marked by a relative détente between the virus and the host immune system. This phase can be as short as several weeks, but has been seen to last longer than 20 years. The adaptive immune response lowers the amount of virus present in the blood, but many cells, including CD4+ T cells, macrophages, and monocytes can remain latently infected. In most patients, there is still a slow decline in CD4+ T-cells, but at a rate much lower than that seen during acute infection.

At some point, in most HIV-infected individuals, the immune response can no longer control replication of the virus and viral loads again start to rise to levels seen in early infection. As a result, CD4+ T cells drop precipitously. This leaves the body susceptible to many infections that a healthy immune system would be able to fight off. At this point, patients begin to show signs of opportunistic infections, which mark the onset of acquired immunodeficiency syndrome (AIDS).

*IV. Natural History of HIV Infection in Children*

The dynamics of HIV-1 infection in children (summarized in Fig. II) are markedly different than in adults. This is not surprising given that the immune system develops in the first few years of life. Thus, in children born with HIV, the immune system has not had the chance to mature in the absence of a virus whose main target *is* the immune system. Also as a result of the developing immune system, infants have a much higher number of CD4+ lymphocytes than adults, giving HIV a much larger pool of infectable targets early in infection [5].

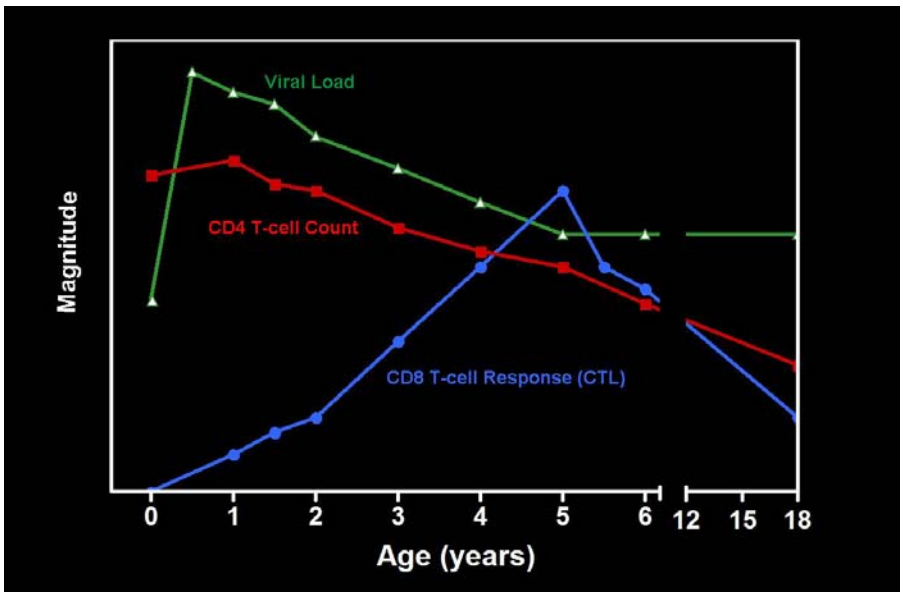


**Figure II. Natural Progression of HIV Disease in Children**

Neonates experience a higher initial viral burst and virus levels decrease slowly over a period of years[6-8], whereas adults generally control the initial viral burst within 3 to 4 months[3, 9-11]. The greater viral burst in neonates is thought to be a result of a combination of higher thymic output, high numbers of circulating CD4+ T cells, and deficient immature virus-specific immune responses.

HIV-specific CD8+ T cell responses are slower to develop in children than in adults, and it has been thought that they reach their peak only after several years as a result of an immature immune system [12, 13]. Recently though, groups have shown that HIV-specific CD8+ T cell responses can be present in the first few days of life [14]. It has also been reported that CD8+ T cell responses in infants can exert selection pressure in well known CD8+ T cell epitopes in the first few months of life, suggesting that the responses are at least partially functional [15-17].

Progression of HIV disease also tends to be more severe in children than in adults [18]. It has been observed that without therapeutic intervention a subset of perinatally infected children, between 15-60% (strikingly different rates exist in developed and developing countries), progress to AIDS very rapidly, within the first few years of life [10, 11, 19]. The factors responsible for this increased severity of disease in children remain unknown, but have been postulated to include destruction of the thymus by HIV [20, 21], HLA Class I sharing between mother and infant [22, 23], and immaturity of the immune system at the time of infection [12, 24]. It is also clear that children infected with HIV are more likely to have a host of other developmental problems (behavioral problems, cognitive deficiencies, stunting of growth, etc.) [25, 26], although exactly how HIV affects these other areas of development is not fully understood.

*V. HIV-specific Immune Responses*

The CD8+ T cell response in the setting of HIV-1 infection plays an important role in determining the course of disease. Many studies have established evidence for the importance of CD8-mediated immunity in controlling HIV-1 infection. Importantly, the appearance of HIV-1 specific CD8+ T cells is temporally associated with the initial drop in viral load seen in acute infection [27, 28]. A direct association between disease progression and class I HLA types has been found in many studies [29, 30]. Additionally, there is a strong base of evidence that cytotoxic T lymphocytes (CTL) exert a selective pressure on HIV-1, as illustrated by the high rates of mutation in epitopes recognized by CTLs [22, 31-33]. Evidence from the simian immunodeficiency virus (SIV) model has shown that the depletion of CD8+ T cells in rhesus macaques results in a rapid and dramatic rise in viremia that is reduced upon reintroduction of CD8+ T cell compartment [34]. These studies highlight the important lines of evidence that suggest a direct role for HIV-specific CD8 responses in controlling the virus.

For reasons that are still poorly understood, the HIV-specific CD8+ T cell response eventually loses control over viral replication, which typically results in disease progression [35, 36]. One of our main research goals was to clearly delineate aspects of the HIV-specific CD8+ T cell immune response that lead to successful control of viral replication. This information could then be used to design therapeutic vaccines that would encourage "protective" immune responses against HIV-1.

*VI. HIV-specific Immune Responses in Children*

Just as there are striking differences in the natural history of HIV infection between adults and children, there are also differences in the HIV-specific immune

response between the two groups. The well documented association between HIV-specific T cell responses and control of viral replication seen in adult acute infection [27, 28, 31] is less clearly established in pediatric infection. It was initially thought that HIV-specific CD8 T cell responses in early pediatric infection were infrequent and hard to detect [37-39], but recent work has shown that HIV-specific CD8+ T cell responses can be found in the first few days of life in most in-utero infected children [14]. Several studies investigating the earliest HIV-specific T cell responses observed that there was no immediate clinical benefit to infants generating responses [24, 39], suggesting that CD8 T cell responses were not as effective in children as compared to adults. In contrast though, studies have seen that HIV-specific CD8 responses in infants can exert selection pressure in known CD8+ T cell epitopes [15-17] and that the HLA-B alleles B27 and B57 are associated with slowed disease progression in pediatric infection [16, 40]. This evidence as a whole suggests that CTL responses generated in infancy are at least partially effective. It has also been shown that the pediatric immune system can exhibit more plasticity than adults in responding to a constantly mutating virus, allowing children to develop *de novo* responses to mutated virus more easily than adults. [16]

The majority of studies investigating pediatric HIV-specific T cell responses have focused on infants and very young children. There is much less known about the role the immune response plays in chronic pediatric infection, where the immune system has developed in the presence of the virus. It has been established that there is the same negative correlation between viral load and CD4 T cells present in untreated adolescents that is seen in adults [41]. The breadth and magnitude of CD8+ T cell responses in older children appears to be similar to those seen in adults [42], but it seems that the proteins

predominantly targeted change from Env and Rev in acute infection to Gag, Pol, and Nef in chronically infected children [14]. The magnitude of HIV-specific CD8 T cell responses in children has been seen to correlate strongly with the presence of HIV-specific CD4+ T cell responses[43]. The relationship between viral load and activation levels well documented in adults [44] is often, but not always, observed in children [43, 45]. It has also been reported that the antigen driven maturation of T cells seen in adults is markedly less pronounced in children[46]. Taken together, these studies suggest that there are some profound differences in the way the pediatric and adult immune systems respond to HIV infection.

*VII. Study of Pediatric HIV-Specific Immune Responses in Minority Children*

Unlike the adult HIV epidemic in the US, which was initially concentrated in Caucasian men, the pediatric HIV epidemic has always been centered in minority populations. The vast majority of research on the adult HIV-specific immune response has been focused on people of Northern European descent, although non-Caucasian populations are now bearing the greatest burden of HIV infection. There is evidence of immunological differences between individuals of different races and it is not unlikely that HIV-specific immune responses might differ between ethnicities as a result. Research in children is not only important to explore the interactions between the developing immune system and HIV, but is also provides an opportunity to investigate the HIV-specific immune response in non-Caucasian populations. As these individuals will be the main target for any future therapeutic intervention, especially a vaccine, it is crucial to understand the HIV-specific immune response in minority populations.

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# Chapter 1:

# Racial Variations in the Pediatric HIV-Specific CD8+ T Cell Response

Adapted from the previously published article: Sharp ER, Barbour JD, Karlsson RK, Jordan K, Sandberg JK, Wiznia A, Rosenberg MG, Nixon DF. Higher frequency of HIV-1 Specific T cell Immune Responses Observed Among African American Children Vertically Infected with HIV-1. J Infect Dis. 2005 Nov 15;192(10):1772-80.

## Introduction

There is evidence for immunological differences between races in a number of disease settings. African-Americans show higher incidence and morbidity rates of systemic lupus erythematosus [1-3]. There is increased incidence of transplant rejection in African-American transplant recipients compared to other ethnic groups[4, 5]. It has been proposed that these observations can be explained by a more robust T cell response in African-Americans than other ethnic groups, perhaps due to enhanced co-stimulation[6], or cytokine polymorphisms[7]. In hepatitis C virus (HCV) infection, African-Americans more often develop chronicity and liver cancer than Caucasians, as well show a decreased responsiveness to IFN-α therapy, although they tend to have a more benign outcome. These differences are thought to be the result of an increased and broader HCV CD4+ T cell response in African-Americans compared to Caucasians[8, 9]. To date, there have been few studies investigating differences in HIV-specific immune response between ethnic and gender groups.

Variations in HIV disease progression and plasma viremia have also been reported between different ethnic groups. For example, African-Americans and Hispanics have a faster progression to AIDS and shorter survival times than other ethnic groups, although this may be attributable to socioeconomic factors and lack of access to quality health care[10]. Paradoxically, several studies have shown that at a given CD4+ T cell count, Black persons infected with HIV-1 present with lower viral loads than individuals of other races[11, 12]. Other studies have shown that, compared to Caucasians infected with HIV-1, Blacks experience a slower decline in CD4+ T cell counts[13, 14]. However, this is a complex area of study as several other reports have found contradictory results.

For example, Brown *et al*. found no racial differences in CD4+ T cell levels or viral load when all races were socioeconomically equal and had similar quality of health care[15]. Therefore, the basis for observed differences in progression is unclear, although it leaves open the question of whether there are differences in immune responses to HIV-1 based on race or ethnicity. The studies described in this proposal have the unique opportunity to study ethic groups from geographical location, socioeconomically groups, all with access to the same health care.

There is still a paucity of data, compared to that on adults, on the relationship between the immune response, viral load, and disease progression in children vertically infected with HIV-1. As mentioned earlier, the dynamics of HIV-1 infection are markedly different in children than in adults. Neonates experience a higher initial viral burst and virus levels decrease slowly over a period of years [16, 17], whereas adults generally control the initial viral burst within 3 to 4 months [18, 19]. The greater viral burst in neonates is thought to be a result of higher thymic output and high numbers of circulating CD4+ T cells which serve as targets for HIV-1[20]. The strength and breadth of HIV-specific CD8+ T cell response in perinatally infected children has been shown to be influenced by the state of the CD4+ T cell compartment as well as age [21].

Based on data suggesting there are differences in the immune response between races we hypothesized that African American children will mount an HIV-specific immune response of higher magnitude compared to age and disease-matched Hispanic children.

To explore this hypothesis we paired perinatally infected African-American children with Hispanic children, matched by age, VL, and CD4 characteristics, and directly compared the CD8+ T-cell response to HIV antigens between the two groups.

## Materials and Methods

*Subjects and Matching*

The 41 subjects, all of whom were perinatally infected, were attendees of the Pediatric HIV clinic at Jacobi Medical Center, Bronx, NY. Patients were classified as Hispanic or African-American by clinician determination. Heparinized whole blood was collected from patients on a multi-weekly basis after they or their guardians provided informed consent, in accordance with local institutional review board–approved protocols. Blood was then shipped overnight to UCSF, where PBMC were isolated using a Ficoll density gradient, frozen, and stored at -180°C until use.

In order to make a balanced comparison, one Hispanic patient was matched with one African-American patient, based on proximity of age, viral load, and either CD4+ T cell count or percentage. In all but two pairs, gender was matched as well (Table 1.1). Five samples (three Hispanic, two African-American) were excluded in the final analysis due to inadequate cell number or failure to respond to the positive control. The resulting unpaired samples were included separately in the data analysis, as they did not differ from the balance of the cohort with respect to viral load, age, and CD4+ T cell count.

**Table 1.1.** Matched Patient Characteristics

| Patient ID | Pair # | Race | Sex | Age(m) | HLA-A1 | A2 | B1 | B2 | C1 | C2 | Viral load | LVL | CD4% | CD4 | Gag | Nef | Tat | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| JMC-001 | 1 | B | F | 71 | 31 | 80 | 7 | 40 | 3 | 7 | 29,608 | 4.5 | 32 | 941 | 1752 | 1960 | 900 | 4612 |
| JMC-002 | 1 | H | F | 68 | 24 | 29 | 7 | 39 | 7 | 7 | 30,141 | 4.5 | 26 | 879 | 1392 | 118 | 33 | 1542 |
| JMC-003 | 2 | B | F | 162 | 3 | 74 | 42 | 58 | 6 | 17 | 19,633 | 4.3 | 14 | 485 | 810 | 690 | 50 | 1550 |
| JMC-004 | 2 | H | M | 182 | 11 | 66 | 35 | 58 | 4 | 6 | 28,447 | 4.5 | 30 | 685 | 705 | 895 | 50 | 1,650 |
| JMC-005 | 3 | B | F | 209 | 1 | 32 | 8 | 40 | 2 | 7 | 79,792 | 4.9 | 22 | 246 | 978 | 660 | 155 | 1,793 |
| JMC-006 | 3 | H | F | 188 | 30 | 80 | 42 | 44 | 4 | 17 | 68,510 | 4.8 | 22 | 338 | 10 | 110 | 30 | 150 |
| JMC-007 | 4 | B | F | 138 | 30 | 66 | 44 | 58 | 7 | 8 | 1,157 | 3.1 |  |  | 530 | 233 | 345 | 1,108 |
| JMC-008 | 4 | H | F | 142 | 26 | 68 | 7 | 39 | 7 | 12 | 866 | 2.9 | 18 | 357 | 182 | 90 | 10 | 282 |
| JMC-009 | 5 | B | F | 126 | 30 | 66 | 44 | 58 | 7 | 8 | 140,800 | 5.1 | 22 | 790 | 2,882 | 283 | 285 | 3,450 |
| JMC-010 | 5 | H | F | 146 | 3 | 23 | 7 | 14 | 8 | 15 | 160,134 | 5.2 | 21 | 407 | 63 | 288 | 23 | 373 |
| JMC-011 | 6 | B | F | 111 | 1 | 68 | 7 | 58 | 2 | 15 | 33,564 | 4.5 | 38 | 686 | 1,785 | 482 | 0 | 3,267 |
| JMC-012 | 6 | H | F | 101 | 1 | 68 | 39 | 50 | 6 | 12 | 42,409 | 4.6 | 16 | 158 | 20 | 0 | 3 | 23 |
| JMC-013 | 7 | B | F | 59 | 26 | 30 | 37 | 35 | 2 | 16 | 50 | 1.7 | 44 | 1,078 | 0 | 3 | 0 | 3 |
| JMC-014 | 7 | H | F | 66 | 24 | 24 | 40 | 40 | 3 | 3 | 50 | 1.7 | 44 | 2,003 | 18 | 0 | 30 | 48 |
| JMC-015 | 8 | B | F | 16 | 33 | 74 | 8 | 14 | 3 | 7 | 4,412 | 3.6 | 46 | 1,563 | 338 | 170 | 75 | 583 |
| JMC-016 | 8 | H | F | 14 | 3 | 33 | 35 | 35 | 4 | 4 | 2990 | 3.5 | 35 | 1521 | 63 | 190 | 20 | 273 |
| JMC-017 | 9 | B | M | 138 | 36 | 74 | 18 | 53 | 2 | 4 | 134,488 | 5.1 | 19 | 331 | 10 | 385 | 90 | 485 |
| JMC-018 | 9 | H | F | 140 | 68 | 74 | 15 | 58 | 3 | 6 | 110,714 | 5.0 | 14 | 228 | 175 | 0 | 10 | 185 |
| JMC-019 | 10 | B | M | 60 | 20 | 23 | 42 | 45 | 6 | 17 | 15,762 | 4.2 | 27 | 628 | 1,702 | 485 | 0 | 2,188 |
| JMC-020 | 10 | H | M | 57 | 2 | 23 | 44 | 53 | 4 | 4 | 17,948 | 4.3 | 37 | 783 | 359 | 577 | 17 | 951 |
| JMC-021 | 11 | B | M | 67 | 33 |  | 15 | 35 | 4 | 14 | 400 | 2.6 | 23 | 1,125 | 44 | 553 | 0 | 598 |
| JMC-022 | 11 | H | M | 71 | 2 | 26 | 35 | 44 | 4 | 5 | 400 | 2.6 | 39 | 1,326 | 13 | 233 | 15 | 260 |
| JMC-023 | 12 | B | M | 79 | 23 | 30 | 15 | 42 | 5 | 17 | 4,902 | 3.7 | 32 | 1,142 | 375 | 172 | 20 | 567 |
| JMC-024 | 12 | H | M | 97 | 2 | 11 | 51 | 63 | 1 | 14 | 4,495 | 3.7 | 32 | 1,257 | 533 | 752 | 107 | 1,392 |
| JMC-025 | 13 | B | M | 134 | 2 | 30 | 44 | 57 | 5 | 7 | 50 | 1.7 | 17 | 295 | 220 | 630 | 0 | 850 |
| JMC-026 | 13 | H | M | 134 | 2 | 11 | 40 | 57 | 3 | 6 | 50 | 1.7 | 13 | 201 | 0 | 127 | 0 | 127 |
| JMC-027 | 14 | B | M | 169 | 3 | 68 | 3 | 57 | 2 | 18 | 3,368 | 3.6 | 33 | 695 | 468 | 40 | 60 | 568 |
| JMC-028 | 14 | H | M | 171 | 68 | 68 | 14 | 57 | 7 | 8 | 3,761 | 3.6 | 18 | 394 | 35 | 75 | 30 | 140 |
| JMC-029 | 15 | B | M | 109 |  |  |  |  |  |  | 44,387 | 4.6 | 24 | 616 | 85 | 310 | 0 | 395 |
| JMC-030 | 15 | H | M | 106 | 2 | 2 | 45 | 51 | 15 | 16 | 79,052 | 4.9 | 19 | 307 | 13 | 45 | 25 | 83 |
| JMC-031 | 16 | B | M | 104 | 68 | 38 | 35 | 35 | 2 | 12 | 31,254 | 4.5 | 22 | 625 | 220 | 513 | 450 | 1,183 |
| JMC-032 | 16 | H | M | 105 | 24 | 24 | 35 | 35 | 12 | 16 | 34,343 | 4.5 | 14 | 194 | 625 | 1,220 | 0 | 1,845 |
| JMC-033 | 17 | B | M | 142 | 29 | 30 | 8 | 53 | 3 | 15 | 87,804 | 4.9 | 1 | 9 | 35 | 45 | 20 | 100 |
| JMC-034 | 17 | H | M | 135 | 3 | 25 | 7 | 18 | 7 | 12 | 89,732 | 5.0 | 25 | 629 | 46.9 | 111 | 0 | 158 |
| JMC-035 | 18 | B | M | 28 | 2 | 2 | 44 | 45 | 7 | 16 | 10,658 | 4.0 | 36 | 2,136 | 573 | 165 | 25 | 763 |
| JMC-036 | 18 | H | M | 34 | 31 | 68 | 39 | 40 | 3 | 12 | 16,538 | 4.2 | 33 | 1,335 | 130 | 25 | 0 | 155 |
| JMC-037[a] | 19 | B | F | 124 | 30 | 30 | 14 | 53 | 6 | 8 | 109,376 | 5.0 |  | 1,732 | 270 | 1,052 | 30 | 1,352 |
| JMC-038[a] | 20 | B | F | 98 | 23 | 30 | 42 | 58 | 6 | 17 | 7,889 | 3.9 | 34 | 550 | 1,021 | 0 | 7 | 1,028 |
| JMC-039[a] | 21 | H | F | 18 | 2 | 30 | 42 | 52 | 16 | 17 | 248,947 | 5.4 | 34 | 997 | 58 | 468 | 3 | 528 |
| JMC-040[a] | 22 | H | F | 102 | 2 | 23 | 18 | 52 | 2 | 3 | 48,125 | 4.7 | 27 | 699 | 145 | 45 | 58 | 247 |
| JMC-041[a] | 23 | H | M | 26 | 2 | 26 | 8 | 51 | 7 | 15 | 50 | 1.7 | 36 | 967 | 4 | 28 | 48 | 80 |

[a] Unmatched patients (see Materials and Methods)

*Detection of HIV-specific T cell responses*

Quantification of HIV-specific T cell responses using thawed viable PBMC was performed using the cytokine amplified ELISPOT assay[22]. PBMC were stimulated with synthetic peptides (15 amino acids in length. 15mers) of the clade B consensus sequences derived from either Gag, Nef, or Tat (1μg/ml) with IL-7 and IL-15 (2.5ng/ml). Staphylococcus enterotoxin B (SEB) was used as a positive control and media alone, with IL-7 and IL-15, was used as a negative control. IFN-γ spot-forming units (SFUs) were visualized, counted, and standardized to SFU/$10^6$ PBMC. The addition of IL-7 and IL-15 at 2.5ng/ml did not lead to the detection of false-positive spots above the positive threshold in control ELISPOTS performed with PBMC from HIV-seronegative donors. We termed the *total* HIV-specific response as the sum of the responses to HIV Gag, Nef, and Tat peptides

*HLA typing*

DNA was extracted from PBMC and HLA typing was performed using PCR with sequence-specific primers.

*Statistical Methods*

We used median (interquartile range) as a measure of central tendency for continuous variables. We employed the Wilcoxon Two Sample test for simple comparison tests, and the Spearman Rank correlation test to explore associations between 2 continuous variables. Single and multivariable linear regression modeling was

performed using Proc Reg in the SAS system. All statistical analyses were performed in the SAS System version 8.2 for Windows (SAS Institute, Cary, NC).

## Results

*African-Americans show higher total HIV-specific immune response within age and disease-matched pairs*

We compared the HIV-specific T cell response to HIV clade B consensus peptide pools of Gag, Nef, and Tat as measured by a cytokine enhanced IFN-γ ELISPOT, between Hispanics and African-Americans matched for age and disease state. We termed the *total* HIV-specific response as the sum of the responses to HIV Gag, Nef, and Tat peptides. The total HIV-specific T cell immune responses of the paired samples are shown in Figure 1. In 13 out of 18 matched pairs, the African-American patient showed a higher immune response than the corresponding Hispanic patient (Figure 1.1). Within Hispanic subjects, only 5 patients had strong total HIV-specific responses (>1000 SFU/$10^6$ PBMC), and the majority of Hispanic subjects showed low responses (<300 SFU/$10^6$ PBMC).

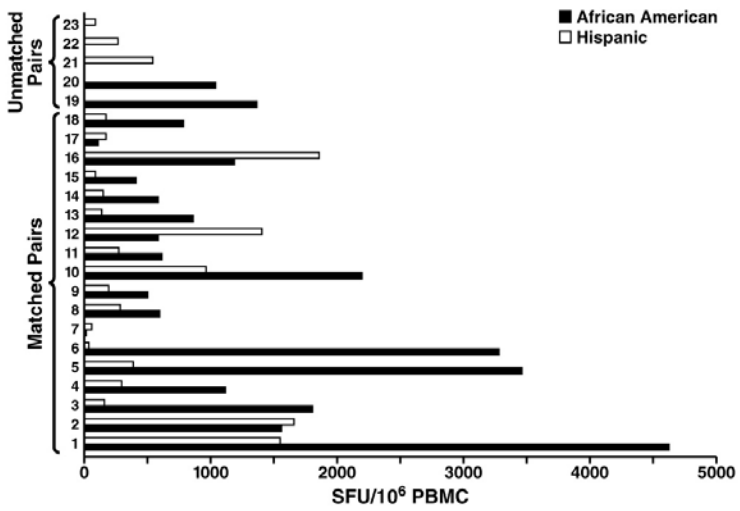


**FIGURE 1.1. Total HIV-1 specific T cell response in study subjects. African American (■) and Hispanic (□) subjects were matched by age, viral load and CD4+ T cell count or percentage. Five unmatched subjects did not have a corresponding pair (see materials and methods). See Table 1 for subject and pair characteristics.**

*Higher total HIV-specific immune response in African-Americans is driven by higher Gag response*

When considered as a group, African-American patients showed a significantly higher *total* HIV-specific IFN-γ response as compared to Hispanic patients (p=0.004) (Figure 1.2D). This difference in response was driven heavily by the higher Gag-specific response and to a lesser degree by the Nef-specific response. Ninety percent of African-Americans demonstrated a positive Gag-specific response (>30 SFU/$10^6$ PBMCs) compared to 70% of Hispanic patients. This pattern was also seen in Nef-specific responses, while the proportion of positive Tat responders was similar the 2 groups. The magnitude of the Gag, Nef, and Tat-specific T cell response was higher in the African-American group compared to the Hispanic patients, but only the difference in Gag-specific response was significant (p=0.01) (Figure 1.2A-C). On average the African-American patients made a Gag-specific response that was 486 SFU/$10^6$ PBMC higher than Hispanic children (p=0.01). Although the Nef-specific responses followed a similar trend as the Gag-specific responses, the overall difference was not statistically significant (p=0.06) (Figure 1.2B). There were very few Tat responders in either group and no significant difference between groups was seen (p=0.3) (Figure 1.2C).

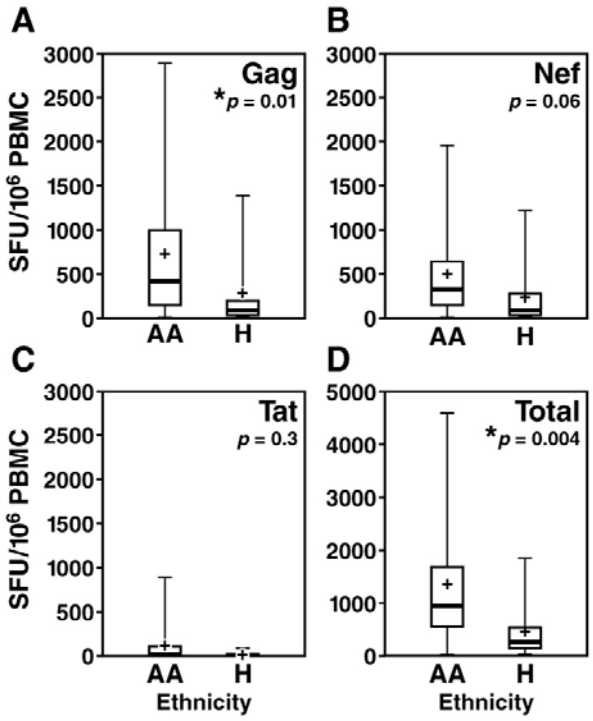


**FIGURE 1.2. HIV Gag, Nef, Tat and Total specific T cell responses from African American (AA) and Hispanic (H) subjects. IFN-γ secretion in response to stimulation with HIV Gag (*A*), Nef (*B*), Tat (*C*) or Total (*D*). Boxes encompass the 25th-75th percentile, with the median (-) and mean (+) displayed. Whiskers denote the entire range of responses.**

*African-American females near the age of puberty show the highest HIV-specific immune response among all racial/gender groups.*

When the subjects were divided into racial and gender groups, it was clear that the highest Gag-specific and total response was seen in African-American females, with African-American males having the second highest response, the Hispanic males next, and the lowest response seen in Hispanic females (Figure 1.3A-B). A positive Gag-specific response was seen in 90% of African-American females and males, 70% of Hispanic females, and 63% of Hispanic males. We concluded that the higher total HIV and Gag-specific response seen in the African-American group was significantly driven

by the high response in African-American females. This general trend held true for Nef and Tat-specific responses as well (data not shown). Although the subjects were initially matched for viral load, we again verified that there was no significant difference in viral load between the 4 groups that could be leading to the observed difference in responses (Figure 1.3C).

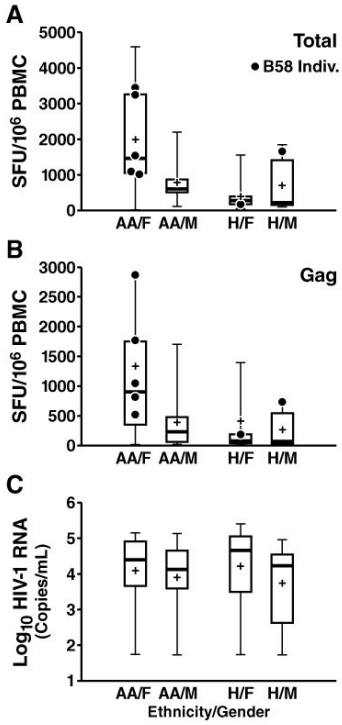


**FIGURE 1.3. Total HIV and Gag-specific T cell immune response and viral load among racial and gender groups. *A*, African American females had the highest total HIV-specific T cell immune response among all ethnic/gender groups. *B*, African American females also had the highest Gag-specific T cell immune response. *C,* Viral loads, were similar among all ethnic/gender groups. Responders carrying HLA-B*58 allele (●) are denoted in both Gag and Total responses**

African-American females showed the highest response of all the ethnic/gender groups tested. Within this category the highest responders 6 African-American females

clustered around puberty, with a median age of 10.8 years (Figure 1.4A). African-American females enter puberty at an earlier age than other races, estimated to be around 8.5 years [23].

*Relationship between Gag-specific responses, age, CD4 count, and viral load*

Interestingly, we observed a non-linear relationship between Gag-specific responses and each of the parameters we matched for: age, CD4+ T cell count and viral load. Gag-specific responses rose with age until about the age of 5, plateauing until the age of 9, after which the responses decreased (Figure 1.4A). Immune responses rose until a CD4+ T cell count of approximately 800 cells/μl, after which they declined (Figure 1.4B). With respect to viral load, Gag-specific responses positively correlated with viral load until 30,000 copies/ml. Subjects with viral loads higher than 30,000 copies tended to show a decreased immune response (Figure 1.4C).

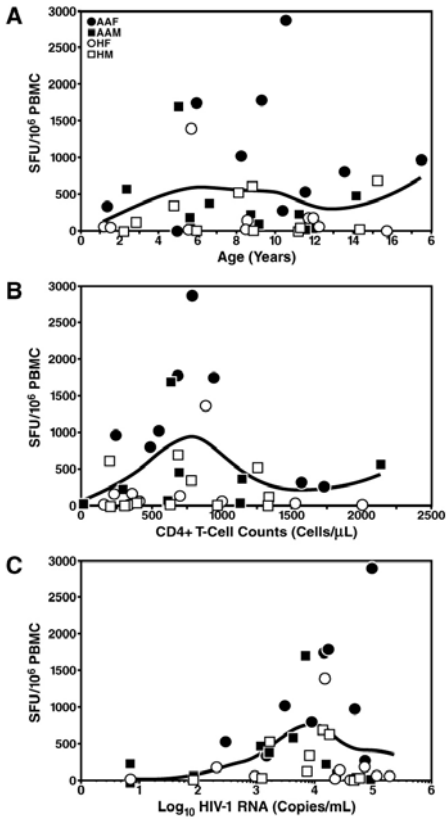


**FIGURE 1.4. Relationships between Gag-specific T cell immune response and age, CD4+ T cell count and plasma viral load. The trend of the values is modeled as a line in the relationships between HIV -Gag-specific T cell response and age (A), CD4+ T cell count (B) and plasma viral load (C).**

*Relative influence of race, gender, age, viral load, CD4+ T--cell count on Gag-specific responses*

In order to further delineate the relative influence of ethnicity, gender, age, HIV-1 RNA levels and CD4+ T cell count on HIV-Gag specific T cell responses, we performed multi-variable linear regression analyses. To compare groups of patients we formed gender and ethnic categories, by the use of indicator variables, for which African-American males were arbitrarily chosen as the reference group. The multivariable regression analysis demonstrated that compared to African-American males, the African-

American females, had an average 671.9 SFU/$10^6$ PBMC higher response to Gag peptide (p=0.02). The Hispanic females and Hispanic males were estimated to have on average –244.6 and –151.2 SFU/$10^6$ PBMC lower responses than the African-American males, although these differences were not significant (p=0.36 and p=0.56 respectively). In this model, neither CD4+ T cell count (–0.1 SFU/$10^6$ PBMC per CD4+ T cell), age in years (-17.3 SFU/$10^6$ PBMC per year older), or $\log_{10}$ HIV-1 RNA (145 SFU/$10^6$ PBMC per 1 $\log_{10}$ higher HIV-1 RNA), at time of study, were associated with IFN-γ response to Gag peptides (p=0.69, p=0.56 and p=0.11 respectively).

*HLA-A*66 and -B*58 haplotypes associated with higher Gag-specific response*

We then sought to determine the association of Class I alleles with the observed IFN-γ responses to HIV peptides. HLA typing revealed a wide range of haplotypes, shown in Table 1.1. Of the known Class I allele associations we examined those known to have the strongest association with disease progression on the population level (HLA-A*66, -B*27, -B*35, -B*51, -B*57, -B*58, -Cw*02, and -Cw*14) [24, 25], reasoning that these would be the most apparent associations in a small observational cohort study. Among these alleles, we observed two, HLA-A*66 and -B*58 which associated with higher Gag response (p=0.07 and p=0.007, respectively). The 3 subjects with HLA-A*66 also expressed HLA-B*58. The immune responses of the HLA-B*58 positive individuals are highlighted (Figure 1.3A-B). There were 7 subjects with the HLA-B*58 haplotype (6 females). African-American females comprised the highest number of HLA-B*58 individuals among any ethnic/gender group, with 5/10 African-American females carrying the HLA-B*58 allele.

## Discussion

There is mounting evidence that racial differences in HIV-specific immune responses may exist [11-14]. To the best of our knowledge, this is the first study to observe and compare the HIV-specific T cell immune response in vertically infected children of 2 different races that live in close geographic proximity. Age, HIV viral load, and CD4+ T cell levels are thought to strongly influence the magnitude of the immune response in children [16, 21, 26, 27]. We matched African-American and Hispanic patients for these three parameters, enabling better direct comparisons between the two groups. In this cohort of HIV-1 infected pediatric patients, African-American children mounted a stronger HIV-specific T cell immune response compared to age and disease matched Hispanic children, when stimulated with HIV-Gag. Gag was the most frequently recognized antigen, and led to the strongest responses in both ethnicities, followed by Nef, and there were very few Tat responders. In a linear regression model, when accounting for viral load, CD4+ T cell count and age, African-American females showed the highest response. We examined associations between immune response and HLA alleles that are strongly linked with advanced or decreased disease progression, and found that HLA-B*58 was strongly associated with higher Gag-specific IFN-γ responses. We observed a non-linear relationship between immune response and each of the three matched parameters: age, viral load, and CD4 count.

The relationship between the magnitude of the T cell response and plasma viral load is complex and often changes with differences in treatment and/or disease level of the subjects studied. Previous studies in adults, have found a negative correlation between viral load and Gag-specific proliferative responses and cytokine secretion [28-30], while

more recent studies have reported either no correlation between Gag-specific immune response and viral load [31, 32], or even a positive correlation between the two [33]. One pediatric study found no correlation between the magnitude/breadth of HIV-1 specific immune response and viral load [34]. Other investigators have postulated a bell-shaped relationship between Gag-specific T-cell-responses and viral load [35]. Our results support this, and suggest that Gag-specific T cell responses positively correlate with viral load at lower levels of viremia, when the immune response is driven by antigenic exposure. With higher viremia, the immune response is most likely depleted, inhibited, or overwhelmed, leading to a negative correlation between viral load and immune response.

It is known that expression of certain HLA Class I molecules can influence HIV disease progression, although the exact mechanism is unknown. Some alleles, such as HLA-B*57 and -B*27 have been strongly associated with delayed disease progression, and others, such as B*35, associated with an accelerated progression to AIDS [25, 36-39]. The HLA-B*58 molecule has a very similar binding motif to that of HLA-B*57, one of the HLA alleles most significantly associated with delayed disease progression. HLA-B*57 and -B*58 are both classified in the B58 supertype group [40], and the two alleles are known to present identical epitopes [41]. HLA-B*57 has previously been associated with stronger CTL immune responses in acute HIV-1 infection [42, 43]. It is possible that, along with HLA-B*57, B*58 restricted immune responses target a dominant epitope, leading to a stronger, more effective HIV-specific immune response and slower disease progression. Recent studies have shed some light on this issue, finding that in individuals with protective HLA alleles, the majority of the HIV-specific CD8+ T cell response in primary infection is restricted through the protective allele [44]. These data

suggest that HLA-B*57 and -B*58 alleles can contribute to delayed disease progression, at least partially, through shaping the immune response.

The HLA-B*58 allele was enriched for in the African-American female population in our cohort, with 50% of the African-American females in our study carrying the HLA-B*58 allele. HLA-B*58 is mainly found in the African-American population, at a frequency of approximately 6-10% [45, 46]. The higher frequency of HLA-B*58 among African-American females in our cohort suggests a survival advantage mediated by HLA-B*58.

Socioeconomic and environmental differences are two other possible factors that could lead to the disparate responses observed. However, all the study subjects received care at the same clinic, and most patients live in close geographic proximity and are of a similar socioeconomic level. Cultural differences could also play a role, such as different dietary practices, including the use of herbal and other non-prescription dietary supplements. Major virological differences are unlikely, given that all of the children are infected with clade B HIV. Finally, variability in genes controlling immune function might also contribute to variable immune responses. Genes such as those for cytokines, toll-like receptors, complement proteins, and stress-inducible proteins show race-related patterns of genetic polymorphisms [47-53].

Although the children have variable treatment regimens and adherence levels, almost all were receiving some form of antiretroviral therapy. Data from the physicians at the pediatric clinic in the Bronx shows that 36 patients were prescribed HAART regimens at the time of study, while 4 patients were on less than three drugs, with one or two nucleoside analogs, and 1 patient was not being treated. Of the 40 children on

treatment, only 5 patients had excellent adherence, with the remaining 35 having only variable adherence. The poor treatment adherence is not surprising, as these children were expected to take complex regimens. Therefore, we believe that the differences in immune response that we see are not driven by therapy *per se* but a reflection of the host-viral relationship.

The data presented here suggests a relationship between HIV-specific immune responses, race, and gender, but the small number of study participants precluded us from applying these findings to the general population. The finding of a higher magnitude of Gag-specific response in a group of African American girls, most of whom express HLA-B*58, is intriguing and raises the question of the influence of epitope-specific responses.

In our cross-sectional study there was a wide range of viral loads and CD4+ T cell characteristics, even among those individuals with protective alleles. This suggests that even among those expressing alleles known to associate with slower disease progression there are differential levels of immune control, which has been documented by other groups as well [38]. We hypothesize that the HLA allele/s that restrict the Gag-specific response are very important in the eventual level of disease progression in an individual. More specifically, HIV-specific CD8+ T cells that are restricted by protective alleles are associated with viral control than HIV-specific CD8+ T cells that are restricted by non-protective alleles. An example of this can be seen in two individuals who shared two of the same HLA alleles (A*30 and B*58). One individual who was unable to control the virus directed their response through the HLA allele A*30. The other subject who controlled their virus directed their CD8 response through the HLA-B*58 allele. This

preliminarily suggests that the immune responses restricted by protective alleles are of critical importance in viral control.

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# Chapter 2:

# Dysregulation of Jak/STAT Signaling Associated with HIV Infection in Perinatally Infected Children

## Introduction

Our earlier finding that perinatally HIV-infected African American children displayed an HIV-specific CD8+ T cell response of higher magnitude than age and disease-matched Hispanic children led us to hypothesize about the possible causes of this observed difference in immune response. There are a myriad of factors that can influence the HIV-specific immune response in any given individual, including: viral strain, viral mutations, innate immune response, antigen processing, antigen presentation, T cell receptor expression, and phenotype of responding T cells, among many others. We decided to focus on one aspect, that of innate immune response, and specifically monocytes.

Monocytes are an important intermediary between the innate and acquired immune response as a result of their ability to phagocytose and present antigen to effector T cells, as well as produce and secrete important cytokines[1]. It has been long observed that HIV infection results in defective monocyte activity, including reduced antigen uptake and altered cytokine secretion [2, 3]. Variable monocyte function between African American and Hispanic subjects could cause the difference in HIV-specific immune response that we observed in our earlier study.

A previous study had shown varied IFN-induced STAT signaling among racial groups of HCV-infected donors [4], and so we explored whether immune cell signaling also varied among ethnically diverse groups of HIV-1 infected subjects. In collaboration with Dr. Andrew Lee at Stanford University, we utilized the newly developed technique of using flow-cytometry to interrogate phosphorylation states of signaling intermediates (termed phospho-flow)[5, 6], to study the Jak/STAT signaling pathway within monocytes

of our perinatally HIV-infected cohort. We initially hypothesized that we would observe Jak/STAT signaling differences between African-American and Hispanic subjects, but our initial screen revealed a general HIV-related dysfunction that did not appear to be associated with race. We then expanded our study to probe MAPK signaling and expanded our cell subset analysis to include T and B cells. In the resulting study we identified a significant defect in Stat5 activation and enhancement of ERK signaling within the monocytes of HIV-1 infected pediatric subjects.

This study identified a defect in monocyte signaling associated with HIV infection, and implies the importance of monocyte function in the immunopathogenesis of HIV. This understanding of altered monocyte signaling during HIV-1 infection could lead to therapeutic approaches that compensate for the deficiency.

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# Single-Cell, Phosphoepitope-Specific Analysis Demonstrates Cell Type- and Pathway-Specific Dysregulation of Jak/STAT and MAPK Signaling Associated with In Vivo Human Immunodeficiency Virus Type 1 Infection▽

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**Despite extensive evidence of cell signaling alterations induced by human immunodeficiency virus type 1 (HIV-1) in vitro, the relevance of these changes to the clinical and/or immunologic status of HIV-1-infected individuals is often unclear. As such, mapping the details of cell type-specific degradation of immune function as a consequence of changes to signaling network responses has not been readily accessible. We used a flow cytometric-based assay of signaling to determine Janus kinase/signal transducers and activators of transcription (Jak/STAT) signaling changes at the single-cell level within distinct cell subsets from the primary immune cells of HIV-1-infected donors. We identified a specific defect in granulocyte-macrophage colony-stimulating factor (GM-CSF)-driven Stat5 phosphorylation in the monocytes of HIV-1+ donors. This inhibition was statistically significant in a cohort of treated and untreated individuals. Ex vivo Stat5 phosphorylation levels varied among HIV-1+ donors but did not correlate with $CD4^+$ T-cell counts or HIV-1 plasma viral load. Low Stat5 activation occurred in HIV-1-infected donors despite normal GM-CSF receptor levels. Investigation of mitogen-activated protein kinase (MAPK) pathways, also stimulated by GM-CSF, led to the observation that lipopolysaccharide-stimulated extracellular signal-regulated kinase phosphorylation is enhanced in monocytes. Thus, we have identified a specific, imbalanced monocyte signaling profile, with inhibition of STAT and enhancement of MAPK signaling, associated with HIV-1 infection. This understanding of altered monocyte signaling responses that contribute to defective antigen presentation during HIV-1 infection could lead to immunotherapeutic approaches that compensate for the deficiency.**

The hallmark of uninhibited human immunodeficiency virus type 1 (HIV-1) infection is a well-documented, progressive immune cell dysfunction. Widespread T-cell activation, elevated inflammatory cytokine secretion, and hypergammaglobulinemia, to name a few examples, prominently occur in infected individuals. HIV-1 may affect immune cell function directly by inducing cell signaling through binding to cell surface receptors (8, 41) or by manipulating the cellular response to physiologic stimuli. For example, HIV-1-infected lymphocytes exhibit an hyperactive response to TCR stimulation, in a tat-dependent manner (30), that leads to potentiated, reflexively inappropriate signaling events that drive T-cell dysfunction. As part of the systemic changes induced by HIV-1, it is possible there will be alterations in intracellular signaling networks or feedback mechanisms that lead to dysfunctional immune cell responses to cytokines, further contributing to HIV-1 pathology in the host. Thus, direct or indirect effects upon a normal "set point" of immune cell homeostasis would lead to aberrant cell signaling and consequential immune dysfunction.

The Janus kinase/signal transducers and activators of transcription (Jak/STAT) pathways are essential for signaling by cytokines, including such important intracellular mediators as interferons (IFNs), interleukins, and colony-stimulating factors, as well as certain hormones (reviewed in reference 12). A recently described genetic mutation in humans that results in Stat1 deficiency is associated with fatal disseminated viral infection (14), highlighting the importance of STAT signaling for antiviral immunity. Among a diverse group of pathogens, HIV-1 has been shown to alter Jak/STAT signaling, although the exact mechanism is unclear, with some studies showing STAT hyperactivation (2, 4) and others reporting inhibition of STAT-driven transcriptional activity (26). Such paradoxical effects may be explained, at least in part, by effects upon distinct target cell types, different viral strains, or alternative pathways

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used in these studies. Thus, in the absence of a cell-by-cell analysis of signaling biochemistry, clear interpretation of the underlying mechanistic disorders driven by HIV-1 is difficult to formalize.

The development of flow cytometry-based approaches to the identification of aberrant signaling profiles, via measurement of phosphorylation of signaling intermediates (termed "phospho-flow"), has allowed for accelerated association of network topologies with disease states (19–22). Our previous work led to the realization that triggering cells to respond to environmental stimuli, such as cytokine or drug action, and the activation phenotypes associated with such perturbations, allows for clearer resolution of the underlying networks of protein activation states and allows for more distinct classification of signaling-associated disease outcomes (25). Signaling is a dynamic event, and as such static views of basal states would be considered insufficient for determination of a network's structure, therefore rendering correlations to clinical outcomes less meaningful. Phospho-flow is particularly well suited to address cell signaling in the context of HIV-1 disease because it can simultaneously discern multiple signaling events in an individual cell within complex cellular populations. The ability to monitor cell signaling in primary patient samples such as peripheral blood mononuclear cells (PBMC), allowed us to measure signaling pathway activation ex vivo. This novel approach obviates the use of potentially biased extended in vitro-cultured cells and allows for more physiologic interpretations in situations, for instance, where immune action depends on natural context for study of antigen presenting cells. In addition, phospho-flow technology allowed us to closely monitor signaling states within HIV-1-infected individuals, correlated to disease stage and treatment.

Here we compare the phosphorylation, and hence the activation status, of three Stat proteins (Stat1, Stat3, and Stat5) in response to a panel of cytokine stimuli, within three immune cell populations (T cells, B cells, and monocytes) from HIV-1-infected and uninfected pediatric subjects. We previously observed gender and ethnic differences in HIV-1-specific immunity in this cohort (35). Since we have also observed varied IFN-induced STAT signaling among racial groups of HCV-infected donors (18), we explored whether immune cell signaling also varied among ethnically diverse groups of HIV-1-infected subjects. The results of the initial phospho-flow screen then led us to investigate mitogen-activated protein kinase (MAPK) pathways. We identified an imbalanced signaling profile in response to model stimuli within the immune cells of HIV-1-infected pediatric subjects, with defective STAT activation and enhanced MAPK signaling. This cross-sectional study represents an critical step toward linking signaling changes found in HIV-1-infected individuals to recognized immune dysfunctions that occur in HIV-1 infection and disease progression.

## MATERIALS AND METHODS

**Clinical samples, isolation, storage, and thawing of primary cells.** Peripheral blood samples were obtained from perinatally HIV-1-infected and exposed-but-uninfected (EU) pediatric subjects (both groups born to HIV-1+ mothers) monitored at the pediatric HIV clinic at Jacobi Medical Center, Bronx, NY. The race and ethnic background of this cohort is reflective of the population of the Bronx, NY, where the residents are predominantly African-American (35.6%) and Hispanic (40.4%) (2000 census). The absolute total lymphocyte count, absolute $CD4^+$ T-cell count and percentage, HIV-1 plasma viral load (Amplicor HIV-1 monitor; Roche Diagnostic Systems), and clinical data, including antiretroviral drug therapy history and adherence assessments, were recorded for samples from the HIV-1-infected patients. Peripheral blood samples from HIV-1-infected adults were obtained from an ongoing study of compartmental shedding in chronically infected adults conducted at San Mateo County Medical Center and Health Department. Peripheral blood samples from uninfected adults were obtained from the Stanford University Blood Bank. Institutional review boards at Jacobi, San Mateo County site, University of California at San Francisco (UCSF), and Stanford University approved this study. PBMC were isolated via density gradient separation (Ficoll-Paque Plus; Amersham Biosciences AB, Uppsala, Sweden). PBMC were pelleted and resuspended in either 90% fetal bovine serum (HyClone, South Logan, UT) or 90% human AB serum (Irvine Scientific, Irvine, CA) and 10% dimethyl sulfoxide (Sigma, St. Louis, MO) and then stored in liquid nitrogen. The blood samples from infected and uninfected pediatric subjects were handled and processed in parallel.

For signaling studies, PBMC were thawed in RPMI with 5% human AB serum, counted, pelleted, resuspended at $5 \times 10^6$ ml cells per ml, and allowed to rest at 37°C for 2 h in a 5% $CO_2$ tissue culture incubator. PBMC isolated from buffy coat preparations from uninfected adults (Stanford University Blood Bank) were similarly isolated and stored in multiple aliquots from the same donor, which served as internal controls to test for the integrity of the stimulation and staining procedures. In separate experiments, repeat testing on multiple aliquots of the same normal donor showed low variance of phospho-flow responses ($n = 4$, $\sigma^2 < 0.1$).

**Stimulation, fixation, and permeabilization for detection of cell signaling.** PBMC were stimulated with vehicle or 4 ng of granulocyte-macrophage colony-stimulating factor (GM-CSF)/ml, 20 ng of IFN-γ/ml, 10 ng of interleukin-3 (IL-3)/ml, 20 ng of IL-7/ml, 100 ng of IL-10/ml, and 1 μg of lipopolysaccharide/ml (LPS) (from *E. coli*; Sigma, St. Louis, MO) and phorbol myristic acetate (PMA)-ionomycin (50 ng/ml and 1 μM, respectively; Sigma) for 12 min at 37°C. All stimuli were titrated to maximum signaling activity. All of the cytokines except GM-CSF were obtained from Peprotech (Rocky Hill, NJ). Clinical-grade GM-CSF (Sargramostim; Immunex, Seattle, WA) was obtained from the Stanford University Hospital Pharmacy. At the indicated times, paraformaldehyde (PFA; Electron Microscopy Services, Fort Washington, PA) was added to a 2% final concentration for 10 min at room temperature to arrest signaling activity. Cells were washed and permeabilized with 90% ice-cold methanol for 10 min on ice until staining for flow cytometry.

**Cell surface and intracellular phospho-specific flow cytometry.** Directly conjugated antibodies against human CD3 (UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD16 (3G8), CD20 (cytoplasmic, H1), CD33 (P7.6), and CD116 (GM-CSF receptor α chain, M5D12) were obtained from Becton-Dickinson (BD; San Jose, CA). Unconjugated anti-IL-3/IL-5/GM-CSF receptor β chain was obtained from Chemicon (Temecula, CA) and goat anti-mouse-phycoerythrin conjugate was obtained from Molecular Probes (Eugene, OR). Surface marker staining, both before and after the addition of methanol, was compared to the results obtained on unfixed, unpermeabilized cells. Since we determined that the traditional marker for monocytes, CD14 (M5E2), was not stable under methanol treatment, we identified monocytes by using CD33, a member of the sialic acid-binding immunoglobulin-like lectin (SIGLEC) family (reviewed in reference 9). In untreated cells, we confirmed that the two markers identified the same population and that the $CD33^+$ cells were GM-CSFrα positive (Fig. 1A and B). We also confirmed the stability of CD33 and CD3 under different fixation-permeabilization conditions (Fig. 1C). Although CD33 also stains peripheral blood dendritic cells (16), this cell type comprises ca. 0.1% of all PBMC and is likely to make a minor contribution to the gated populations.

PFA-fixed, methanol-permeabilized PBMC were washed twice and then resuspended in fluorescence-activated cell sorting staining buffer (phosphate-buffered saline plus 0.5% bovine serum albumin) and stained in final volume 100 μl for 30 min at room temperature. Antibodies obtained from BD were against phospho-Stat1(Y701), phospho-Stat3(Y705), phospho-Stat5(Y694), phospho-p38(T180/Y182), and extracellular signal-regulated kinase 1/2 (ERK1/2; pT202/pY204). Rabbit anti-phospho-p44/42 MAPK (T202/Y204) was obtained from Cell Signaling Technologies (Danvers, MA), and goat anti-rabbit-Alexa 647 conjugate was obtained from Molecular Probes (Eugene, OR).

**Fluorescence-activated cell sorting acquisition and data analysis.** Events were acquired on a FACScalibur or an LSR II (BD). To minimize day-to-day variation in cytometer settings, control beads with fluorescence in all channels (Spherotech, Lake Forest, IL) were tested at the beginning of each acquisition run. Fluorescence values varied by <10% of target values. Flow cytometry data was analyzed with FlowJo software (Tree Star, Ashland, OR), Microsoft XL

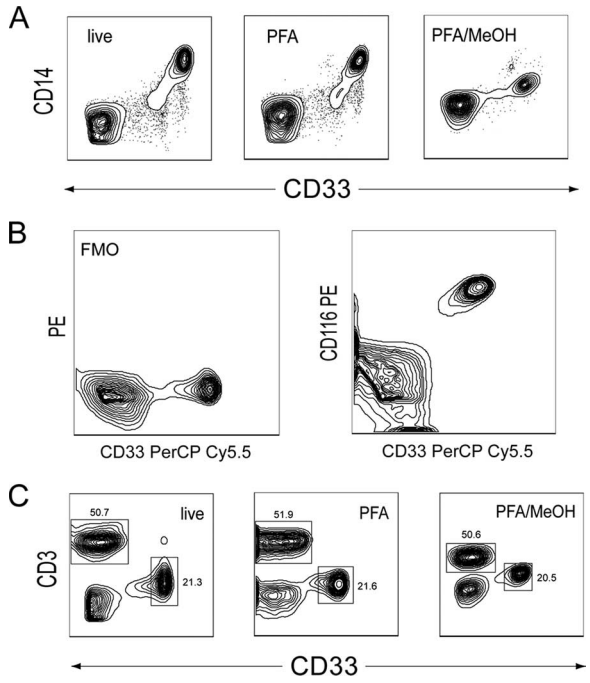


FIG. 1. CD33 and CD14 recognize overlapping populations of cells within human PBMC. (A) Cells from adult HIV-1-negative donors were either fixed with 2% PFA, PFA fixed and permeabilized with 90% methanol (PFA/MeOH), or left untreated (live) prior to staining for flow cytometry with anti-CD14 and anti-CD33 antibody. (B) Untreated PBMC were stained with anti-CD33 (FMO, fluorescence minus one control), or anti-CD33 and anti-CD116 (GM-CSF receptor, alpha chain) antibody. (C) Cells treated as in panel A were stained with anti-CD3 and anti-CD33 antibody. The percentage of live events is shown next to each gate. Representative results of three independent experiments are shown.

(Microsoft, Redmond, WA), and Prism 4.0 (GraphPad Software, San Diego, CA). Heat maps were generated with Spotfire DecisionSite (Tibco, Palo Alto, CA).

**Purification of monocytes.** Anti-human CD33 microbeads were obtained from Miltenyi Biotec (Auburn, CA) and used according to the manufacturer's instructions. The purity was determined by flow cytometry and was >90% $CD33^+$.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting.** Isolated $CD33^+$ cells were lysed in IPLS buffer containing 50 mM Tris-HCl (pH 8), 120 mM NaCl, 5 mM EDTA, and 0.5% (wt/vol) NP-40 and supplemented with fresh 1× protease inhibitor cocktail (Calbiochem, San Diego, CA). The protein concentration of each lysate was determined by using either a BCA assay (Pierce, Rockford, IL) or by Nanodrop technology. Lysates, matched for protein concentration, were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% Tris-glycine gels (Bio-Rad) before being transferred to a nitrocellulose membrane. Membranes were immunoblotted with STAT5-specific polyclonal antibodies (1:1,000) overnight at 4°C and visualized by chemiluminescence.

**Quantitative reverse transcription-PCR.** After stimulation for the indicated times, PBMC were lysed with TRIzol (Invitrogen, Carlsbad, CA). RNA was isolated and treated with RNase-free DNase (amplification grade; Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 500 ng of RNA using oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Real-time reverse transcription-PCR was performed on an Applied Biosystems (Foster City, CA) 7900 HT Fast Real-Time PCR system with a compatible Sybr green PCR master mix (Applied Biosystems). As a control for RNA quantity, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected by using specific primers: 5′-TGGGCTACACTG AGCACCAG-3′ (sense) and 5′-GGGTGTCGCTGTTGAAGTCA-3′ (antisense). The primer sets for human CIS1 (5′-TCCTCTGCGTTCAGGGACCT-3′ [sense] and 5′-ACACTAGGCGCATCCTCCTT-3′ [antisense]) and pim-1 (5′-CGAGC ATGACGAAGAGATCAT-3′ [sense] and 5′-TCGAAGGTTGGCCTATCTG A-3′ [antisense]) were made to order by Operon Technologies (Huntsville, AL). Each of the PCR assays were run in duplicate, and the gene copy numbers were estimated from standard curves, using the S.D.S. software supplied with the PCR cycler.

## RESULTS

**Phospho-flow screening identifies multiple unresponsive STAT signaling nodes associated with advanced HIV-1 disease.** HIV-1 is known to alter cell signaling, including the Jak/STAT pathway, although with conflicting results reported (2, 26). We monitored cell signaling in primary patient samples, via phospho-flow cytometry, in an attempt to resolve whether cell subset signaling differences could be an explanation for the differences observed. We stimulated PBMC ex vivo (derived from uninfected and HIV-1-infected pediatric subjects) with each of the cytokines IL-7, IL-10, GM-CSF, and IFN-γ. We assayed for increases in phospho-Stat1, phospho-Stat3, and phospho-Stat5 (pStat1, pStat3, and pStat5, respectively) and represented this as a ratio of induction over baseline levels (fold change) as the readout. In aggregate form, this information was then represented as heat maps, as described by Eisen et al. (15). We confirmed that STAT phosphorylation in uninfected PBMC correlated with increased target gene transcription (mean 14.5-fold increase in cytokine-inducible SH2-domain protein [CIS], a Stat5 target gene, after GM-CSF stimulation; A. Lee et al., unpublished results).

As a first step toward determining whether HIV-1-associated cell signaling changes might be affected by disease state, we compared STAT activation in the group of HIV-1+ pediatric subjects that had robust responses to highly active antiretroviral therapy (HAART), i.e., low viral load and high $CD4^+$ T-cell counts (categorized as "+T" for HIV-1+ on treatment), versus pediatric subjects with advanced HIV-1 disease, i.e., high viral load and low CD4 counts (categorized as "+A" for advanced HIV-1+). Table 1 lists the clinical and demographic data for the pediatric subjects screened.

As shown in Fig. 2A, we identified B cells ($CD20^+$), monocytes ($CD33^+$), and T lymphocytes ($CD3^+$ $CD4^+$ and $CD3^+$ $CD4^-$ [i.e., $CD8^+$]) within fixed and permeabilized PBMC by standard surface marker characterization and gating. The phospho-protein fold changes for each immune cell type, in response to the panel of cytokines, are shown in a heat map representation in Fig. 2B. Of the 60 unique signaling nodes (combination of cell type, stimulus, and phospho-protein) surveyed in these experiments, we identified three with very high fold changes ($\geq 3$ $\log_2$ fold change, highlighted in red) in uninfected (control) pediatric subjects. Interestingly, comparison of these signaling nodes across the donor categories showed "normal" STAT activation in HAART-treated, HIV-1-infected pediatric subjects (+T columns) and the absence of STAT phosphorylation in pediatric subjects with advanced HIV-1 disease (+A columns). Figure 2C shows the underlying data in histogram form for the three nodes with the highest fold change in uninfected pediatric subjects, compared across the different donor groups. In total, these results indicated that in advanced HIV-1 disease, multiple STATs in different immune cell types had lost responsiveness to upstream stimuli, whereas in HIV-1-infected donors with a good response to treatment, Jak/STAT signaling was preserved.

TABLE 1. Clinical and demographic features of HIV-infected and exposed, uninfected pediatric subjects

| Figure | Status | Donor identification no. | CD4% | CD4 ABS[a] | Viral load (copies/ml) | Treatment[b] | Age (mo)[c] | Sex | Race[d] |
|---|---|---|---|---|---|---|---|---|---|
| Fig. 2 | Advanced HIV+ | 0802 | 4 | 62 | 527,298 | | 111 | F | NHB |
| | | 0408 | 4 | 100 | 150,000 | d4T, 3TC, ddI EC, ATV | 124 | M | NHB |
| | | 1903 | 11 | 251 | 24,008 | PTI on ABC/ZDV/3TC | 143 | M | H |
| | Treated HIV+ | 1013 | 35 | 617 | 364 | d4T, ddI, EFV | 161 | M | H |
| | | 1007 | 36 | 831 | <400 | ABC/ZDV/3TC and NFV | 171 | F | H |
| | | 0303 | 48 | 815 | <50 | ZDV, 3TC, LPV/RTV | 139 | F | NHB |
| | | 2602 | 48 | 1,401 | 135 | d4T, ddI, EFV | 93 | F | NHB |
| | HIV− | 1006U | | | | | 84 | M | H |
| | | 1813U | | | | | 37 | M | NHB |
| | | 2212U | | | | | 55 | M | H |
| Fig. 3 | HIV+ | 319 | 4 | 67 | 5,245 | | 74 | M | NHB |
| | | 610 | 6 | 19 | 511,000 | ABC/ZDV/3TC, ddI EC, ATV | 187 | F | NHB |
| | | 718 | 17 | 444 | 183,083 | | 164 | M | H |
| | | 1310 | 18 | 542 | 6,630 | | 112 | F | NHB |
| | | 1013 | 18 | 595 | 60,300 | | 92 | F | H |
| | | 1819 | 22 | 251 | 10,100 | ZDV/3TC + LPV/RTV | 105 | F | H |
| | | 1007 | 25 | 1,067 | 10,439 | d4T/3TC | 35 | F | H |
| | | 1019 | 26 | 418 | 3,134 | | 63 | M | NHB |
| | | 1001 | 31 | 620 | 6,358 | | 58 | F | NHB |
| | | 416 | 31 | 856 | 6,390 | d4T/3TC | 53 | M | H |
| | | 1303 | 31 | 583 | 111,682 | | 68 | F | NHB |
| | | 302 | 32 | 695 | 14,715 | | 50 | F | NHB |
| | | 510 | 34 | 1,153 | <50 | ABC/ZDV/3TC, TDF, ATV/RTV | 187 | F | NHB |
| | | 103 | 36 | 912 | 31,116 | d4T/3TC | 110 | M | NHB |
| | | 1316 | 44 | 2400 | 521 | d4T/3TC | 20 | F | H |
| | | 1302 | 49 | 1,776 | 33,854 | 3TC, LPV/RTV, TDF | 108 | F | NHB |
| | HIV− | 1018U | | | | | 12 | M | H |
| | | 1006U | | | | | 16 | F | H |
| | | 1103U | | | | | 69 | M | NHB |
| | | 2212U | | | | | 49 | M | H |
| | | 1023U | | | | | 53 | F | NHB |
| | | 0402U | | | | | 57 | M | H |
| | | 0316U | | | | | 52 | F | H |
| | | 1823U | | | | | 171 | M | NHB |
| | | 0118U | | | | | 91 | M | H |
| | | 1302U | | | | | 87 | F | NHB |
| | | 0323U | | | | | 107 | M | NHB |

[a] Absolute $CD4^+$ T-cell count in cells/$mm^3$.
[b] Medication and treatment abbreviations: 3TC, lamivudine; ABC, abacavir; ATV, atazanavir; d4T, stavudine; ddI EC, didanosine, enteric coated; EFV, efavirenz; LPV/RTV, lopinavir/ritonavir; RTV, ritonavir; TDF, tenofovir; ZDV, zidovudine. PTI, partial treatmentinterruption.
[c] Figure 2: $P = 0.23$, NS, for Student $t$ test of ages of HIV+ ($n = 16$) versus HIV− ($n = 11$) pediatric subjects.
[d] H, Hispanic; NHB, non-Hispanic black.

**Directed phospho-flow testing reveals a significant HIV-1-associated decrease in GM-CSF-stimulated Stat5 activation in monocytes.** The initial phospho-flow screen indicated that in pediatric subjects with advanced HIV-1 disease, there was marked inhibition of Stat1 activation in monocytes and Stat5 activation in both T lymphocytes and monocytes (Fig. 2C). In order to determine the statistical significance of our initial observations, we repeated phospho-flow testing on these particular signaling nodes, selecting a random sample of uninfected and HIV-1-infected donor PBMC from among >2,000 potential samples contained within the Jacobi Medical Center database. We tested for signaling differences among ethnic groups within the HIV-1-infected pediatric cohort and found that cytokine-driven STAT phosphorylation did not differ significantly between Hispanics and non-Hispanic blacks (E. R. Sharp et al., unpublished results). Clinical and demographic data for the selected donors are shown in Table 1. In this larger sample, IL-7 stimulation of pStat5 in $CD3^+$ T lymphocytes and IFN-γ activation of pStat1 in $CD33^+$ monocytes did not significantly differ between uninfected and infected pediatric donors (Fig. 3A and B, respectively). In contrast, phosphorylation of Stat5 after GM-CSF stimulation was significantly inhibited in monocytes from HIV-1-infected pediatric subjects ($P = 0.03$ by two-tailed Student $t$ test, Fig. 3C). Only 2 of the 16 HIV+ donors in this sample had very advanced HIV disease (<10% $CD4^+$ T cells, Table 1), arguing against selection bias influencing these results. Titration of GM-CSF at up to 20

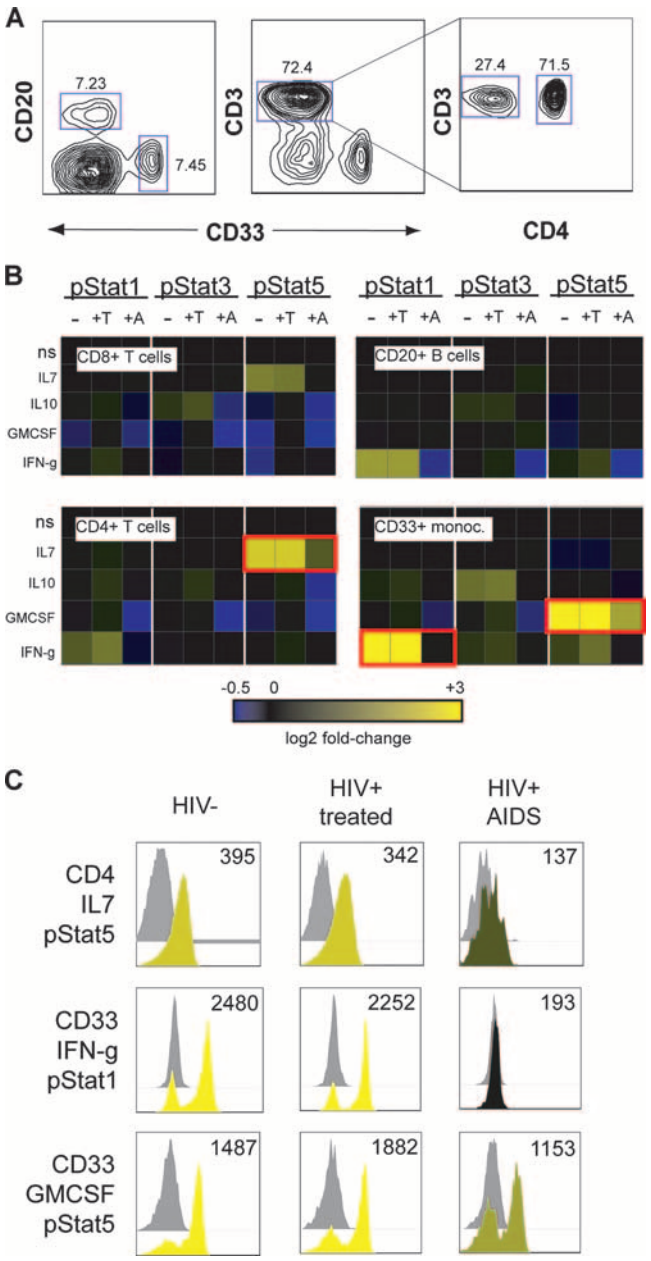


FIG. 2. Flow cytometry screening reveals loss of STAT responsiveness in multiple immune cell types in advanced HIV-1 infection. Clinical and demographic data for the donors shown in Table 1. PBMC were stimulated ex vivo with cytokines, fixed, permeabilized, and stained for surface and intracellular phospho-epitope-specific flow cytometry as described in Materials and Methods. Briefly, PBMC were left untreated or exposed to 10 ng of IL-7/ml, 100 ng of IL-10/ml, 4 ng of GM-CSF/ml, or 20 ng of IFN-γ/ml for 12 min at 37°C; fixed with 2% PFA; permeabilized with 90% methanol; and stained for surface and phospho-specific markers. (A) Surface staining and gating hierarchy of fixed and permeabilized cells. B cells were identified as $CD20^+$, monocytes were identified as $CD33^+$, and T lymphocytes were identified as $CD3^+$ $CD4^+$ and $CD3^+$ $CD4^-$. The percentage of events is shown for each gate. One representative result from 10 experiments is shown for an HIV-1− pediatric donor. (B) Comparison of phospho-Stat1, -Stat3, and -Stat5 levels in immune cell populations for (i) HIV-1− pediatric subjects, (ii) treated HIV-1+ pediatric subjects with a good response, and (iii) pediatric subjects with advanced HIV-1 infection. A heat map representation of the fold change in phospho-protein levels after cytokine stimulation, normalized to unstimulated controls, is shown. The fold change was converted to $\log_2$ and colored according to the scale shown. Each quadrant shows the results for gated cell type ($CD8^+$ and $CD4^+$ T lymphocytes, B cells, and monocytes). Each column is labeled according to donor group ("−" for HIV-1−, "+T" for HIV-1+ with good treatment response, "+A" for HIV-1+ with advanced HIV-1+). Each row shows the ex vivo stimulus added (ns = no stimulus control). Three signaling nodes (cell type-stimulus-phosphoprotein combination) with high fold changes (>3 $\log_2$ fold change) in the HIV-1− donors are outlined in red. One representative of three experiments is shown. (C) Histogram data underlying the highlighted nodes outlined in the heat map representation shown in panel B. Nonstimulated cells are shown as gray histograms, while stimulated cells are shown in colors corresponding to the heat map scale. The median fluorescence intensities for the stimulated cells are shown. For cell populations with two peaks, the median fluorescence intensities for the higher peak are shown.

ng/ml did not correct the HIV-associated inhibition of Stat5 phosphorylation (A. Lee and E. R. Sharp, data not shown).

To delineate whether this was a GM-CSF pathway-specific inhibition of the phosphorylation of Stat5, we examined IL-3-stimulated Stat5 activation in $CD33^+$ monocytes. GM-CSF, IL-3, and IL-5 are closely related members of the $\beta_c$ cytokine family, with common β receptor subunits that signal through the activation of Jak2 and Stat5. IL-7, in contrast, signals through Jak3 and Stat5 (31). We found that IL-3-mediated activation of Stat5 was also inhibited in the $CD33^+$ monocytes of HIV-1+ pediatric subjects ($P = 0.006$, Fig. 3D). Our observation that Stat5 activation is inhibited in response to IL-3 and GM-CSF in $CD33^+$ monocytes, but not by IL-7 in $CD3^+$ T lymphocytes, suggests that HIV-1 infection is associated with a cell type- and pathway-specific inhibition of Jak2 and Stat5 activation and that whatever feedback or regulatory mechanism that results in this "nonresponsiveness" is enabled specifically in monocytes.

**Lack of correlation between clinical parameters and GM-CSF-mediated Stat5 phosphorylation.** We noted the broad range in GM-CSF-stimulated pStat5 responses among the HIV-1-infected donors (0.8- to 8.7-fold change, Fig. 3C) and explored whether clinical or virologic parameters correlated with Stat5 responsiveness. As shown in Fig. 4, there was no detectable correlation between either CD4% or plasma viremia and GM-CSF-stimulated pStat5 fold change (Fig. 4A and B, respectively). Since previous reports have described correction of HIV-1-related immune dysfunction in response to antiretroviral therapy (1), we also compared pStat5 responses in HAART-treated pediatric subjects versus patients that had been off HAART for at least 6 weeks. Although there was a trend toward higher pStat5 responses in treated compared to untreated pediatric subjects (mean, 4.9 versus 2.7, respectively, Fig. 4C), the differences were not statistically significant ($P = 0.08$). Taken together, these results suggest that low pStat5 responses are the result of complex factors, beyond HIV-1 disease progression and viral burden. In agreement with prior reports, we found that HIV-1-associated immune dysfunction (in this case low Stat5 phosphorylation) generally improved with antiretroviral treatment.

**GM-CSF receptor levels are not affected by HIV-1.** Our results left open the possibility that reduced GM-CSF receptor levels could result in reduced signaling through Jak2/Stat5.

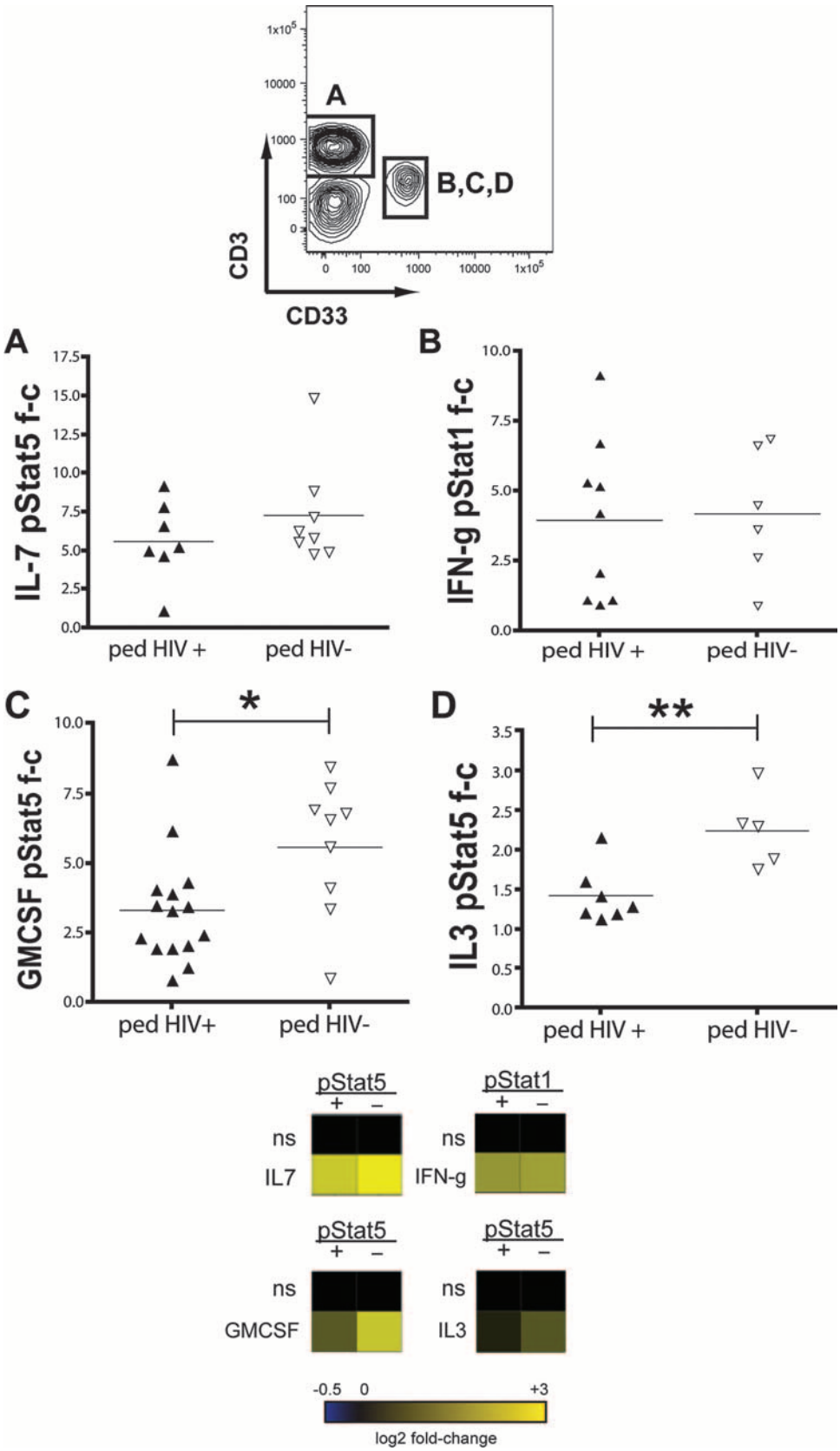


FIG. 3. Identification of a specific inhibition of cytokine-driven Stat5 activation in monocytes associated with HIV-1 infection. Clinical and demographic data for the donors shown in Table 1. PBMC were stimulated and fixed as in Fig. 2. The data were compiled from 10 independent experiments. (Top panel) Representative surface staining showing the populations gated for the phospho-protein fold change (f-c) results shown in panels A to D. (A) IL-7-stimulated phospho-Stat5 f-c in $CD3^+$ T lymphocytes. ($P$ = NS by two-tailed Student $t$ test). (B) IFN-γ-stimulated phospho-Stat1 f-c in $CD33^+$ monocytes ($P$ = NS). (C) GM-CSF-stimulated phospho-Stat5 f-c in $CD33^+$ monocytes (*, $P < 0.05$). (D) IL-3-stimulated phospho-Stat5 in f-c $CD33^+$ monocytes (**, $P < 0.01$). (Bottom panel) Heat map summary of mean fold changes for repeated experiments shown in panels A to D. The color scale is as described for Fig. 2.

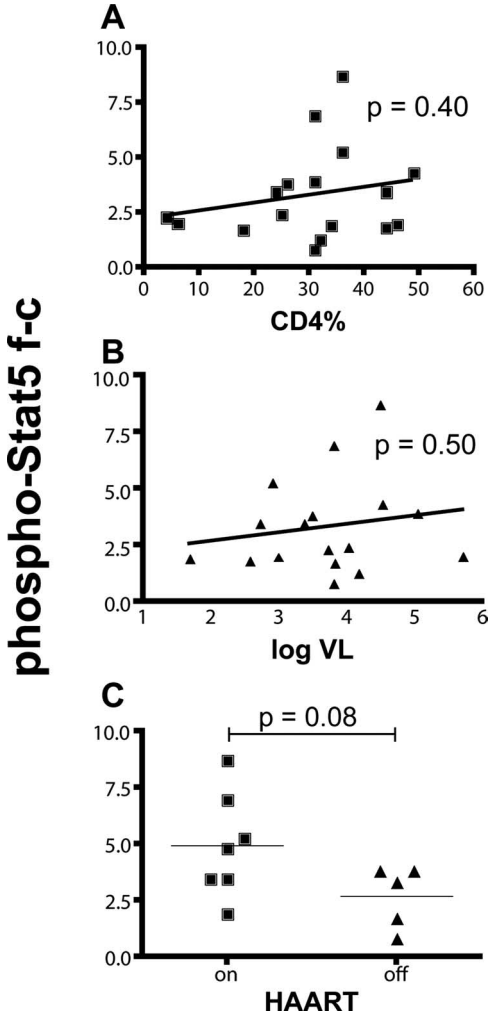


FIG. 4. Comparison of Stat5 activation with clinical, virologic, and treatment status of HIV-1+ pediatric subjects. (A) GM-CSF-stimulated phospho-Stat5 fold change in monocytes versus %$CD4^+$ of peripheral T cells. ($P = 0.40$, NS, by two-tailed Student $t$ test). (B) Phospho-Stat5 fold change versus $\log_{10}$ HIV-1 viral load ($P = 0.50$, NS). (C) Phospho-Stat5 fold change for treated versus untreated HIV-1+ pediatric subjects (mean = 4.9 and 2.7, respectively, $P = 0.08$ [NS]).

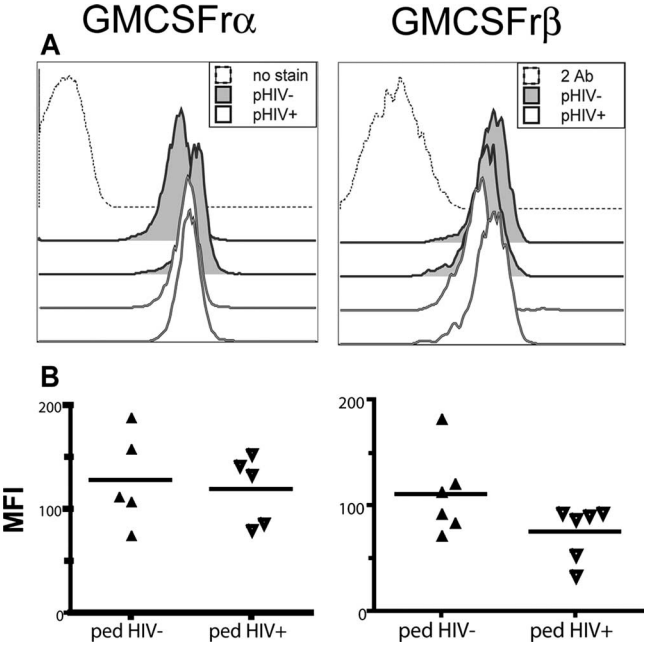


FIG. 5. HIV-1 does not significantly affect GM-CSF receptor levels. GM-CSF receptor α (left column) and β (right column) chain levels as determined by flow cytometry in monocytes from uninfected and HIV-1-infected pediatric subjects. (A) Representative histograms of receptor staining from two HIV-1− and two HIV-1+ pediatric subjects. Only events from $CD33^+$ cells are shown. Controls (dotted histograms) were unstained samples for the α chain and secondary antibody only for the β chain. (B) Summary of MFI (median fluorescence intensities) for all donors ($P$ = NS).

Since ligand binding to receptor immediately precedes Jak2 phosphorylation, we compared GM-CSF receptor levels in uninfected and HIV-1-infected donors. The complete GM-CSF receptor consists of an α/β heterodimer, with the α chain conferring ligand specificity, while the β chain is responsible for signal transduction (11). Since the α chain is found in excess, we determined the levels of both receptor subunits on monocytes by flow cytometry. GM-CSF receptor α and β levels did not differ significantly between uninfected and HIV-1-infected donors ($n = 5$ for each, $P$ = not significant [NS], Fig. 5). This finding supports a conclusion that a specific intracellular mechanism is responsible for the lack of responsiveness in these cells.

**HIV-1-associated potentiation of ERK phosphorylation in monocytes.** As a growth factor for myeloid cells, GM-CSF provides a pleiotropic stimulus for multiple signaling pathways, including Jak/STAT, PI-3 kinase, Akt, p38 MAPK, and Ras-raf-ERK (reviewed in reference 13). As a result of our observation that HIV-1 infection blocks GM-CSF signaling through Stat5, we hypothesized that other GM-CSF signaling arms, such as the MAPKs p38 and ERK, might be enhanced in a compensatory manner. We first tested if we could detect GM-CSF stimulation of ERK phosphorylation with flow cytometry. In normal adult blood donors, GM-CSF was a very potent stimulus for Stat5 phosphorylation (6.6-fold change at 0.5 ng/ml) but relatively weak for ERK phosphorylation (<2-fold change at 0.5 to 10 ng/ml, Fig. 6). We therefore expanded our phospho-flow screen to include other known stimuli for ERK and p38 phosphorylation, separately and in combination with GM-CSF. Comparison of MAPK signaling between HIV-1-infected and uninfected pediatric subjects revealed a striking HIV-1-associated enhancement of ERK phosphorylation in monocytes in response to bacterial LPS (mean fold change of 6.3 versus 1.4, $P = 0.04$, Fig. 7) and the combination of GM-CSF plus LPS (mean fold change of 3.3 versus 1.3, $P = 0.02$). These stimuli also resulted in tumor necrosis factor alpha (TNF-α) secretion by PBMC and purified monocytes from both uninfected and HIV-infected donors ($n = 12$; A. Lee, data not shown), arguing for the physiological relevance of the signaling results.

In contrast, ERK activation in response to phorbol ester treatment with PMA-ionomycin did not significantly differ in HIV-1 ($P$ = NS, Fig. 7 and data not shown). These results indicate that HIV-1 enhances the monocyte signaling response to a natural innate immunity ligand such as LPS, with the

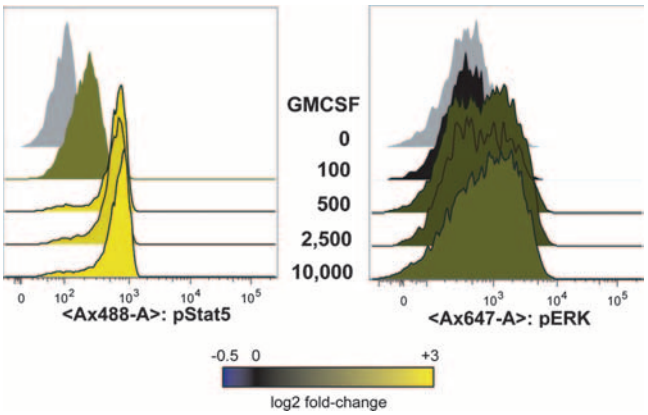


FIG. 6. GM-CSF more potently activates Stat5 compared to ERK in normal adult donors. PBMC from HIV-1-negative adult donors were stimulated, fixed, and stained as in Fig. 2. Phospho-Stat5 and phospho-ERK were simultaneously detected with antibodies conjugated to Alexa-488 and Alexa-647, respectively. Histogram data are shown for $CD33^+$ cells only. The GM-CSF concentrations used in the titration (0 to 10,000 pg/ml) in the center of the figure are shown at the same level as the corresponding histogram data. The data shown are representative of two independent experiments with similar results.

potential to increase host inflammatory responses to pathogens. Thus, HIV-1 induced reduction of signaling potential is related to antiviral immunity (as represented by pStat5), while increasing signaling related to inflammatory responses (as represented by pERK) in monocytes, a cell type that HIV-1 interacts with extensively throughout the course of infection.

## DISCUSSION

This study delineates the effects of in vivo HIV-1 infection on the signaling behavior of single immune cells interacting within a complex cellular milieu. Interrogation of phospho-epitopes within pathogen-altered intracellular signaling networks at the primary cell level allowed linkage of network states with virologic and clinical parameters. In this cross-sectional study, our comparison of the Jak/STAT pathways within prominent peripheral blood immune cell populations revealed a specific defect in GM-CSF-stimulated Stat5 phosphorylation within monocytes associated with HIV-1 infection. Expansion of the phospho-flow screen to include other phospho-proteins activated by GM-CSF led to the observation that LPS-stimulated ERK phosphorylation was enhanced in HIV-1 infection. Reduced signaling potential of "antiviral" STAT pathways, and enhanced signaling of "inflammatory" MAPK pathways are consistent with long-standing observations of monocyte dysfunction in HIV-1 infection, including reduced antigen uptake and hyperactive inflammatory cytokine secretion (5, 39). These results provide a specific molecular basis for the prior observations of dysfunction.

In order to put these results into context, it may be helpful to organize the complex body of literature on HIV-1 and Jak/STAT signaling into studies of pathway activation state (i.e., activated or depressed signaling) and studies of pathway responsiveness (i.e., the ability to activate in response to an upstream stimulus). Activation state studies have shown increased phospho-Stat5 after in vitro HIV-1 infection or exposure of both $CD4^+$ T-cell and monocytic cell lines (24) and constitutive activation of a truncated form of Stat5 (Stat5Δ) in a majority of HIV-1-infected donors (4). In contrast, studies of Jak/STAT responsiveness in HIV-1 have shown a reduced ability to phosphorylate Stat5 after IL-2 stimulation of $CD8^+$ T cells from HIV-1-infected adults (26), as well as after GM-CSF stimulation of monocyte-derived macrophages infected with HIV-1 in vitro (43). Preexisting activation through viral antigen stimulation or immune activation may desensitize the pathway to further stimulation. This could occur through negative feedback mechanisms, for instance, as mediated by the

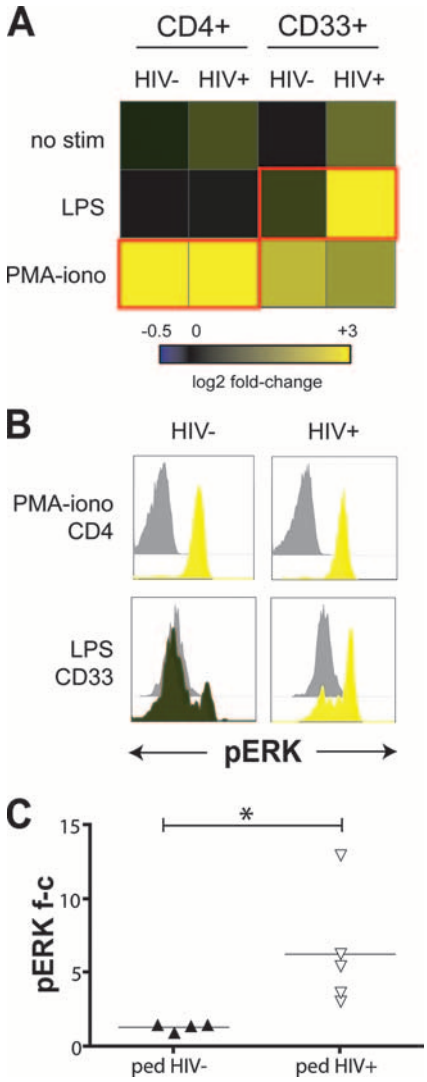


FIG. 7. Enhanced ERK phosphorylation in monocytes of HIV-1+ pediatric subjects. (A) Heat map of flow cytometry analysis of ERK phosphorylation in PBMC from HIV-1− and HIV-1+ pediatric subjects. Cells were stimulated ex vivo with 1 μg of LPS/ml or 50 ng of PMA/ml and 1 μM ionomycin, for 12 min. (B) Histogram data underlying the heat maps outlined in red in panel A. Phospho-ERK levels are shown for $CD4^+$ T cells stimulated with PMA-ionomycin (top row) and for $CD33^+$ monocytes stimulated with LPS (bottom row). Unstimulated controls are shown in gray. One representative result from four experiments is shown. (C) Summary of phospho-ERK fold-changes for all donors. The data were compiled from four independent experiments (*, $P < 0.05$).

suppressor of cytokine signaling (SOCS) family (17, 29, 36), among others. Except for donors with advanced HIV-1 infection, our results were not consistent with a systemic decrease in Jak/STAT responsiveness across all cell types and pathways but rather a specific inhibition of Jak2/Stat5 signaling in response to related $\beta_c$ cytokines (GM-CSF and IL-3) in monocytes. Since our results are based on blood samples obtained from HIV-1-infected donors, we both validate and extend the significance of similar observations based on in vitro model infection systems (43), providing here a physiologic and in vivo characterization of the effect.

By studying a cohort of perinatally HIV-1-infected pediatric subjects and their exposed, uninfected controls, we were able to take advantage of closely matched socioeconomic status and environment. In addition, the drawing, processing, and storage of the blood samples were performed completely in parallel, providing ideal controls for sample handling conditions. Our results are not limited to pediatric disease but bear significance to HIV-1 in general. In separate experiments, we found that GM-CSF induced Stat5 phosphorylation was significantly reduced in HIV-infected adults compared to uninfected controls (67% of control, $n = 8$ [HIV+], $n = 4$ [HIV−], $P = 0.02$).

In addition to the advantage of monitoring cell signaling in primary immune cells ex vivo, phospho-flow screening allowed the integration of signaling results with the clinical and virologic parameters of the HIV-1-infected donors under study. In the initial comparison of treated HIV-1+ pediatric subjects (low VL and high CD4s) versus those with advanced HIV-1 disease (high VL and low CD4s), STAT activation was normal in the former group, whereas the latter group could not activate multiple STATs, suggesting a correlation between severe disease and nonresponsive STATs. However, repeat testing in a larger cohort of HIV-1-infected pediatric subjects did not reveal a clear relationship between low Stat5 activation and HIV-1 disease state, as measured by CD4% or HIV-1 viral burden. We also noted improvement in low Stat5 responses associated with HAART, as seen with other HIV-1-related immune dysfunctions, although the difference was not statistically significant (Fig. 4C).

We also tested our HIV-1-infected pediatric subjects for differences in signaling among different ethnic groups, taking advantage of the ethnic diversity reflected in this urban cohort. We did not find significant differences in ex vivo-stimulated STAT phosphorylation between Hispanics and African-Americans, despite an earlier demonstration of variable HIV-1-specific immunity in this cohort (35). Our results also show that reduced Stat1 responses to IFN stimulation in blacks, compared to whites, in the context of HCV infection (18) does not extend to other viruses such as HIV-1. As such, it is obvious the different disease contexts, as well as different ethnic groups, preclude a direct comparison of studies.

Testing the idea that a GM-CSF signaling block at Stat5 might lead to enhanced signaling of other GM-CSF-stimulated pathways led to a second significant finding, namely, that MAPK signaling in monocytes is potentiated in HIV-1 in response to specific natural ligands of innate immunity, but not to broadly active, small molecule inducers of cell signaling. As with the STAT activation observations, we found that the HIV-1-related increase in MAPK activity was specific, i.e., only in response to LPS or the combination of LPS and GM-CSF. MAPK signaling is essential for inflammatory cytokine secretion, and monocytes play a key role in secretion of these molecules. The data supports a hypothesis that HIV-1 increases the ERK response in monocytes specifically, in order to promote aberrant inflammatory cytokine secretion in response to Toll-like receptor (TLR) signals.

We did not find evidence for reduced GM-CSF receptor levels in HIV-1 as a potential explanation for low phosphorylation of Stat5 (Fig. 4). In addition, HIV-1 infection was not associated with lower total Stat5 levels by Western blotting (E. R. Sharp et al., unpublished results), arguing that the low pStat5 response we observed resulted from a reduction in steady-state phosphorylation. Although we considered the possibility that a reduction in the fold increase in pStat5 after stimulation might arise from elevated baseline phosphorylation due to chronic activation in the setting of HIV-1 infection, we did not find any significant difference in basal pStat5 between HIV-1− and HIV-1+ ($P = 0.81$, $n = 11$ [HIV-1−], $n = 14$ [HIV-1+]). The recent observation that HIV-1 Nef induces SOCS, which negatively regulates pStat1 and pStat3 activation in B cells (32) is potentially relevant to our findings. However, an upregulation in negative feedback mechanisms should result in generalized inhibition of STAT signaling, not a specific inhibition of $\beta_c$ cytokine-driven Stat5 activation, as we observed. Thus, a simple explanation of upregulation of broadly active SOCS inhibition is not the cause, but the results do not obviate a mechanism involving cell type specific utilization of SOCS.

Exposure to several HIV-1 proteins is known to result in ERK hyperphosphorylation. Nef exposure resulted in ERK activation in $CD4^+$ T cells (33), increasing target cell activation and infectivity (44), and endothelial cells (38). HIV-1 gp120 binding to CCR5 on macrophages resulted in the activation of both ERK and phosphatidylinositol-3 kinase, which were required for gp120-stimulated TNF-α secretion (27). Extrapolating from the work above, one might predict that HIV-1 infection would result in elevated ERK phosphorylation ex vivo. However, we found that basal pERK levels were nearly equivalent ($P = 0.56$, $n = 6$ [HIV-1−], $n = 10$ [HIV-1+]), and instead we observed significant enhancement of LPS-stimulated ERK phosphorylation by HIV-1 (Fig. 7). LPS stimulation of ERK in monocytes signals through TLR4, whereas HIV-1 signals through TLR7/8 (3, 28), raising the possibility that HIV-1 infection enhances ERK activation by providing additional or complementary TLR signals (40).

What are the implications for the altered signaling responses we have demonstrated in this group of HIV-1-infected pediatric subjects? Although full-length Stat5 binds to the HIV-1 LTR and increases viral production in $CD4^+$ T cells (34), a constitutively activated, truncated form of Stat5 is present in HIV-1-infected individuals and negatively regulates HIV-1 expression (10). Although we cannot distinguish isoforms with the phospho-specific Stat5 antibody, reduction in pStat5 is likely to counteract the antiviral effect of truncated Stat5. Variability in GM-CSF-mediated pStat5 response in HIV-1+ donors (Fig. 3C) may have contributed to observed differences in effects of GM-CSF therapy on HIV-1 viral load in clinical trials (7, 23). Nonresponsiveness to GM-CSF in a proportion of subjects has been observed in these and other studies of HIV-infected and uninfected adults.

MAPKs, including ERK, are integral for inflammatory cytokine secretion in response to a variety of stimuli. Enhancement of LPS-stimulated ERK phosphorylation by HIV-1 represents a possible mechanism for increasing inflammatory cytokine secretion, thus contributing to the development of generalized immune activation. Notably, HIV-1 gp120 may cause TNF-α secretion from macrophages in a CCR5- and Lyn (a member of the src family kinases)-dependent manner (37). Although "traditional" $CD14^+$ monocytes lack CCR5 (42), $CD16^+$ monocytes in HIV-1 have elevated CCR5 expression (up to 40% CCR5 in HIV-1+ donors) (16). It will be of interest to determine whether the enhancement of LPS-stimulated ERK in monocytes we have identified here depends on CCR5.

The potentiation of LPS-stimulated signaling in HIV-1 infection is of special interest, in light of the recent work by Brenchley et al. demonstrating a strong correlation between elevated serum LPS levels (resulting from gut microbial translocation) and HIV-1-related chronic immune activation (6). Chronic in vivo LPS stimulation also led to reduced ability of monocytes to secrete TNF-α ex vivo. The potentiation of LPS-induced ERK phosphorylation may represent a strategy used by HIV-1 to counter the desensitizing effects of chronic LPS stimulation and maintain persistent immune activation through inflammatory cytokine secretion.

In summary, we have applied phospho-specific flow cytometry to determine the extent of altered cell signaling profiles in HIV-1-infected individuals. In this cross-sectional study, we identified a significant defect in Stat5 activation and enhancement of ERK signaling within the monocytes of HIV-1-infected pediatric subjects, with intriguing implications for the role of monocytes in the immunopathogenesis of HIV-1 infection. These findings could guide further investigations into long-described immune cell dysfunctions that occur in HIV-1 infection but have eluded understanding at a mechanistic level. In the era of "personalized medicine," one can envision how phospho-epitope-specific analysis could be applied to larger and/or longitudinal studies to further bridge our understanding of how HIV-related signaling abnormalities may affect disease progression and clinical outcomes.

## ACKNOWLEDGMENTS

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# Chapter 3:

# Immunodominance Patterns and Differentiation Profiles of HIV-1-Specific CD8+ T Cell Responses and Association with Disease Progression in Perinatally Infected Children

# Introduction

### *The Influence MHC Class I on HIV-1 Disease Progression*

Host factors have a strong influence on the HIV-specific CD8+ T cell responses and the consequent level of control exerted on the virus. Of particular importance are the genes contained within the MHC, especially the human lymphocyte antigen (HLA) alleles. Each individual can express up to six different HLA class I alleles: two from each loci HLA-A, -B, and -C. Each allele can bind a different repertoire of cytoplasmically expressed peptides, such as those derived from infecting viruses. This allows for efficient recognition of virus-infected cells by CD8+ T cells. Individuals who are homozygous at any of the three HLA loci are much more likely to have a rapid progression to AIDS, compared to those who are heterozygous at these alleles, as the peptide repertoire displayed is limited [1]. This suggests that it is advantageous for an individual to have a diverse range of HIV-specific CD8+ T cell responses. Interestingly, the general consensus currently is that the breadth and/or magnitude of the HIV-specific response is not associated with the control of viral replication [2-7]. The overall CD8+ T cell response consists of many oligoclonal responses that differ in their specificities and functionality.

Potentially, viral control may be limited to only a few specific epitopes. This concept is strengthened by the consistent association of certain HLA Class I alleles with disease progression. Several HLA alleles have been repeatedly associated with long-term non-progression (HLA-B*27, HLA-B*57, and HLA-B*5801), while other alleles (HLA-B*35Px and B*53) have been associated with rapid disease progression [8-11]. Taken together, these data suggest that although HIV-specific CD8+ T cells are important for

the partial control of HIV, and that certain specificities of CD8+ T cell responses are more effective than others.

### *Gag-specific CD8+ T cell Responses*

Gag is the major structural protein of HIV-1 and contains the viral matrix (p17/MA), capsid (p24/CA), and nucleocapsid (p7/NC) proteins as well as a protein (p6) important in endosomal targeting and viral budding. As discussed in Chapter 1, it does not seem that there is a simple relationship between the magnitude of Gag-specific responses and disease progression. However, the presence of a Gag-specific CD8+ T cell response has been repeatedly associated with a better clinical outcome. In contrast, Env-specific CD8+ T cell responses have been associated with a more severe disease progression [12]. It has been shown in the SIV model that epitopes derived from the Gag protein are some of the first viral peptides to be presented to CD8+ T cells after infection [13]. It is also known that there is a high viral fitness cost resulting from CTL escape mutations in Gag [14-16]. Several studies in chronically infected patients have shown that individuals whose immune response is preferentially targeted against Gag progress more slowly and/or have a lower viral load [2, 12, 17, 18]. More recently, studies have shown that individuals who control their virus preferentially target Gag derived epitopes during acute infection, and this is also seen in individuals who express the protective alleles HLA-B*27 or HLA-B*57 [19-21]. These data strongly suggest that T cell responses specifically directed against the Gag protein are beneficial and may contribute to slower HIV disease progression.

Along with the magnitude of Gag-specific response, there have been conflicting reports on the association between the breadth of the Gag-specific response and disease

progression. Early studies showed a negative association between the breadth of the HIV-specific response and disease progression [17, 22, 23], while more recent studies have repeatedly observed no relationship [2-4, 6]. Originally it was thought that targeting a broad range of HIV epitopes was important for slower disease progression. However current thinking is now shifting. Recent investigations have found that individuals with protective HLA alleles have CD8+ T cell responses that predominantly target specific regions within the Gag protein during acute infection [19-21]. These data suggest that the overall breadth of response is not as important as which epitopes are targeted, and specifically regions in Gag. Based on these findings as well as our previous results, we focused this study specifically on Gag derived epitopes.

***Immunodominance of Epitope-Specific Responses***

CD8+ T cells recognize viral peptides presented on HLA Class I molecules, and the specificities of an individuals CD8+ T cells are mainly limited by the HLA alleles expressed by that person. Although there are 3 different HLA loci: A, B, and C, the HLA-B loci has been implicated as being the dominant allele through which CD8+ T cell responses are restricted [24-26]. Therefore the HLA-B alleles can have an important influence on the rate of HIV disease progression [27]. It has been shown that HLA-B alleles restrict the majority of HIV-specific responses [12]. As mentioned before, several HLA-B alleles (HLA-B*27, -B*57, -B*58, -B*15, -B*63) have been associated with slower disease progression [1, 8-11, 28, 29]. One possible explanation for these associations that has been raised is that the HLA-B allele can strongly influence the quality and efficiency of the immune responses generated against HIV [27]. This would suggest that the delayed progression in people expressing these protective alleles is a

result of HIV-specific CD8+ T cell responses restricted specifically through these alleles, a theory that has been raised in many studies [19, 30-32].

In spite of a near countless number of potential HIV peptides that could be presented on HLA Class I molecules, and thus give rise to an epitope-specific CD8+ T cell response, only a small fraction are ultimately targeted by an individual's CD8+ T cell response. In most cases, CD8+ T cell responses are directed relatively narrowly, targeting a very small number of possible epitopes dictated by the HLA type of the individual. The size of each response generated by each epitope gives rise to a hierarchical order of responses [33, 34]. Within the hierarchy, the greatest response can be considered as *immunodominant*, while the weaker responses are *subdominant*. [35]. It is becoming clear that the possession of certain immunodominant responses could be an important factor in establishing control over HIV [34]. The development of immunodominance is dependent on many factors; including, the kinetics of viral protein expression, the autologous sequence of the infecting virus, and most importantly the HLA alleles expressed by the individual [35]. Unsurprisingly then, not all individuals expressing the same HLA allele will have the same immunodominance patterns. The pattern of immunodominant responses in an individual is dependent on the combination of all six HLA Class I alleles. Whereas one individual might have an immunodominant response restricted through a specific HLA allele, another individual expressing the same allele might restrict their immunodominant response through a different allele not expressed in the first person. This suggests that responses restricted through certain alleles dominate over responses restricted through other alleles. This phenomenon, termed immunodomination, has been

observed in individuals that express the "protective" alleles HLA-B*27 and -B*57 [19, 32, 36].

The majority of research on immunodominance of certain epitope-specific responses has been performed in Northern European populations and HLA-B*27 and -B*57 epitope-specific responses have been some of the most well studied. Although the HLA-B*57 allele is strongly overrepresented in long-term non-progressors (LTNP) cohorts, it is also known that the expression of this allele alone is not sufficient to confer slow disease progression. Migueles *et al*. compared the HIV-specific immune response between HLA-B*57 LTNPs and HLA-B*57 typical progressors and observed that in contrast to progressors, the LTNPs mainly focused their response on peptides known to be HLA-B*57-restricted [31]. Consequent studies of immune responses during acute and chronic infection have supported this finding. Indeed, the immunodominance of HLA-B*57 restricted immune responses has been associated with control of viremia [19, 20, 30, 32]. This suggests that it is not the expression of the protective allele that is important in maintaining viral control, but the generation of dominant immune responses through the protective allele.

### *CD8+ T Cell Differentiation Phenotype*

The lack of association between viral control and quantitative measures of the HIV-specific immune response has been difficult to explain. Recently, the qualitative aspects of the HIV-specific CD8+ T cell response have become the focus of intense study. One of these qualitative aspects is the differentiation phenotype of HIV-specific CD8+ T cells. It has been suggested that the inability of HIV-specific CD8+ responses to control viremia is due to a failure of these cells to fully differentiate [37].

One model that has been proposed to describe the maturation of antigen-specific CD8+ T cells is the "linear differentiation" model and is based on the expression of the lineage markers CD45RA and CCR7 (see Figure 3.1). In this model, antigen-specific CD8+ T cells mature in a mostly linear fashion from a naïve ($T_N$) (CCR7+, CD45RA+) to a central memory ($T_{CM}$) (CCR7+, CD45RA-) to an effector memory ($T_{EM}$) (CCR7-, CD45RA-) to a terminally differentiated effector memory ($T_{EMRA}$) (CCR7-, CD45RA+) phenotype [38].

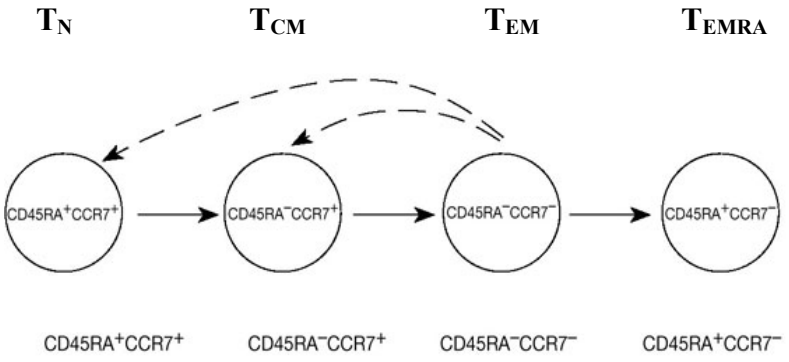



**Figure 3.1: The linear differentiation model, describes the linear differentiation of Naïve T cells ($T_N$) to Central Memory ($T_{CM}$), to Effector Memory ($T_{EM}$), to the final highly differentiated Effector Memory CD45RA+ T cell ($T_{EMRA}$) (figure adapted from [38]) .**

These different subsets of CD8+ T cells have been shown to have different functional capabilities [38, 39]. For example, $T_{EM}$ cells are known to display direct antiviral functions such as IFN-γ secretion. On the other hand, $T_{CM}$ cells are not thought to have direct antiviral effects, but to act by stimulating other T cells through the secretion of IL-2 and proliferation. Fully differentiated CD8+ T cells, $T_{EMRA}$, express lower levels of lymphocyte migration markers than naïve or central memory cells and

appear to contain more perforin than all the other subsets [38]. This suggests that $T_{EMRA}$ cells might be the most effective CD8+ T cell subset in the control of viral infection.

***Maturation Phenotype of HIV-specific CD8+ T cell Responses***

As compared to other chronic viral infections, HIV infection seems to result in a maturational block in the generation of the HIV-specific CD8+ T cell responses [37]. As compared to the immune response to cytomegalovirus, the HIV-specific CD8+ T cell response seems to be skewed toward an effector memory, $T_{EM}$, phenotype, with an overall decrease in the frequency of fully differentiated effector memory, $T_{EMRA}$, cells [37, 40]. It has been proposed that this block in maturation of HIV-specific CD8+ T cells is a major cause of poor viral control. This theory is logical, as different subsets of CD8+ T cells have different phenotypes and, correspondingly, different capacities to respond to antigen stimulation [38, 39]. The contribution of different CD8+ T cell subsets to the entire HIV-specific response may well correlate to overall success of the immune response in controlling the virus.

Several studies have recently addressed these topics by analyzing the maturation phenotype of HIV-specific CD8+ T cell responses. Hess *et al.* were the first to address the possible importance of CD8 $T_{EMRA}$ cells. They observed that a small group of individuals who were successful in controlling virus without treatment had HIV-specific immune responses that consisted of higher frequencies of $T_{EMRA}$ cells compared to those who could not control virus [41]. Research from our group and others confirms the beneficial influence of HIV-specific $T_{EMRA}$ cells in the control of virus. Addo *et al.* performed work in a larger cohort and also observed that HIV-specific $T_{EMRA}$ cells were more frequently detectable in those who control the virus without treatment than in those

who progress [4]. Our group has shown that both the frequency and absolute numbers of HIV-specific $T_{EMRA}$ cells in early infection was negatively correlated with the future viral load set point [42]. Since this study was performed in early infection, it suggested that a highly differentiated immune response was the cause of viral control, and not vice versa. Taken together, these studies strongly suggest that HIV-specific $T_{EMRA}$ cells are more efficient at controlling virus than other CD8+ T cell subsets.

***CD8+ T cell Maturation in HIV Infected Children***

The vast majority of studies investigating differentiation of HIV-specific CD8+ T cell responses have been performed on adults. As mentioned earlier, the natural history of pediatric HIV infection varies from that observed in infected adults as well as several aspects of the HIV-specific immune response. Our group has investigated the differentiation status of HIV-specific CD8+ T cells in HIV infected children versus infected adults, using pediatric patients from the Jacobi cohort [43]. Notably, Jordan *et al.* observed that children and adults differ in their CD8+ T cell differentiation driven by HIV infection [43]. It has been observed that HIV infection in adults drives a broad activation and differentiation of CD8+ T cells, resulting in markedly higher levels of fully differentiated CD8+ $T_{EMRA}$ cells, compared to uninfected adults. This study observed that this phenomenon is markedly weaker in children, with similar levels of fully differentiated $T_{EMRA}$ cells present in both infected and uninfected children [43]. These data suggest that the maturation phenotype of HIV-specific CD8+ T cells could also be different between adults and children.

***Maturation Phenotype of Epitope-Specific Responses***

Until recently, most studies focused on the maturation phenotype of global responses (i.e. responses to pools of peptides). There has been limited work on how the epitope-specific maturation profile of CD8+ T cell responses differs between patients and even between epitopes in the same patient. Hess *et al.* explored a limited number of epitope-specific responses and found that expansion of the $T_{EMRA}$ subset within the immunodominant response was associated with viral control [41]. Addo *et al.* expanded on this work and looked at the CD8+ T cell response to a wide spectrum of epitopes, restricted by a variety of HLA alleles. They found that within one individual the maturation phenotype differed among epitope-specific responses. Interestingly, they also observed that the differentiation phenotype of a given epitope-specific response could not be predicted by the HLA allele that restricted it. Thus, total CD8+ T cell responses to epitopes restricted by HLA-B*57 or -B*27 were not globally more differentiated than responses restricted by less protective alleles [4]. This indicates the importance of understanding the HIV-specific CD8+ T cell response at the individual epitope level. Saez-Cirion *et al.* looked specifically at a small group of long-term controllers and found that their epitope-specific responses had more of an effector phenotype than progressors. Again, this group found that the maturation state of epitope-specific responses was extremely heterogenous, between different individuals and also between different epitopes in the same individual [44]. These studies suggest that the association between viral control and maturation phenotype might be much more complex than previously appreciated. One area not explored in these studies is a comparison of the differentiation

profile of the immunodominance hierarchy, or a comparison of immunodominant responses between controllers and progressors.

From the reports discussed above, and work generated within our group, it is becoming evident that all responses are not equal. Understanding what constitutes responses associated with slower progression will be of great importance for vaccine design.

This study was performed to test our hypotheses: *in those subjects with no immune suppression, the CD8+ T cells that make up the immunodominant epitope-specific responses will have a more differentiated phenotype than other subdominant epitope-specific responses. Concurrently, in those patients that have severe levels of immune suppression, the maturation profile of the CD8+ T cells that make up their immunodominant response will be less differentiated.*

# Materials and Methods

*Patient Sample Characteristics*

All subjects attend/ed the Pediatric HIV clinic at Jacobi Medical Center in the Bronx, NY. The vast majority of attendees of the Jacobi Pediatric HIV Clinic are either African-American or Hispanic. The pre-exisiting samples were chosen for this study based on clinical characteristics that allowed them to be classified according to previously published CDC guidelines [45], as explained below. Heparinized whole-blood samples were obtained from 58 subjects after informed consent, based on local Institutional Review Board-approved protocols. Plasma HIV-1 RNA was measured with the Amplicor HIV-1 Monitor with a lower limit of quantification at 50 copies RNA/ml (Roche Diagnostic Systems, Branchburg, NJ).

As a result of very limited number cells in each sample (about 5-10 million cells per sample), and the need to perform three separate assays on each patient for this study, we used three timepoints for each patient. We chose three consecutive samples for each patient, coming from three consecutive clinic visits. There was an average of 3.67 months between clinic visits and samples.

All subjects were perinatally infected and over 10 years of age, and can thus be defined as long-term survivors (LTS) [46-48]. None of the subjects received HAART during the first two years of life. All of the patients, with the exception of 2 children, were either taking antiretrovirals (ARVs) or were ARV-experienced, but the vast majority had variable adherence levels. There were no significant differences in treatment regimen or adherence levels between the two clinical groups.

*Clinical Categorization*

The general consensus in the pediatric HIV field is that the CD4% value is the most valuable marker of disease progression and is used by the CDC to classify levels of progression[45]. Therefore, patients were categorized into two groups based on CD4% values, using guidelines set forward and published by the CDC [45]. Subjects with a sustained CD4%≥25% are considered LTS with **No** Evidence of Immune Suppression **(LTS-NS)**. Those with a sustained CD4%≤15% are considered LTS with **Severe** Immune Suppression **(LTS-SS)**.

The entire known patient history was considered when categorizing patients. Only patients that had always possessed CD4% levels very near or above 25% were considered an LTS-NS subject. Patients that possessed CD4% levels very near or below 15% for several clinic visits were grouped as an LTS-SS subject. Figure 3.2 highlights an example from each group. As mentioned earlier, we used three timepoints for each patient, and a window was chosen within which to choose those consecutive samples. We chose a period in time in which samples were available and the patient was as clinically stable as possible. This window is highlighted in orange in Figure 3.2.

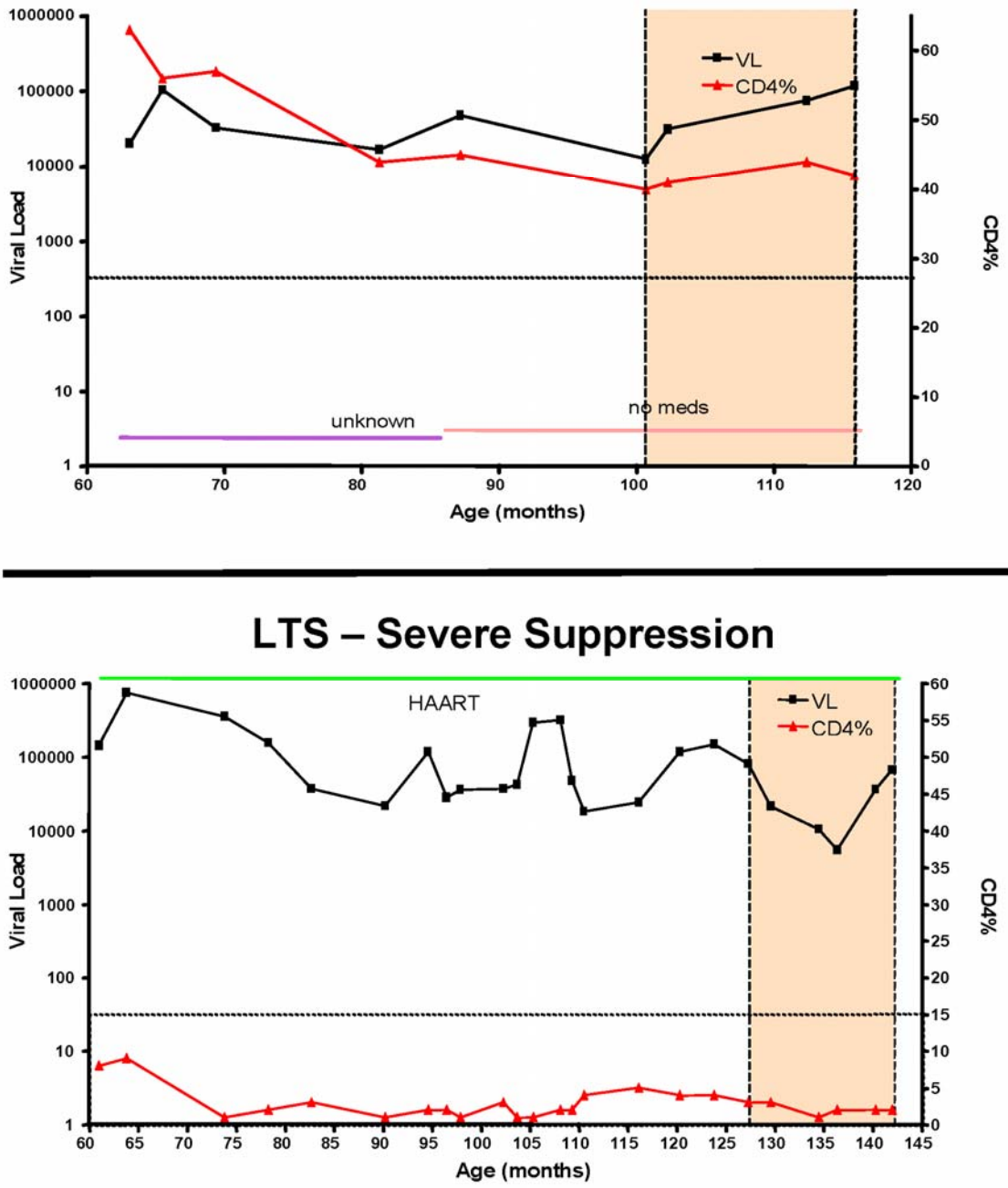



**Figure 3.2** Two examples of patients categorized into either long-term survivors (LTS)-No Immune Suppression (NS) or LTS-Severe Immune Suppression (SS). The dotted line is the boundary of the CD4% value of that progression group. The orange shading marks the window within which we chose samples to use in the following assays.

*HLA typing*

DNA was extracted from PBMC using a QIAamp DNA Mini kit (QIAGEN Inc., Valencia, Calif.). HLA typing used an amplification refractory mutation system with sequence-specific primers as described by the manufacturer (Invitrogen, Carlsbad, CA).

*Determining Epitope-specific Responses*

Due to the limited cell numbers in samples, we needed to determine the repertoire of epitope-specific CD8+ T cell responses using a two-stage ELISpot strategy (see below for description). Antigenic stimulation was achieved through the use of 122 peptides, comprising the entire HIV-1 HXB2 Gag protein: 15-mers overlapping by 10 amino acids. Lyophilized peptides were obtained from the NIH AIDS Research and Reference Reagent Program and resuspended in dimethylsulfoxide (DMSO) and/or PBS.

Initially, the individual peptides were combined to form 12 sequential pools of 10-13 peptides. Patient PBMC were then screened with the 12 sequential pools at 2 μg/ml in an IFN- γ ELISpot in order to identify the broad regions of Gag that were being targeted by each patient.

After we determined which pools each patient responded to, we pursued a targeted approach to identifying the epitopes each individual responded to. Using the HLA type of every patient, we identified potentially reactive epitopes within a positive pool response by using the documented HLA-specific epitope responses provided by the HIV-1 Molecular Immunology Database (Los Alamos National Database). Also, our lab has developed a database that allows the rapid identification of epitope-containing peptides, which can be applied in a HLA specific manner [49]. We then verified these

predicted epitope-specific responses by performing another IFN-γ ELISPOT, using single peptides, at 10 μg/ml, containing the predicted epitopes as antigenic stimulation.

*Detection of Gag-specific CD8+ T-cells by IFN-γ ELISPOT*

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque PLUS density gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden) and cryopreserved. Quantification of HIV-specific T-cell responses using thawed viable PBMC was performed using the IFN-γ ELISPOT assay[50]. Briefly, each well of a 96-well plate (Millipore MAHAS4510, Bedfort, MA) was coated with 50μl of anti-IFN-γ mAb (Mabtech, Stockholm, Sweden) at 5μg/ml. After incubation, each well was washed and blocked with 10% FCS in RPMI (Cellgro). PBMC ($1x10^5$–$2x10^5$) were added to duplicate wells and peptides comprising the HXB2 sequence of Gag (AIDS Research and Reference Reagent Program, NIH) were added at 2 or 10 μg/ml to the cells. As a positive control, the mitogen phytohemagglutinin (PHA) was used at 4μg/ml, and wells with only media added were used as a negative control. After overnight incubation (14-16 hours) at 37°C, plates were washed with phosphate-buffered saline (PBS). Biotinylated anti-IFN-γ mAb 7-B6-1 (Mabtech) was added at 1μg/ml, and incubated at 37° C for 1 hr. Plates were washed with PBS + 0.1% Tween 20 and treated with streptavidin-bound alkaline phosphatase. After 1h incubation, plates were washed with PBS + 0.1% Tween 20 and developed using Alkaline Phosphatase Substrate Kit III (Vector Laboratories, Burlingame, CA). IFN-γ spot-forming units (SFUs) were visualized and counted using an AID EliSpot reader (Autoimmun Diagnostika GMBH, Germany). Spots were standardized to SFU/$10^6$ PBMC. Spots formed in the presence of media alone were considered non-specific background and subtracted from the SFU in stimulated wells. A

reading of 30 SFU/$10^6$ PBMCs after the subtraction of non-specific background spots was considered positive, as previously defined by pediatric studies, using the same or a similar assay, performed by our lab [43, 51-53]. We defined the immunodominant response as the strongest response detected in the ELISpot assay.

*Multi-parameter flow cytometry*

Single peptides identified as containing targeted epitopes were used as the antigenic stimulus. Our negative control was media alone and our positive control was staphylococcal enterotoxin b (SEB). We also stimulated with a peptide pool consisting of well-identified HLA class I restricted CMV, EBV and Influenza epitopes (CEF) in patients that had previously demonstrated reactivity.

Briefly, cryopreserved PBMC were thawed and cells were stimulated for one hour with either: media alone, antigen, or positive control. The antibody, CD107 a/b-PECy5 was added with stimulation. Brefeldin A (Sigma-Aldrich, St. Louis, MI, USA) was then added at a concentration of 5μg/ml and cells incubated overnight. The next day, PBMC were washed and stained with antibodies, in combination, against CD4-Alexa700, CD8-Pacific Blue, CD45RA-biotin, CCR7-PECy7, CD57-PECy5, and a live/dead marker emitting in the aqua wavelength for 20 minutes at 4°C. Cells were washed twice and stained with the secondary antibody streptavidin-Qdot655 for 20 minutes at 4°C. Cells were then washed and fixed in 2% paraformaldehyde. The cells were permeabilized using FACS Perm solution (BD Biosciences), washed and stained using antibodies, in combination, against CD3-ECD, IFN-γ-APC, TNF-α-FITC, and MIP-1β-PE for 30 minutes at 25°C. Following staining, the cells were washed, fixed in 1%

paraformaldehyde, and collected on a BD LSR-II using FACS DIVA software (BD Biosciences). Data was analyzed using FlowJo (TreeStar).

*Gating Strategy*

In all analyses we first used a forward scatter (FSC)-height versus FSC-area plot to exclude all cell conjugates. We then excluded dead cells by only gating on cells negative for the live/dead marker. A FSC-area vs. side scatter (SSC)-area plot was used to define the lymphocyte gate. T cells were selected by gating on CD3+ lymphocytes, followed by selection of CD8+ cells by gating on CD3+CD8+ cells. CD4+ cells were defined as CD3+CD8- cells. In panels with CD3, CD4, and CD8 antibodies, we verified that, on average, 93% of CD3+CD8- cells were CD4+. CD57+ cells were defined using a FITC "fluorescence minus one" (FMO) sample. Quadrant gates were set for expression of CCR7 and CD45RA by using a QDot655 FMO and a PECy7 FMO. IFN-γ+ cells were defined using an APC FMO. IFN-γ+ cells were further analyzed for expression of T-cell memory markers in a CCR7 versus CD45RA.

*Statistical Methods*

A median (interquartile range) was used as a measure of central tendency for continuous variables. We employed the Mann-Whitney two-tailed t-test for all simple comparisons between two groups. The Spearman Rank correlation test and linear regression analyses were used to explore associations between 2 continuous variables. Differences between categorical data were calculated using Fisher's exact test. We considered a p-value of $<0.05$ significant. All statistical analyses were performed using the GraphPad Prism 4.03 software package (La Jolla, CA).

# Results

*Subject Cohort Characteristics*

We initially analyzed peripheral blood samples from 58 children and adolescents with vertically acquired HIV-1. As described in Materials and Methods, these children were divided into two groups of immunological progression based on CDC guidelines. The characteristics of both groups as well as the total cohort are shown in Figure 3.3 and described in Table 3.1.

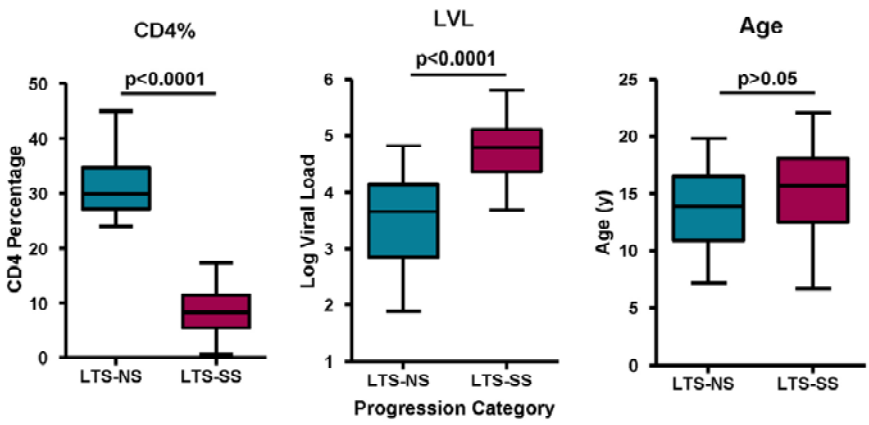



**Figure 3.3** Clinical characteristics of the two progression groups: LTS-NS and LTS-SS

**Table 3.1**

| Immunological Category | N | CD4% | LVL | Age (y) | Sex | Race |
|---|---|---|---|---|---|---|
| No immune suppression (LTS-NS) (CD4% ≥ 25) | 30 | 29.8% (27.1; 34.8) | 3.653 (2.845; 4.138) | 13.8 (10.9; 16.6) | M = 15<br>F = 15 | H = 9<br>AA = 20 |
| Severe immune suppression (LTS-SS) (CD4% <15) | 28 | 8.25% (5.5; 11.5) | 4.786 (4.365; 5.106) | 15.6 (12.5; 18.1) | M = 11<br>F = 17 | H =13<br>AA= 13 |
| TOTAL | 58 | 24.5% (8.25; 31.0) | 4.278 (3.605; 4.806) | 14.3(11.6; 17.52) | M = 26<br>F = 32 | H = 22<br>AA = 33 |

**Table 3.1** Clinical characteristics of the total cohort and two progression groups. Data is displayed as: median (interquartile ranges)

As the children were categorized according to CD4%, it was not surprising find a strong statistically significant difference in the viral loads. Of particular note, all of the patients had some level of ongoing viral replication, as none of them had consistently undetectable viral loads. The long-term survivor (LTS)- no immunological suppression (NS) group was comprised of more African-Americans than the long-term survivor (LTS)- severe immunological suppression (SS) group, but not significantly. Fifty six of the 58 children were on various treatment regimens, but the vast majority had variable adherence levels. There were no significant differences in treatment regimen or adherence levels between the two clinical groups.

*HLA Allele Distribution among Progression Groups and Associations with Clinical Characteristics*

All subjects were initially HLA typed in order to determine the HLA Class I distribution among a mainly minority population. HLA typing revealed a wide range of HLA-A and HLA-B alleles among the two progression groups, shown in Figure 3.4A and B. We used previously published data from the National Marrow Donor Program to calculate the expected allele frequency in a African American and Hispanic population [54]. The National Marrow Donor Program calculated allele frequencies using data from over 14,000 individuals from 4 different ethnicities.

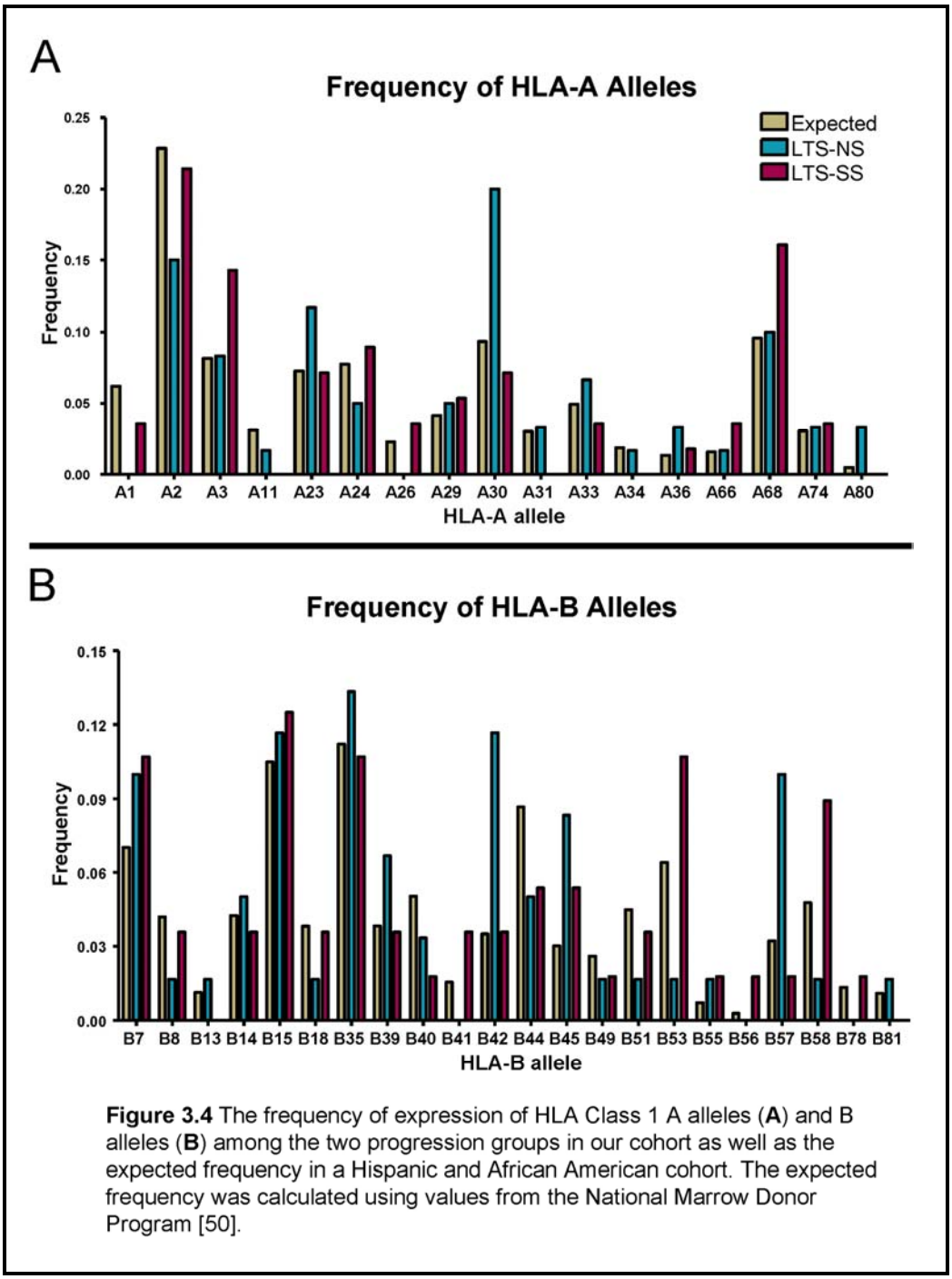
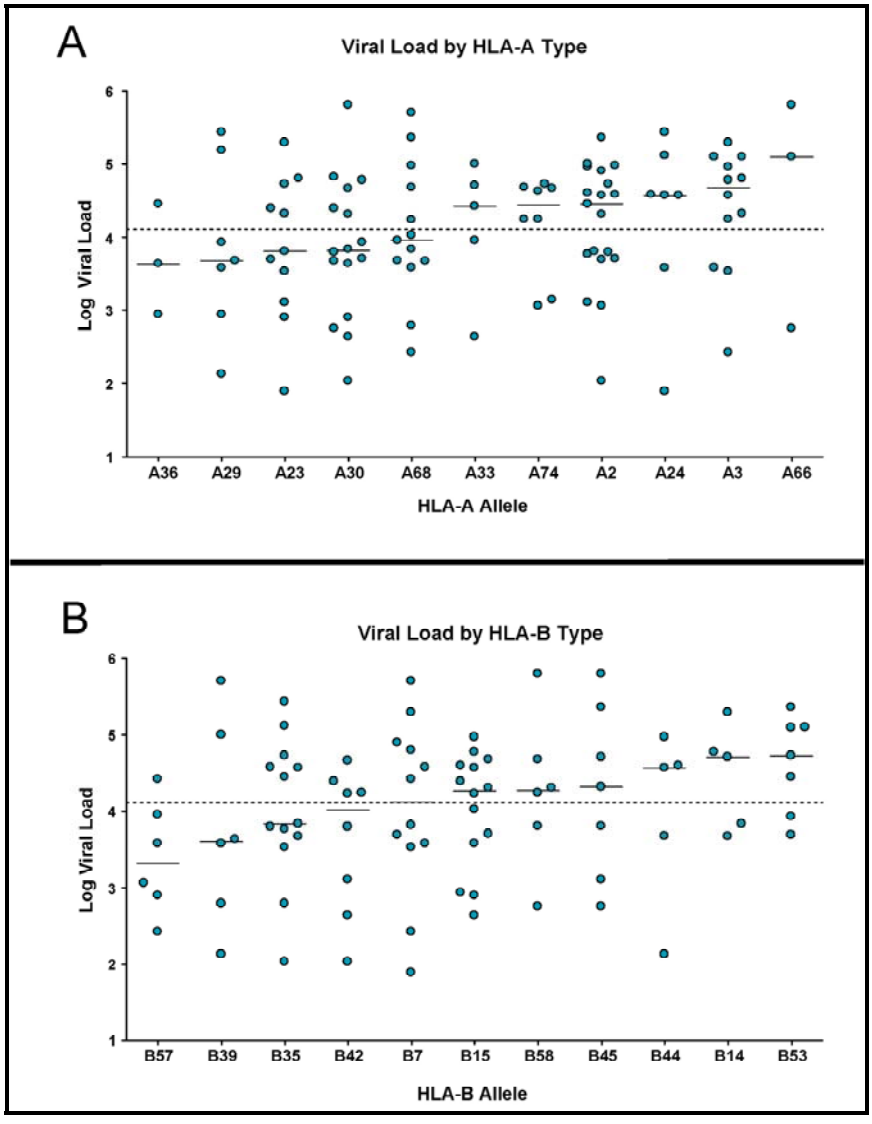



**Figure 3.4** The frequency of expression of HLA Class 1 A alleles (**A**) and B alleles (**B**) among the two progression groups in our cohort as well as the expected frequency in a Hispanic and African American cohort. The expected frequency was calculated using values from the National Marrow Donor Program [50].

Among the alleles expressed by our cohort, we observed several that had uneven distribution among the LTS-NS and LTS-SS groups. Those that were more frequently present in the LTS-NS group were A*30, B*42, and B*57 (p=0.06, 0.16, and 0.11, respectively. Two-tailed Fisher's exact test). The alleles B*53 and, interestingly, B*58

were overrepresented in the LTS-NS group (p=0.055 and 0.10 respectively). None of these associations reached statistical significance, but they suggest influences of HLA alleles on disease progression.

We then sought to determine if specific HLA alleles were associated with CD4% or viral load (Figure 3.5 A-D). As expected, those alleles overrepresented in the LTS-NS group tended to have the highest CD4% and lowest viral loads. Certain HLA-B alleles such as B*57, B*35, B*39, and B*42 were associated with low viral loads and high CD4% levels.



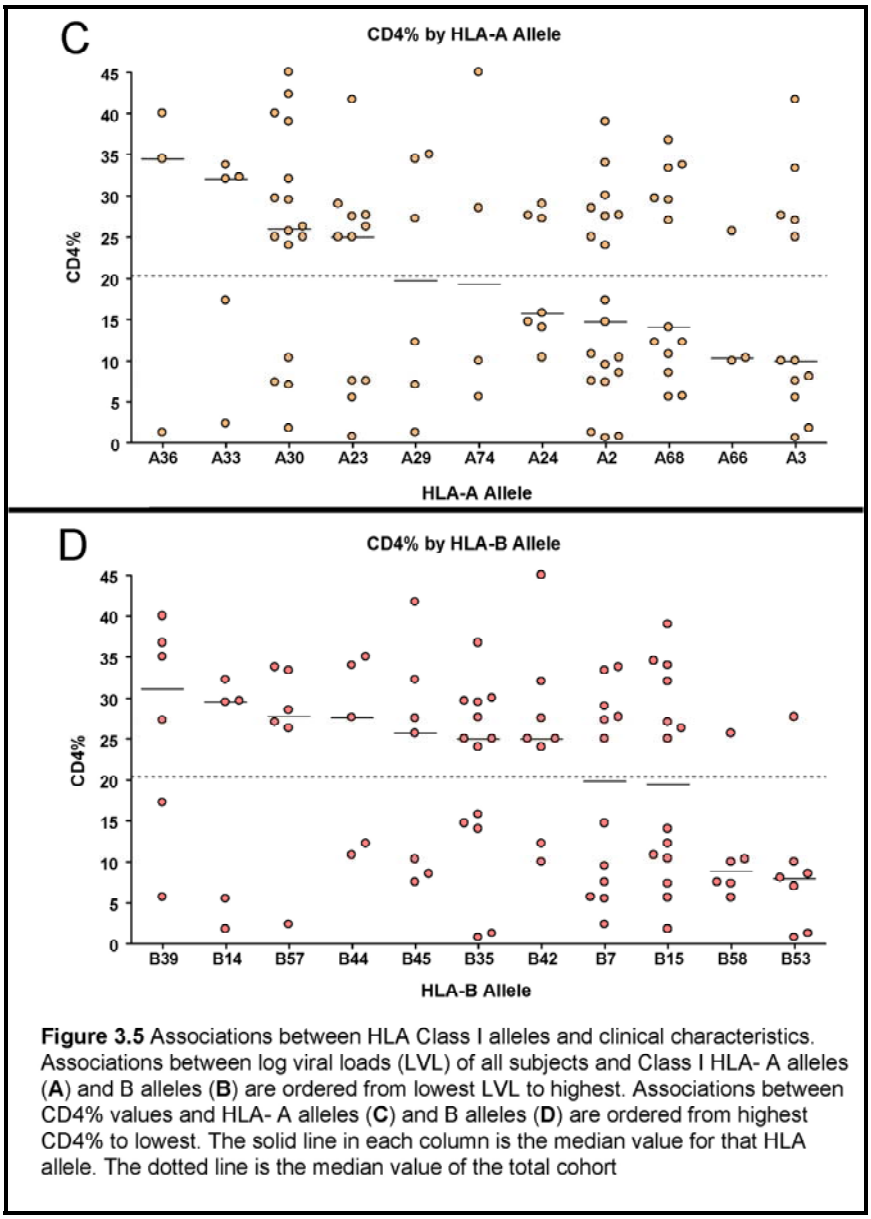



**Figure 3.5** Associations between HLA Class I alleles and clinical characteristics. Associations between log viral loads (LVL) of all subjects and Class I HLA- A alleles (**A**) and B alleles (**B**) are ordered from lowest LVL to highest. Associations between CD4% values and HLA- A alleles (**C**) and B alleles (**D**) are ordered from highest CD4% to lowest. The solid line in each column is the median value for that HLA allele. The dotted line is the median value of the total cohort

*Regions of Gag Targeted by CD8+ T cell Responses*

Next, we examined the specific regions of Gag that were being targeted by each individual and compared the regions targeted between the progression groups. This was accomplished by IFN-γ ELISPOT on cryopreserved PBMC, stimulated with sequential pools of overlapping Gag peptides. Other experiments using cytokine flow cytometry demonstrated that over 95% of IFN-γ was produced by CD8+ T cells (data not shown). Five subjects (8.7% of cohort) did not display any Gag-specific response in the first

screening ELISPOT, and 2 subjects displayed a Gag response, but did not have sufficient samples for further experiments, leaving 51 subjects.

Of the three subunits of Gag, the p24 region was the most frequently targeted region, with 100% of the LTS-NS subjects and 80% of the LTS-SS subjects recognizing this region. This was followed by p17, recognized by 66% of LTS-NS and 67% of LTS-SS subjects. The least recognized Gag subunit was p15, with only 24% of LTS-NS and 38% of LTS-SS subjects displaying a response to this region.

Once the broad regions of response were identified for each subject we then predicted the single peptides within the pool that an individual could respond to, based on HLA-restricted epitope data in the Los Alamos National Database CTL epitope maps. A second IFN-γ ELISPOT assay was performed using single 14-mer peptides to identify the exact regions of Gag targeted by the individual.

There was no difference in overall magnitude or breadth of the Gag-specific CD8+ T cell response between the two progression groups (Fig. 3.6A and B). Within the LTS-NS group, there was a statistically significant negative correlation between overall magnitude of the Gag-specific CD8+ T cell responses and LVL (Spearman r= -0.396, p=0.0369) (Fig. 3.6C). Within the LTS-SS group though, there was no correlation between the overall magnitude of the Gag-specific CD8+ T cell response and LVL (Fig. 3.6D).

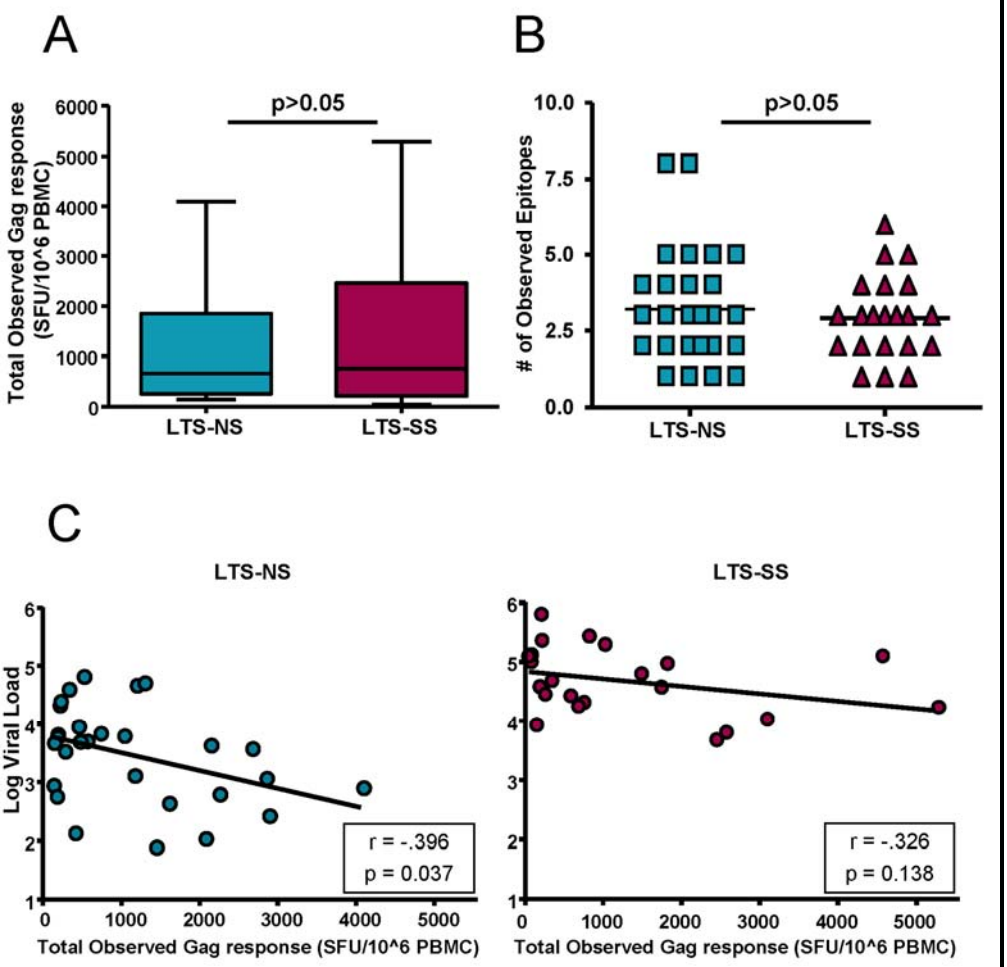
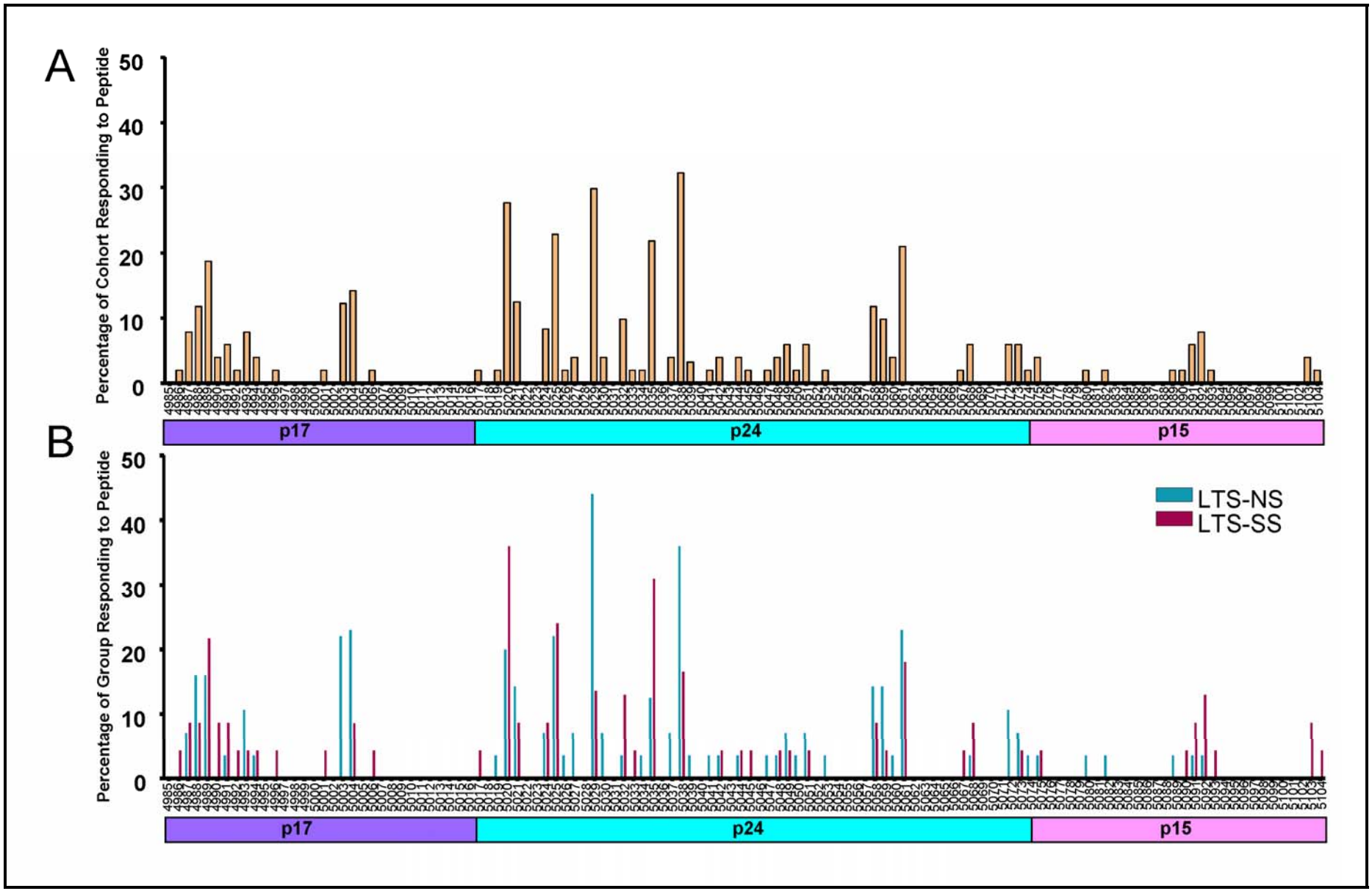



**Figure 3.6** Association between Gag-specific response and disease progression. The magnitude of the total observed Gag-specific response (the sum of all the epitope-specific responses) is compared between the two groups in **A**. The breadth of the Gag-specific response is compared between the two groups in **B**. Correlations between LVL and the magnitude of the total observed Gag response are displayed for both progression groups in **C**.

There were several areas of Gag that were recognized by a large frequency of our cohort (Figure 3.7A and Table 3.2). Of all the peptides, 5038 was the most frequently targeted, being recognized by 32% of subjects with an HLA allele that could present the peptide. Peptides 5029 (30% responding), 5020 (28%), 5025 (23%), 5035 (22%), 5061 (21%), 4988/4989 (20.8%), and 5003/4 (20.4%), were also targeted by over 20% of those with the HLA types able to present the peptide. These peptides are detailed on Table 3.2.


A
Percentage of Cohort Responding to Peptide
50
40
30
20
10
0
p17
p24
p15
B
Percentage of Group Responding to Peptide
LTS-NS
LTS-SS
p17
p24
p15

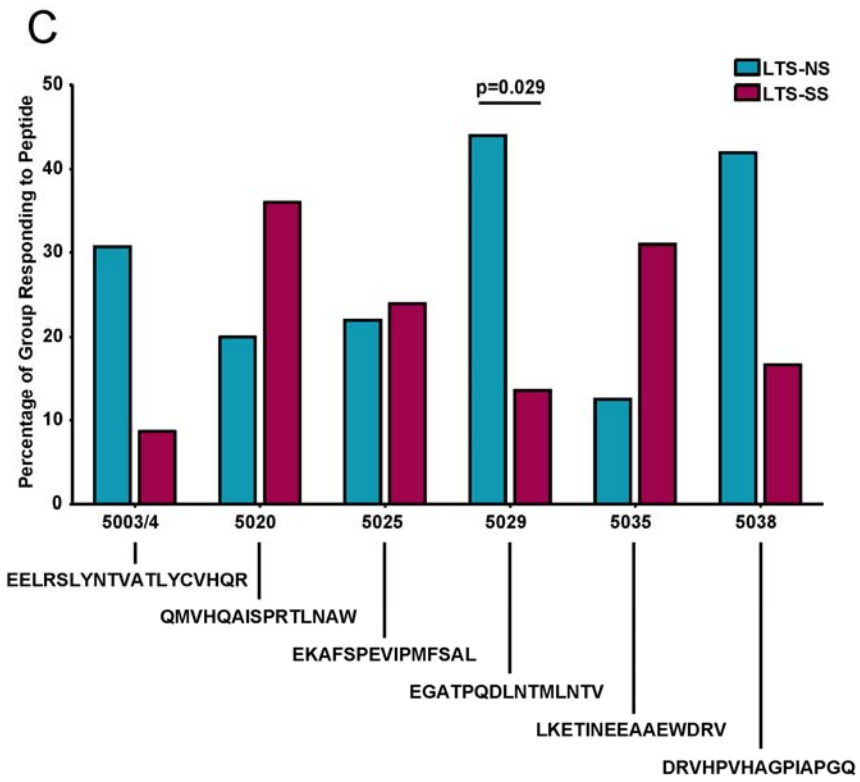



**Figure 3.7** Areas of Gag targeted by CD8+ T cell responses. **A.** The frequency of recognition of single peptides in the total cohort. A diagram of the protein regions of Gag is underneath the peptides that comprise the region. **B.** A comparison of the frequency of recognition of single peptides between the two progression groups. **C.** A more detailed view of some of the most frequently recognized single peptides and/or those that were more frequently recognized by one progression group compared to the other.

| Peptide Number | Location protein (aa-aa) | Amino Acid Sequence |
|---|---|---|
| 4988/9 | p17 (13-31) | LDRWEKIRLRPGGKKKYKL |
| 5003/4 | p17 (73-91) | EELRSLYNTVATLYCVHQR |
| 5020 | p24 (9-23) | QMVHQAISPRTLNAW |
| 5025 | p24 (29-43) | EKAFSPEVIPMFSAL |
| 5029 | p24 (45-59) | EGATPQDLNTMLNTV |
| 5035 | p24 (69-83) | LKETINEEAAEWDRV |
| 5038 | p24 (81-95) | DRVHPVHAGPIAPGQ |
| 5061 | p24 (173-187) | RAEQASQEVKNWMTE |

**Table 3.2** Details of frequently targeted Gag peptides.

*Specific Regions of Gag Targeted Unequally Between Progression Groups*

There were striking differences in the areas of Gag targeted by the two groups, with several peptides in particular targeted more frequently by one group than the other (Figure 3.7B and C). Notably, peptide 5029 was recognized by significantly more LTS-NS subjects with an HLA allele able to present the peptide (44%), than LTS-SS subjects (13.6%) ($p=0.029$, Fisher's exact test). Peptides 5003/4 and 5038 were also more frequently targeted by the LTS-NS group than LTS-SS (30.8% vs. 8.7% and 42% vs. 16.7% respectively) although these differences did not quite reach statistical significance ($p=0.08$ and $p=0.2$ respectively). In contrast, peptides 5020 and 5035 were more frequently targeted by subjects in the LTS-SS group compared to the LTS-NS group (36% vs. 20% and 31% vs. 12.5%, respectively).

*B*57 and B*42 Restricted Epitopes are Highly Immunodominant*

With the knowledge of each individual's HLA alleles and the regions of Gag they responded to, we were able to determine the HLA-restriction of the vast majority of responses using data from the Los Alamos National Database. We observed that subjects that expressed the HLA- B*57 or B*42 alleles were extremely likely to restrict their immunodominant response through these alleles. In addition, responses restricted by these alleles made up the vast majority of the total Gag-specific response in these individuals (Figure 3.8).

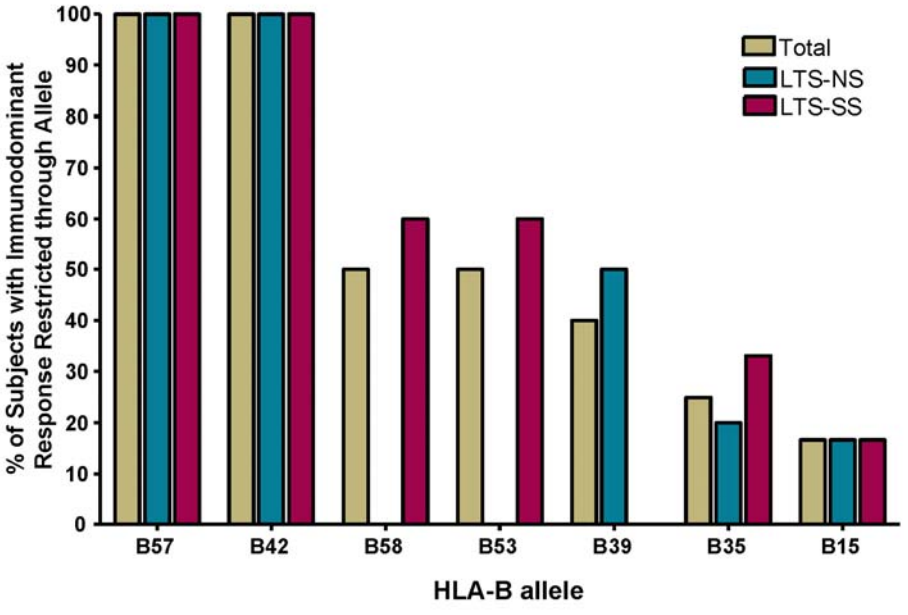



**Figure 3.8** Immunodominance of CD8+ T cell responses restricted by HLA-B alleles. The frequency of several HLA-B alleles restricting the immunodominant response of individuals expressing that allele.

Eight subjects in our cohort expressed at least one HLA- B*42 allele and all 8 restricted their immunodominant response through this allele. B*42 restricted responses accounted for an average of 69% of the total observed Gag-specific response in these individuals. Similarly, five subjects expressed at least one HLA- B*57 allele and again, all 5 of these subjects had an immunodominant response restricted through B*57. In these subjects, B*57 restricted responses accounted for, on average, 84% of their total observed Gag-specific response.

*Comparison of Differentiation Profiles of Bulk and HIV-specific CD8+ T cells Between Progression Groups*

As has been observed in adult studies [4, 41], we hypothesized that we would observe more fully differentiated $T_{EMRA}$ cells in the LTS-NS subjects compared to LTS-

SS subjects, both in the total CD8+ T cell population and in Gag-specific CD8+ T cells. We performed surface staining and intracellular cytokine staining on 17 LTS-NS subjects and 15 LTS-SS subjects, stimulating PBMC with the single Gag peptides that had been identified previously.

Surface staining of the total CD8+ T cell population revealed a significantly higher frequency of naïve T cells (CCR7+ CD45RA+) in LTS-NS subjects (p=0.0066). We observed a trend towards higher levels of $T_{EM}$ (CCR7-CD45RA-) cells in the LTS-SS group although this was not significant (p=0.2). There was no observable difference in the levels of $T_{CM}$ (CCR7+ CD45RA-) or $T_{EMRA}$ (CCR7-CD45RA+) cells between the two groups. (Fig. 3.9)

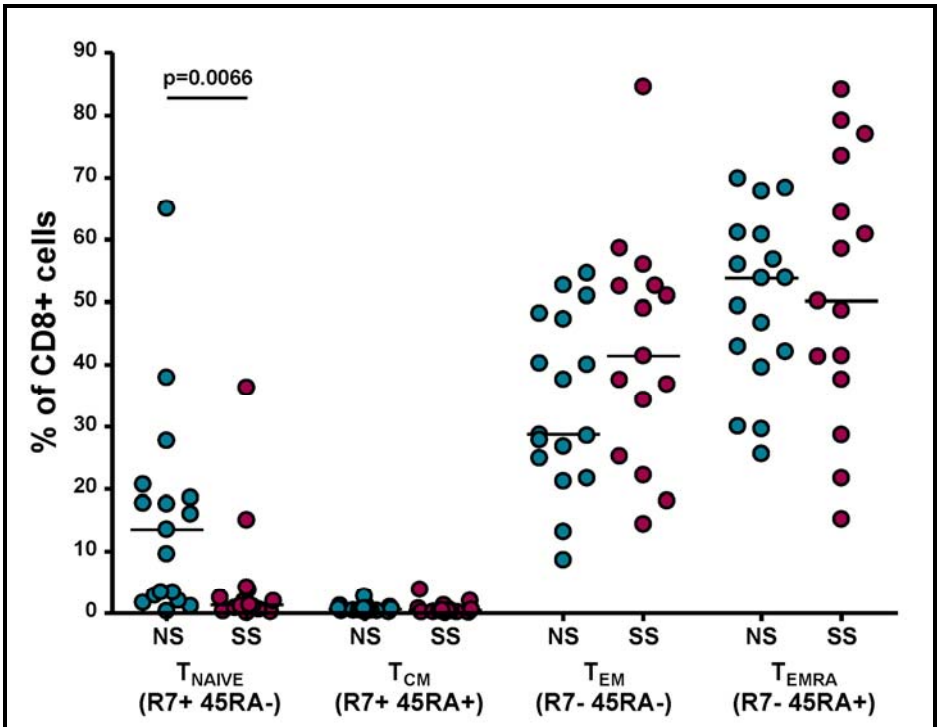



**Figure 3.9** Comparison of differentiation profiles of total CD8+ T cells between progression groups. We used the markers CCR7 and CD45RA to categorize CD8+ T cells into one of four differentiation phenotypes. Blue circles represent LTS-NS subjects and red circles represent LTS-SS subjects. The line in each column represents the median. The differentiation phenotype being compared is beneath each column.

Intracellular cytokine staining allowed epitope-specific CD8+ T cells to be characterized and for the maturation profiles between the two progression groups to be compared. First, all epitope-specific responses were analyzed, regardless of immunodominance. No differences in the maturational profiles of epitope-specific CD8+ T cells between the two groups were observed (Figure 3.10A). Following this, only the immunodominant response from each person was analyzed. Again, there were no statistically significant differences in the maturational profiles, although there seemed to be a trend towards higher levels of Gag-specific $T_{EMRA}$ cells and lower levels of $T_{EM}$ cells in the LTS-NS groups ($p=0.2$ for both) (Figure 3.10B).

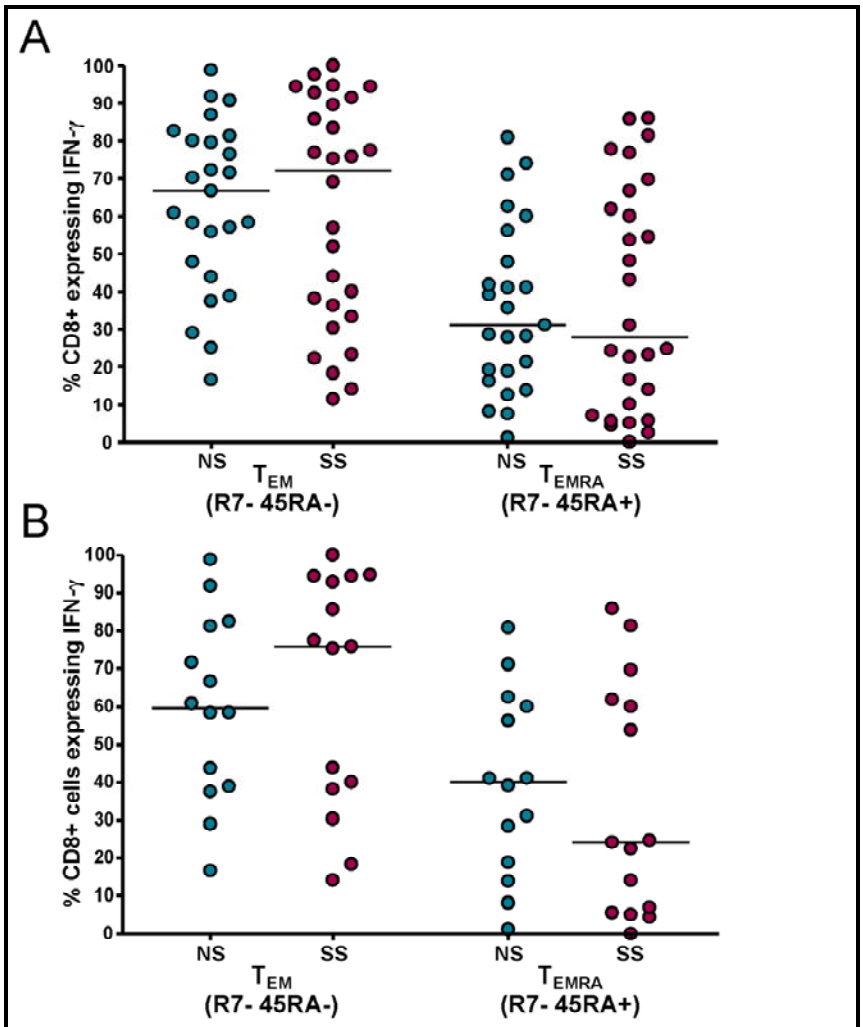



**Figure 3.10** Comparison of differentiation profiles of Gag-specific effector CD8+ T cells between progression groups. **A.** All Gag-specific responses are shown **B.** Only immunodominant responses (the response with the highest magnitude for each individual) are shown. Blue circles represent LTS-NS subjects and red circles represent LTS-SS subjects. The line in each column represents the median. The differentiation phenotype being compared is beneath each column.

*Levels of Cytokine and Degranulation Molecule Secretion in Progression Groups*

In addition to phenotypic characterization of Gag-specific CD8+ cells, the secretion of the cytokines IFN-γ, TNF-α, MIP-1β, and the degranulation marker CD107a/b were also analyzed. The secretion levels of these molecules were analyzed both singly and in different multifunctional combinations. The frequencies of cells secreting any levels of the molecules did not differ significantly between the two

progression groups (Fig. 3.11A). Overall, the frequency of cells secreting TNF-α was quite low, never reaching above .25% of total CD8+ T cells. The levels of cells secreting the other cytokines ranged, with MIP-1β being secreted by the most cells, with a median of 1% total CD8+ T cells secreting MIP-1β.

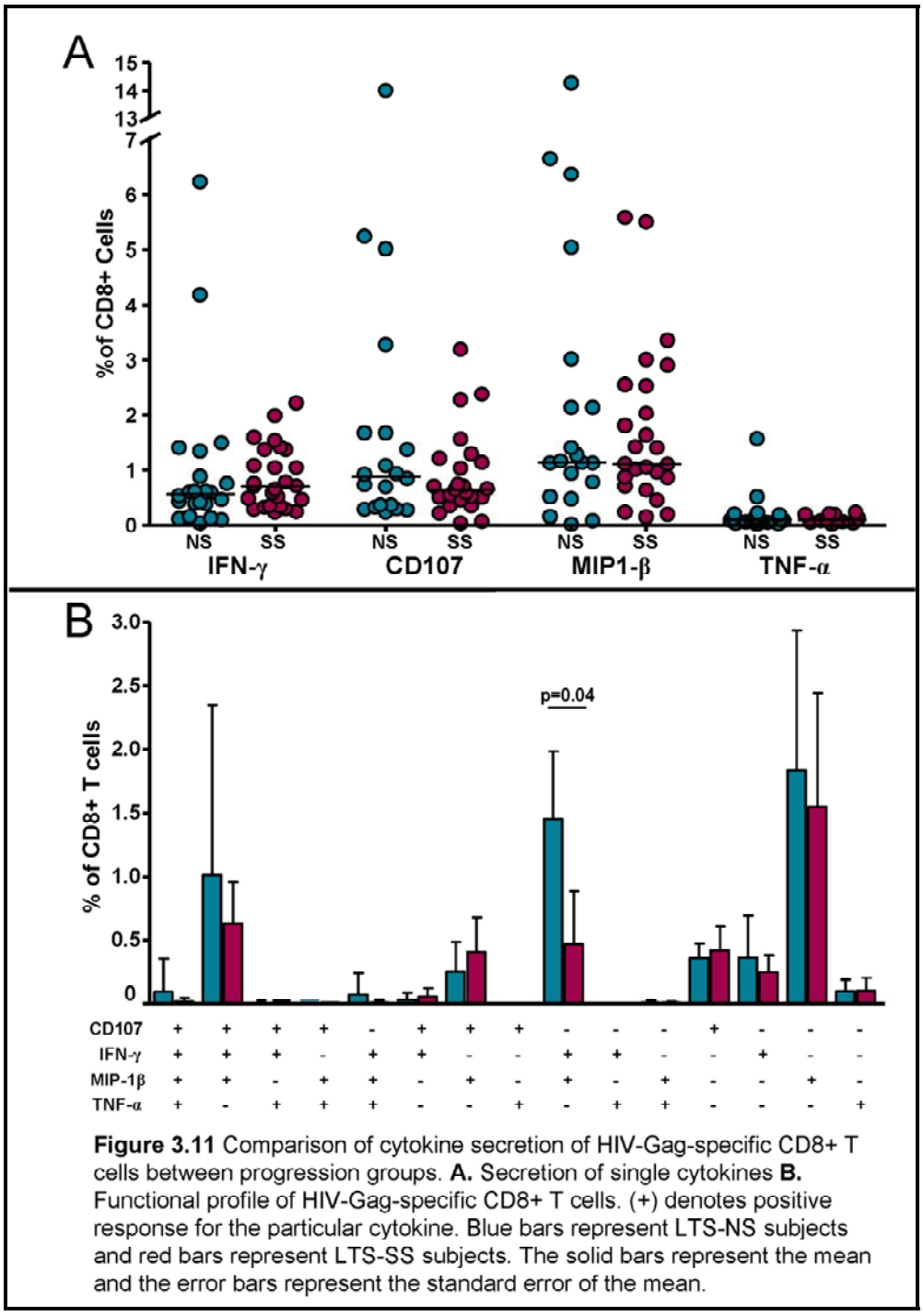



**Figure 3.11** Comparison of cytokine secretion of HIV-Gag-specific CD8+ T cells between progression groups. **A.** Secretion of single cytokines **B.** Functional profile of HIV-Gag-specific CD8+ T cells. (+) denotes positive response for the particular cytokine. Blue bars represent LTS-NS subjects and red bars represent LTS-SS subjects. The solid bars represent the mean and the error bars represent the standard error of the mean.

The vast majority of epitope-specific CD8+ T cells secreted only 1 or 2 cytokine(s) or degranulation marker, out of a possible 4 (Fig. 3.11B). A subset of Gag-specific CD8+ T cells did secrete IFN-γ, MIP-1β, and CD107a/b simultaneously (median of .6% of total CD8+), although there was no difference in the frequency of these cells between the two progression groups. There was a significant difference between the groups in the frequency of cells secreting IFN-γ and MIP-1β simultaneously, with LTS-NS subjects having a higher frequency of these cells ($p=0.04$) than LTS-SS subjects. There was a trend toward LTS-SS subjects having a higher frequency of cells secreting CD107+ and MIP-1β simultaneously compared to LTS-NS subjects, but this was not statistically significant ($p=0.13$).

*Striking Differences in CD4+ T cell Maturational Profiles between Progression Groups.*

In addition to analyzing the maturation of CD8+ T cells we also analyzed the characteristics of CD4+ T cells. As well as being the primary target of HIV, CD4+ T cells provide important help to both CD8+ T cells and B cells. CD4+ cells were defined as CD3+CD8- cells. In a different panel with all three markers we verified that, on average, 95% of CD3+CD8- cells were CD4+. Here remarkable differences in the maturational profiles of CD4+ T cells between the two progression groups were observed. As shown in Figure 3.12, subjects in the LTS-NS groups had much higher frequencies of naïve and central memory CD4+ T cells, which were highly statistically significant ($p=0.0005$ and $p<0.0001$). There was no difference in the levels of effector memory CD4+ T cells ($p=0.984$). Interestingly, subjects in the LTS-SS group had

significantly higher levels of $T_{EMRA}$ cells than LTS-NS subjects, which was also highly statistically significant ($p<0.0001$).

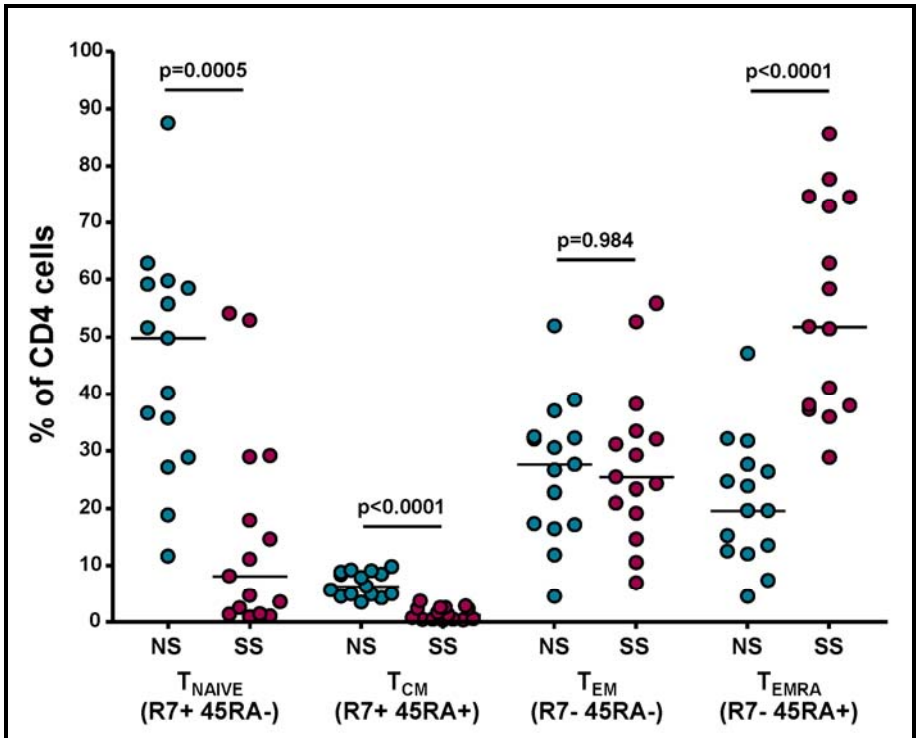



**Figure 3.12** Comparison of differentiation profiles of total CD4 T cells between progression groups. We used the markers CCR7 and CD45RA to categorize CD4 cells into one of four differentiation phenotypes. Blue circles represent LTS-NS subjects and red circles represent LTS-SS subjects. The line in each column represents the median. The differentiation phenotype being compared is beneath each column.

Additionally, the levels of naïve T cells and $T_{CM}$ cells were strongly positively correlated with CD4% (Spearman r= 0.713 and 0.716, respectively, and $p<0.0001$ for both) and negatively with LVL (Spearman r= -0.421, p=0.018; and Spearman r =-0.731, $p<0.0001$, respectively) (Fig 3.13 A-D). In contrast, the levels of $T_{EMRA}$ cells were strongly negatively correlated with CD4% (Spearman r= -0.836, $p<0.0001$) and positively correlated with LVL (Spearman r=0.0026, p=0.0026) (Fig. 3.13G-H).

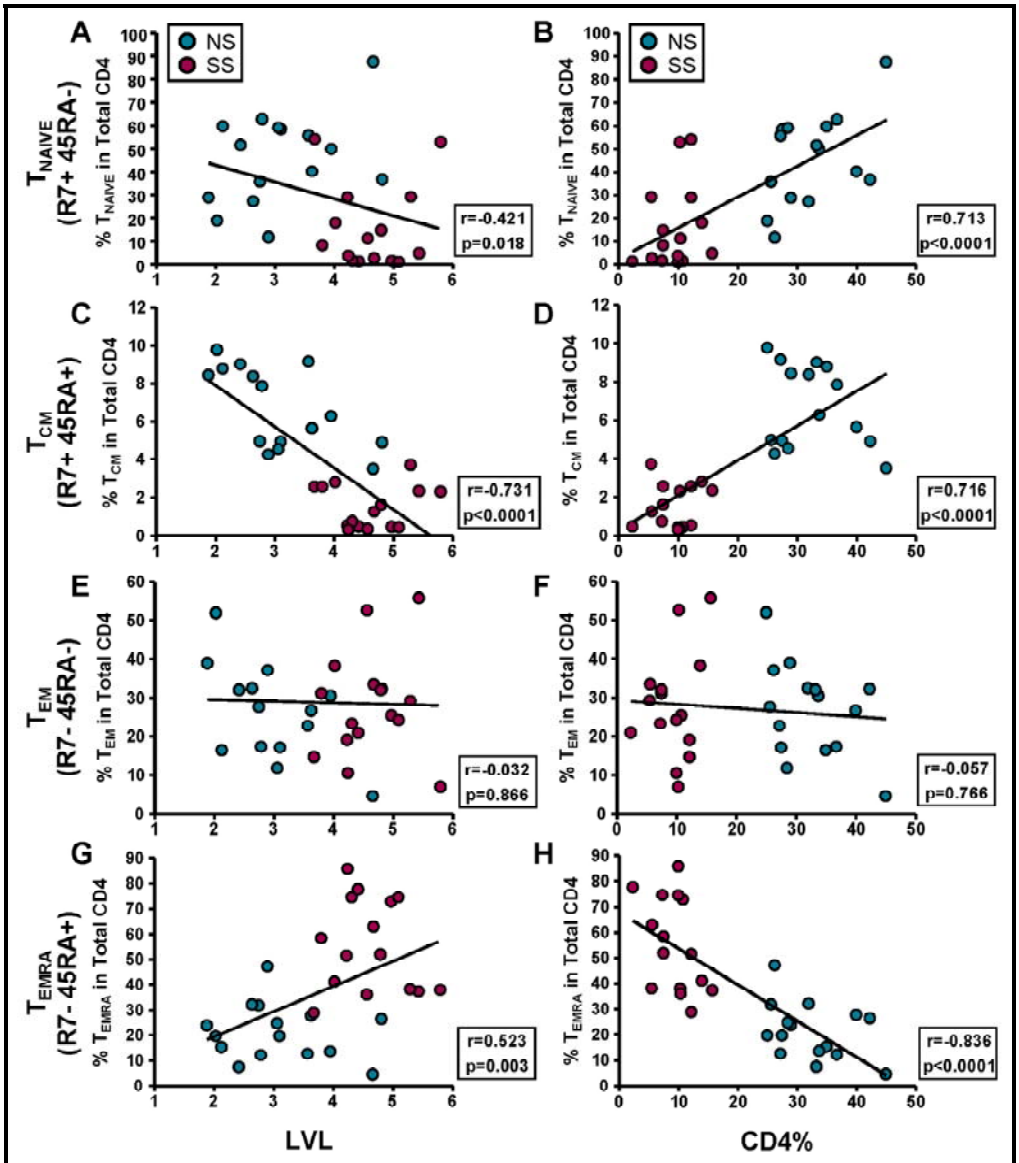



**Figure 3.13** Correlations between frequency of CD4 T cell subsets and clinical characteristics. Log viral load (LVL) versus frequency of CD4 T cell subsets are shown in **A**, **C**, **E**, and **G**. CD4% (of total white blood cells) versus frequency of CD4 T cell subsets are shown in **B**, **D**, **F**, and **H**. The CD4 T cell subset being analyzed is at the beginning of each row.

In order to further characterize CD4+ T cells, levels of CD57 expression were also measured. CD57 has been described a marker of T cell senescence [55]. CD57 expression on CD4+ T cells from LTS-SS subjects was much higher than that seen on CD4 T cells from the LTS-NS group, also strongly statistically significant (p=0.0025) (Fig. 3.14A). The frequency of CD4 T cells expressing CD57 was also negatively

correlated with CD4% (Spearman r= -0.572, p=0.001) and positively correlated with LVL (Spearman r=-0.41, p=0.02) (Fig. 4.14B-C).

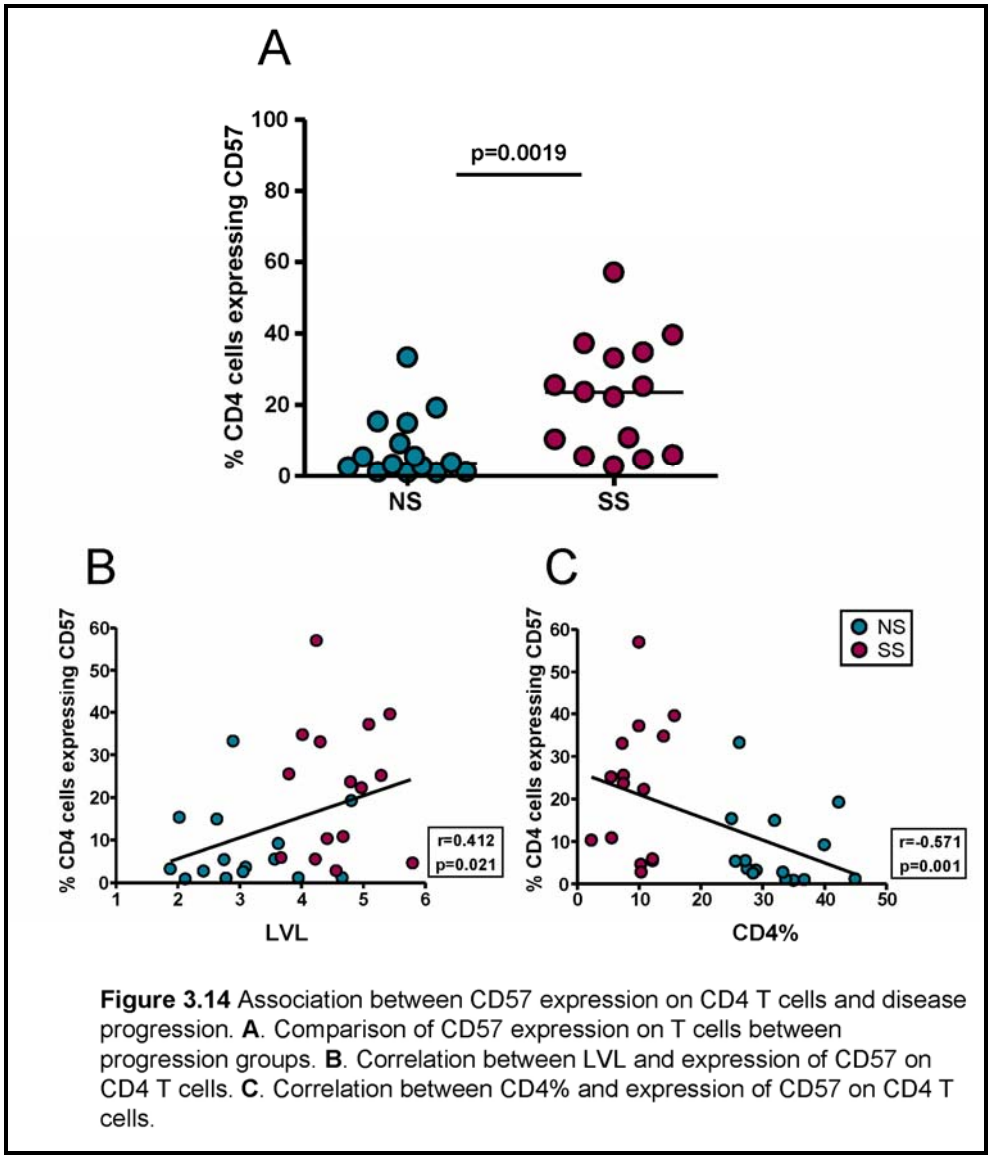



**Figure 3.14** Association between CD57 expression on CD4 T cells and disease progression. **A**. Comparison of CD57 expression on T cells between progression groups. **B**. Correlation between LVL and expression of CD57 on CD4 T cells. **C**. Correlation between CD4% and expression of CD57 on CD4 T cells.

## Discussion

Several recent studies have suggested that qualitative characteristics of the HIV-specific CD8+ T cell are associated with viral control and disease progression [4, 41, 42, 56]. Other studies have suggested that immunodominance patterns of HIV-specific CD8+ T cell responses are of great importance in establishing control of the virus and HIV disease [19, 20, 30, 32]. To our knowledge this is the first study to investigate the intersection of immunodominance patterns and qualitative characteristics of HIV-specific CD8+ T cell responses, namely differentiation patterns and multifunctionality, in perinatally infected children. The children, all older than 10 years of age and thus considered long-term survivors (LTS), were categorized by CD4 percentage levels into those with no immune suppression (LTS-NS) and those with severe immune suppression (LTS-SS), based on previously published CDC guidelines [45].

We observed no difference in the magnitude or breadth of the Gag-specific CD8+ T cell response between the two groups, as measured by IFN-γ production, consistent with other studies [2-4, 6]. In contrast, there were several striking differences in the areas of Gag which were targeted by CD8+ T cell responses by the two progression groups, suggesting that the region of Gag targeted could be important to modulating disease progression. We observed significantly higher levels of naïve CD8+ T cells ($T_{NAIVE}$) in the LTS-NS subjects compared to the LTS-SS subjects ($p=0.0066$), but no differences in any other CD8+ T cell subsets. The differentiation profiles of Gag-specific CD8+ T cells, regardless of immunodominance, were similar between the progression groups. No strong differences in levels of multifunctionality were observed between the two groups. Together, these data suggest that, at least in perinatally infected children, the region of

Gag targeted by CD8+ T cells may have more importance to the rate of disease progression than qualitative features such as differentiation and multifunctionality.

One of the most striking findings of this study was the dissimilarity in CD4+ T cell differentiation profiles between the two progression groups. Children in the LTS-NS group had significantly higher levels of naïve T cells ($T_{NAIVE}$) and central memory ($T_{CM}$) CD4+ T cells than LTS-SS children (=0.0005 and $p<0.0001$, respectively). In contrast, children in the LTS-SS group had significantly higher levels of effector memory RA+ ($T_{EMRA}$) cells ($p<0.0001$). These data suggest that HIV is targeting specific CD4+ T cell subsets, specifically all subsets except for the $T_{EMRA}$ cells.

A recent study by *Oswald-Richter et al.* observed that CD4+ cells with the $T_{EMRA}$ phenotype (CCR7-CD45RA+) were more prevalent in HIV-infected individuals, compared to uninfected individuals [57]. Furthermore, they described that these cells were resistant to infection by CCR5-tropic strains of HIV-1, in spite of robust expression of CCR5. In our study, we observe a striking increase in the frequency of $T_{EMRA}$ cells in LTS-SS subjects, suggesting that these cells are not being lost as quickly as other CD4+ subsets during HIV disease progression, supporting the findings of Oswald-Richter. The frequency of naïve and central memory CD4+ T cells were greatly decreased in the LTS-SS group, which might be a result of earlier thymic destruction, which has been proposed to be a cause of quicker HIV disease progression in young children compared to adults [58, 59].

We observed the strongest difference in immune responses between the two progression groups, not in magnitude or breadth of response or in the differentiation or cytokine secretion profiles of HIV-specific CD8+ T cells, but in the specific areas of Gag

targeted by these cells. This suggests, as others have postulated recently, that it is the region of HIV targeted by the CD8+ T cell response that is the most important factor in mediating the rate of disease progression[12, 21]. Most commonly, CD8+ T cell responses targeting the Gag protein have been associated with improved clinical outcome [2, 12, 17, 18, 20, 21]. The protein subunits of Gag are some of the most numerous proteins in an HIV-1 virion. Around 2,000 p24 subunits make up the capsid core for each viral particle [60]. Mutations in p24 sequence can lead to virions with decreased capsid stability, and thus, less infectious[61]. It is not surprising then that peptides contained within the p24 subunit are some of the most frequently recognized by CD8+ T cells and that these responses are some of the most effective, being associated with slower disease progression. In our study, the majority of peptides most frequently recognized were in p24. The most frequently recognized peptide, 5038, interestingly, contains the cyclophilin-A (CypA) binding domain. Binding of the target cell protein CypA to the viral capsid to the is critical for efficient viral replication [62, 63]. Peptide 5029, which was significantly targeted more frequently by LTS-NS compared to LTS-SS subjects (44% vs. 13.6%), contains the TL9 (TPQDLNTML) epitope, which has previously been identified as a peptide frequently targeted by HLA-B*42 restricted CD8+ T cell responses [64]. The TL9 epitope shares significant homology with the Mamu-A*01-restricted epitope CM9 (CTPYDINQM), which has been implicated in viral control in SIV-infected macaques [65-67]. This suggests that recognition of this area of Gag could be important in establishing immune responses that might slow disease progression in perinatally infected children.

Of the varied MHC Class I alleles that we observed in our cohort, only two, B*57 and B*42, overwhelmingly restricted immunodominant responses. 100% of subjects with at least one copy of these alleles restricted their immunodominant response through that allele (Fig. 3.8). Interestingly, these two alleles were also associated with the LTS-NS group, suggesting that the immune response directed through these alleles plays a major role in mediating slower disease progression. This has been suggested before for HLA-B*57 in adults [19, 31] but not in a perinatally infected population. B*42-restricted responses have been noted for their immunodominance in an African population [64] but have not previously been associated with slower disease progression.

This study suggests that immunodominance patterns are important in the establishment of levels of disease progression in perinatally HIV-infected children, but there are some caveats. The small number of subjects precludes us from applying these findings to the general pediatric population. The subjects were not all on the same treatment regimen, but this is an issue in all pediatric studies. We attempted to minimize this concern by choosing subjects for the study who: 1) did not receive HAART in the first two years of life; 2) had some levels of ongoing viral replication; and 3) were ARV-experienced, except for two patients. Another equalizing factor is that both groups, as a whole, had similar treatment adherence rates.

This study is one of the first to study the patterns of immunodominance and associations with disease progression in a cohort of perinatally infected children. Moreover, unlike previous studies that have focused on individuals of Northern European descent, this work focused on African American and Hispanic populations, two populations that are greatly underrepresented in the seminal studies on the HIV-specific

immune response. We find that in children, as in adults [19], it appears that immunodominance patterns likely influence rates of disease progression. This influence seems to be mainly the cause of focused targeting of the Gag protein, not differentiation or cytokine secretion profiles. In addition, we observed an extremely strong correlation between increased CD4+ $T_{EMRA}$ cells and more severe immunological suppression. Although this suggests that these cells are not being lost in progressive infection, clearly more research into this area is needed. These findings could be of importance to the field of pediatric HIV immunology as well as the larger field of HIV vaccine design.

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# Final Conclusions

Understanding the immunologic mechanisms that play a role in the control of progression of HIV-1 disease is critical for the development of vaccines or new strategies for immunotherapy. Whether infection occurs perinatally, sexually, or through the transmission of blood products, HIV disease ultimately develops in all but a very few individuals. The remarkable variability in the rate of disease progression is dependent on a bevy of factors, both viral and host. Our focus in these studies was specifically on host immunologic responses that were associated with disease progression in perinatally infected children.

In our first study we found suggestions that race and gender play a role in the HIV-specific immune response of perinatally infected children. From this work we also saw hints that there are aspects of the CD8+ T cell response that are influenced by hormones present during puberty. This is of great importance to follow-up, given the large number of adolescent girls worldwide becoming infected or entering puberty perinatally infected.

Our second study focused on the innate immune system, specifically on monocytes. We observed that monocytes from HIV-infected children have impaired cytokine signaling. This finding is important for understanding the general dysregulation of the immune system caused by HIV-1.

Our last study, focused on the intersection of HLA types, immunodominance of CD8+ T cell responses, differentiation phenotypes, and disease progression. We observed that children progressing more slowly targeted p24 Gag more frequently than children progressing more rapidly. Several specific regions of p24 were targeted

more frequently both by those progressing slowly and those expressing HLA alleles that have previously been associated with slower disease progression (with great overlap between the two groups). This suggests that immunodominance patterns and more specifically, the regions of Gag targeted by these dominant responses, do play a role in HIV-1 progression in perinatally infected children. Unexpectedly, we also observed striking differences in the differentiation profiles of the CD4+ T cell pool between the two progression groups, with a marked accumulation of the CCR7- CD45RA+ CD4+ T cell subset in subjects with more severe HIV progression. This finding is intriguing and should be followed up with more detailed studies.

Research on HIV-1 progression can be a difficult task, with the countless different variables that come from studying a human population. Focusing that research on children adds other layers of difficulty. Less is known about pediatric and adolescent HIV, there are additional issues of categorization, and, importantly, almost all children are treated with HAART, but to varying degrees. One concept that is becoming clearer, especially with the studies of elite controllers, is that there is no "one thing" about the host or the virus that makes some individuals progress much more slowly than others [1, 2]. Although this understanding most likely precludes finding outright protection from the disease, it makes studying every aspect of the virus and host that much more important.

Despite seemingly endless grim HIV/AIDS statistics, there are a few that hint that we might be starting to turn the tide on the pandemic. For example, the global percentage of adults living with HIV has leveled off since 2000, the number of deaths from AIDS has fallen over the last several years, and in several countries the

percentage of young pregnant women (15-24 years) living with HIV/AIDS has dropped more than 25% since 2001[3]. Massive spending programs such as PEPFAR, the Global Fund, and the Gates Foundation have vastly increased the resources available for monitoring, prevention, treatment, and care in the countries that need it most.

## Final Conclusions References


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