QUALITATIVE ANALYSIS OF HIV-1-SPECIFIC CD8 T CELL RESPONSES

by

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ABSTRACT

In the absence of antiretroviral therapy (ART), the majority of individuals infected with human immunodeficiency virus-1 (HIV-1) will develop AIDS. HIV-1-infected controllers are exceptions to the rule; without the use of ART, these individuals spontaneously control virus replication. A better understanding of the immune mechanisms that mediate delayed disease progression, as seen in controllers, will provide valuable insight to the design and development of an effective HIV-1 vaccine.

CD8 T cells are important mediators of the antiviral immune response. However, it is unclear which components of the response are critical for long-lasting protection during HIV-1 infection. We first review methods currently used in the analysis of HIV-1-specific CD8 T cell function. We then discuss our analysis of HIV-1-specific CD8 T cell clonal populations that did or did not produce interleukin-2 (IL-2), an effector function associated with delayed disease progression. Having observed a unique clonotypic profile of IL-2 producing CD8 T cells, we predicted that maintenance of IL-2 production identifies a population of CD8 T cells with enhanced ability to (1) produce antiviral soluble factors as observed using multicolor flow cytometry and (2) restrict HIV-1 replication as detected using an in vitro suppression assay (iVSA). To test this hypothesis, we analyzed epitope-specific CD8 T cell function first during chronic HIV-1 infection and subsequently during primary infection.
Using the iVSA, we observed significantly enhanced suppression of HIV-1 replication by CD8 T cells derived from controllers when compared to progressors. Interestingly, the level of suppression correlated with a polyfunctional, IL-2\(^+\) CD8 T cell response. Preliminary results of CD8 T cell effector function during primary HIV-1 disease show that functional, epitope-specific CD8 T cell lines can be expanded and these cells are able to suppress HIV-1 replication \textit{in vitro}, albeit to moderate levels. Both the proliferative and suppressive capacity of CD8 T cells derived from the early stage of disease appeared to increase over time. These studies suggest that suppression of HIV-1 replication and polyfunctional IL-2 production would be promising markers of an effective CD8 T cell response and have important implications for the evaluation of HIV-1 vaccine strategies.

Keywords: HIV-1 infection, CD8 T cell, virus suppression, polyfunctional, IL-2
DEDICATION

I would like to dedicate this dissertation to my parents, Charles Bandele and Margaret Omoyiwola Akinsiku. You instilled in me the importance of hard work and perseverance, teaching by instruction and example. You sacrificed to provide me with all that I ever needed I am so grateful for your love and encouragement. Everything that I have become today stands upon the foundation you built. Daddy, this marks the completion of your doctorate as much as it does mine. I love you both dearly.
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Thank you LORD. You have done me well and I will forever praise your name.
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<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
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<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
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<td>ART</td>
<td>Antiretroviral Therapy</td>
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<td>BLCL</td>
<td>B Lymphoblastoid Cell Line</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation; Cluster of Designation</td>
</tr>
<tr>
<td>CDR3</td>
<td>Complementarity Determining Region 3</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein Diacetate Succinimidyl Ester</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CRF</td>
<td>Circulating Recombinant Form</td>
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<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
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<tr>
<td>E:T</td>
<td>Effector:Target</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>EC</td>
<td>Elite controller</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
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<td>ELISpot</td>
<td>Enzyme-linked Immunospot</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated Cell Sorting</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IL-7</td>
<td>Interleukin-7</td>
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<tr>
<td>LCMV</td>
<td>Lymphocytic Choriomeningitis Virus</td>
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<tr>
<td>LTNP</td>
<td>Long-term Nonprogressor</td>
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<tr>
<td>LTR</td>
<td>Long Terminal Repeat</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
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<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
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<tr>
<td>pVL</td>
<td>plasma viral load</td>
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<tr>
<td>RLU</td>
<td>Relative Luciferase Units</td>
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<td>RT</td>
<td>Reverse Transcriptase</td>
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<tr>
<td>SIV</td>
<td>Simian Immunodeficiency Virus</td>
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<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
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<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-alpha</td>
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INTRODUCTION

Five cases of opportunistic infections in previously healthy, homosexual men in the Center for Disease Control’s June 5, 1981 issue of the Morbidity and Mortality Weekly Report was the first published report of a syndrome hallmarked by severe immunodeficiency in the United States (1). Within months, additional cases were reported among injection drug users as well as blood transfusion recipients (2, 57). Patients presented with symptoms typically observed only among immunosuppressed populations, including pneumocystis carinii infection and mucosal candidiasis. Within two years of the initial report, human immunodeficiency virus (HIV) was identified as the causative agent of this constellation of symptoms now defined as acquired immune deficiency syndrome (AIDS) (9, 29, 53).

By the end of 2009, the World Health Organization reported more than 33 million people were living with HIV infection, with nearly 2.5 million new infections occurring in that year alone (82). Basic research into the pathogenic mechanisms of HIV has driven the development of antiretroviral therapy (ART), which coupled with public health efforts, have significantly reduced morbidity of HIV-1 infection (64, 73). Yet, the pandemic continues seemingly unabated. A critical strategy in efforts to curb the HIV/AIDS pandemic lies in the prevention of new infections. In addition to focused public health initiatives, this demands the identification and treatment of HIV-infected individuals to reduce the spread of disease. It has been demonstrated that patients with lower plasma viral loads (pVL) transmit virus to a lesser degree (63, 68). Successful
administration of ART will reduce pVL to undetectable levels, however, only 30% of individuals who need ART receive it (27). The key strategy in reducing HIV incidence, and primary goal of significant research efforts, would be the development of an effective HIV-1 vaccine. When initial efforts towards a preventive vaccine against HIV-1 failed, the focus shifted to design of a therapeutic vaccine, a product that might delay disease progression. The design of a protective HIV-1 vaccine will require increased knowledge of the nature of both humoral and cell-mediated virus-specific responses and how to appropriately induce these responses during primary and chronic disease.

With significant evidence of the protective role CD8 T cells play in controlling virus replication, it is of great interest to identify the quality of HIV-1-specific CD8 T cells that mediate protection. In these studies, I have investigated the ability of epitope-specific CD8 T cells to produce antiviral soluble factors and mediate suppression of HIV-1 replication in vitro.

**Human Immunodeficiency Virus**

HIV is a member of the retroviridae family, specifically listed in the primate lentivirus group. These classifications indicate important features of HIV—1) the virus has an RNA genome that must first be reverse transcribed to mediate productive infection of a target cell and 2) HIV infection is characterized by a long incubation period, with individuals having been infected for an extended period of time before clinical manifestations of disease. These features of HIV have significant implications for the development of therapeutic strategies to prevent infection and treat disease.
**HIV Species and Subtype Distribution**

Based on genome organization and evolutionary relation to other primate lentiviruses, two species of HIV have been identified, HIV-1 and HIV-2. These distinct species represent separate zoonotic crossover events from apes into humans (30, 31, 38). HIV-1, the species causing the global AIDS pandemic, is a result of the introduction of simian immunodeficiency virus (SIV) from chimpanzees into humans, while HIV-2 originated from SIV-infected sooty mangabeys. HIV-1 strains are classified into three groups, M (main), N (non-M/non-O), and O (outlier) (15).

The HIV-1 M group is further subdivided into clades, viral strains that vary in amino acid sequence, designated A through K. Clades tend to be regionally distributed—clade A is primarily found in northern Asia, clade B infection dominates in the Americas, while the majority of clade C infections are found in sub-Saharan Africa.

HIV-2 is primarily found in West Africa and its distribution is coincident with the habitation of sooty mangabeys. HIV-2 strains are classified into eight groups, denoted A through H, representing eight individual zoonotic transmissions (15). The HIV-2 genome is similar to HIV-1 with one exception, HIV-2 encodes the accessory protein Vpx rather than Vpu. Interestingly, HIV-2 has a milder virulence compared to HIV-1—it is less transmissible and infected individuals often have undetectable pVL (20).

**Genetic Diversity of HIV-1**

The genetic diversity observed among HIV-1 strains is generated in a number of ways. A large contributor to sequence variation is the viral enzyme reverse transcriptase (RT) (28). RT copies the viral genome, creating the double-stranded DNA that will be
incorporated into the host genome during integration. Lacking a proofreading mechanism, RT is highly error-prone, introducing several mutations into every new copy of the viral genome. The high rate of virus replication, in combination with the viral RT, leads to continued emergence of new variants, and establishes viral quasispecies within the infected host. Additional sources of genetic diversity are recombination events between two or more parental strains which generate circulating recombinant forms (CRF). To date, more than 30 recombinant strains have been identified (15). HIV-1 sequence variation presents a formidable challenge to vaccine development; novel mechanisms that can induce protective responses against a wide breadth will be needed.

**HIV-1 Genome and Structure**

The HIV-1 genome is 9.7kb, comprised of accessory genes and three major genes common to all retroviruses—gag, pol, and env (28). The genome encodes four classes of proteins—1) structural proteins matrix, capsid, and nucleocapsid, 2) viral enzymes, reverse transcriptase, integrase, and protease, 3) regulatory and accessory proteins Tat, Rev, Nef, Vif, Vpr, Vpu, and 4) the envelope proteins gp120 and gp41 (Figure 1A). The genomic sequence is flanked by long terminal repeat (LTR) units which contain genetic regulatory elements that govern viral gene expression.

An HIV-1 virion contains two identical RNA strands housed in a conical core made of the capsid protein (Figure 1B). Within this capsid core are multiple copies of nucleocapsid, protease, integrase, and reverse transcriptase. Filling the space between the capsid core and the outer lipid membrane is the matrix protein. The viral membrane contains the envelope proteins, gp120 (surface component) and gp41 (transmembrane
component), arranged in a functional trimeric unit which mediates viral entry into host target cells.

FIGURE 1. HIV-1 genome and structure. Organization of the nine genes encoded by HIV-1 genome. Viral transcripts are read in frames 1, 2, and/or 3. Spliced exons encoding rev and tat are connected using black solid lines. The 9,719 value indicates number of bases in viral strain HXB2. Dashed lines lead to cleaved protein products of the viral polyproteins Gag, Pol, and Env. (B) Structural schematic of an HIV-1 virion.

Gag and Nef are highly immunodominant and command most of the HIV-1-specific CD8 T cell response. Nef expression is high during the early phase of HIV-1 gene expression and is associated with enhanced infectivity, downregulation of CD4 surface expression, and evasion of the host immune response through downregulation of HLA class I alleles, particularly HLA-A and HLA-B molecules (74, 80). While Nef is the
most immunogenic of the HIV-1 proteins, Nef-specific CD8 T cell responses are not associated with control of HIV-1. In fact Nef targeting was shown to correlate with increased pVL (8). In contrast, Gag sequences are highly conserved and a higher number of Gag-specific responses is associated with improved control of virus replication (22, 44).

The potential source of HIV-1 epitopes has expanded even further as we now understand that cryptic epitopes, antigen derived from alternate reading frames of the HIV-1 genome, are recognized and targeted by the host immune system (7, 10). The significance of responses targeting HIV-1 cryptic epitopes is currently being investigated.

**HIV-1 Life Cycle**

HIV-1 preferentially infects activated CD4 T cells (83). However, infection and provirus integration also occurs in non-dividing cells, with virus replication ensuing when the cell becomes activated. Targeting CD4 T cells is a critical factor in HIV-1 pathogenesis as these cells are key regulators of the human immune response, providing help to both remaining subsets of the adaptive response—CD8 T cells and B cells.

The HIV-1 envelope proteins bind to a CD4 molecule at the cell surface, engaging one of two co-receptors, CCR5 or CXCR4, to enter the target cell (Figure 2). Fusion of the viral outer envelope with host cell membrane enables release of the viral core into the cytosol. After uncoating of the core, reverse transcription of the genomic RNA ensues, yielding a pre-integration complex (PIC) made up of viral cDNA, as well as viral and host proteins (67).
After import of the PIC into the nucleus, integrase mediates integration of viral DNA into the host chromosome (67). This establishes the provirus that is a permanent template for virus replication, making total eradication of virus from an infected host nearly impossible (25). The first round of multiply spliced transcripts to be released into the cytosol encodes Tat, Rev, and Nef. Subsequent transport of viral genomic RNA and transcripts encoding the enzymatic, structural, and accessory proteins—all needed for synthesis of new virions—out of the nucleus is dependent on Rev shuttling (19). Assembly of new virions occurs at the plasma membrane, primarily directed by assembly of the Gag polyprotein, facilitating virus budding away from the cytoplasm.

Over 30 medications, targeting critical steps of the virus life cycle (entry/fusion, reverse transcription, integration, protein cleavage) have been developed for treatment of HIV-1 infection. The advent of combination therapy, simultaneous administration of drugs from 2-4 classes, has drastically extended the life expectancy of infected individuals. While ART allows for better control of virus replication, infection is never cleared. Thus, there is a continued need for novel therapeutic strategies to prevent and treat HIV-1 disease.
FIGURE 2. HIV-1 life cycle. HIV-1 entry is mediated by the CD4 receptor and one of two co-receptors. After the virus fuses with the target cell, the capsid core is released into the cytoplasm. The viral enzyme reverse transcriptase generates a DNA copy of the virus RNA genome. The cDNA enters the nucleus and integration occurs. Viral transcripts are exported to the cytosol where HIV-1 (poly)proteins are synthesized on host machinery. Proteins assemble at the plasma membrane and viral particles bud and undergo maturation to become infectious virions.

HIV-1 Immunopathogenesis

Sexual transmission via mucosal surfaces is the most common route of HIV-1 infection. A number of questions regarding transmission of virus in humans and the immediate events thereafter remain unknown and will be difficult to study \textit{in vivo}. Technical advances in molecular cloning of virus isolated within days of infection are providing clues as to the nature of the transmitted/founder virus (77). It is now appreciated that in approximately 80\% of mucosal transmission events, a single HIV-1 virion crosses the mucosal barrier and establishes productive infection (43).

Within two weeks of exposure to HIV-1, the transmitted virus has crossed the mucosal epithelial barrier, infected a small population of CD4 T cells, and expanded locally by infecting additional resting and activated CD4 T cells (36). Having infected a seed population of target cells, the virus is transported to the local draining lymph nodes which contain a large number of target cells, gains access to the bloodstream, and fully disseminates throughout secondary lymphoid organs. By 21-28 days after infection, pVL reaches a peak, with millions of viral copies detectable in patient plasma (61). By this point, the hallmark of HIV-1 infection has been irreversibly established—large numbers of CD4 T cell populations in the host have been depleted.

The earliest detection of HIV-1-specific CD8 T cells coincides with the time at which viral load begins to decrease. The infected host establishes a VL set point, which is predictive of the rate of disease progression; individuals with a low set point tend to have a better disease prognosis (62).

Chronic HIV-1 infection is characterized by a persistent state of immune activation, a critical contributor to disease pathogenesis. On-going HIV-1 replication is
considered one underlying cause of this chronic activation. Host immune cells are subject to continuous stimulation, resulting in increased cell turnover, increased frequency of cells with an activated phenotype, and cell exhaustion (37, 48). More recently, translocation of microbial products was shown to be a contributing factor to this systemic activation (13). Damage to gut epithelium facilitates translocation of microbes and/or microbial products that are normally sequestered in the gastrointestinal tract. The detection of these products was higher during chronic HIV-1 infection and correlated with proinflammatory markers. Treatment strategies to reduce the hyperactive immune state observed during chronic HIV-1 disease could help to mitigate immunological dysfunction and potentially slow progression to AIDS.

Control of HIV-1 Disease

In the absence of ART, the immune system of the majority of HIV-1 infected individuals eventually fails, opportunistic infections develop, and the patient succumbs to AIDS. A number of studies have identified both viral and host factors that are associated with a more rapid or delayed progression to AIDS (42, 50). One noted genetic factor associated with delayed disease progression is the delta 32 mutation, a 32-base pair deletion in the gene encoding the HIV-1 coreceptor CCR5. Individuals homozygous for this deletion fail to express CCR5 at the cell surface and are thus resistant to infection; heterozygotes maintain very low pVLs (40, 54). Infection with viruses that lack a functional Nef protein was also associated with a less pathogenic course of disease (45).

There exists a subset of HIV-1 infected individuals that exhibit spontaneous control of HIV-1 replication in the absence of therapy (46). Originally described as
individuals who maintained CD4 cell counts above 500/μL without the use of ART, long-term nonprogressors (LTNP) were a distinct subset of HIV-1 infected patients who remained healthy for long periods of time. It was later observed that many LTNP do eventually progress, developing clinical signs of disease after 10-15 years of infection (51). Over the years, an even smaller subset of patients arose out of the LTNPs, now commonly referred to as elite controllers (EC) (21). Making up less than 1% of HIV-1 infected individuals, ECs demonstrate remarkable control of virus replication, able to maintain pVL below the level of detection (<50 copies/mL) for as many as 25 years. These individuals provide “proof-of-principle” that natural immunity to HIV-1 exists and protects from disease progression. LTNP and ECs remain the focus of a number of studies to better understand the immune response that affords long-lasting protection (66).

The most common characteristic of HIV-1 controllers is the expression of certain human leukocyte antigen (HLA) class I alleles (16, 34, 42). HLA-B*13, B*27, B*51, B*57, and B*5801 alleles are heavily represented in cohorts of patients with non-progressive HIV-1 disease. The results from a genome-wide association study identified three genomic determinants of the host’s ability to control HIV-1 replication, one of them being expression of the protective allele HLA-B*5701 (24). HLA class I molecules are involved in antigen presentation to CD8 T cells, suggesting that these cells may play an important role in attenuation of HIV-1 disease.
Immune responses Induced during HIV-1 Infection

Cell-mediated immune responses are a critical component of host defense against HIV-1 replication. A successful HIV-1 vaccine will likely have to induce both innate and adaptive immune responses. Hence, understanding the impact of NK cell, B cell and T cell effector functions on the course of HIV-1 disease is critical for defining correlates of long-lasting protection and the development of novel treatment strategies.

Natural Killer Cells

Previous studies have reported the importance of targeting HIV-1 early, as low VL setpoint correlates with delayed disease progression (63). Furthermore, early control of HIV-1 replication achieved by antiretroviral therapy (ART) during primary infection was associated with preserved HIV-specific immune responses (72). As first responders to invasion by microbial pathogens, innate effector cells react to contain and clear infection; hence natural killer (NK) cells are likely an important component of immune protection during the acute stage of disease. Rapid progression to AIDS has been associated with reduced NK cell frequency and function (5). It is also known that HIV-1 infection alters NK cell phenotype, as the subset most responsible for antibody-dependent cell-mediated cytotoxicity (ADCC) is selectively lost during disease (58, 59).

NK cell effector function is regulated by activating and inhibitory receptors expressed at the cell surface (49). One family of NK cell activating receptors consists of three members—NKp30, NKp44, and NKp46— and it was observed that compared to uninfected controls, these receptors were expressed at lower levels in HIV-infected subjects (23). Epidemiologic studies provide further evidence implicating NK cells as
mediators of protection during HIV-1 disease. Five large cohorts of HIV-infected individuals (over 1500 subjects) were analyzed for HIV-1 disease outcome, HLA genotype, and expression of killer immunoglobulin-like receptors (KIR) (55). KIRs comprise a class of NK cell receptors specific for HLA class I molecules. This analysis demonstrated that the co-expression of activating NK cell receptor KIR3DS1 and HLA-B alleles having the Bw4 motif was associated with delayed progression to AIDS. There was no added benefit if the molecules were expressed individually. The impact of NK cell effector function on HIV-1 disease warrants further investigation as innate immunity may impact long-term prognosis of HIV-1 disease during primary infection.

**B cells and HIV-1 Infection**

Humoral responses are an essential component of adaptive immunity. For years, vaccine development focused on the induction of antigen-specific antibodies, as sterilizing immunity to a number of pathogens (measles, hepatitis B) was established if a minimum antibody titer was induced (75). These long-lasting antibody responses are induced by immunization with killed and live attenuated viruses, or recombinant antigens. Safety issues prevent the use of attenuated HIV-1 as a vaccine agent, thus alternate vaccine strategies will be needed to induce appropriate virus-specific humoral responses.

While virus-specific antibodies can be detected within three weeks of infection, HIV-1 neutralizing antibodies (NAbs) are often not detected for 3-4 months after transmission (61), and these NAbs are the first antibodies to which the virus will escape. Adding another level of complexity, NAbs to conserved regions of the HIV envelope
proteins, those likely to provide the most protection, are extremely rare and often not detected for 2-3 years after infection (61).

Pre-existing circulating antibody has been shown to affect disease outcome in rhesus macaques (6, 56). Animals were passively immunized with NAbs and subsequently challenged with chimeric SIV/HIV-1 virus. Compared to controls, macaques receiving antibody infusion showed reduced pathogenicity or a complete block of infection; this protection was observed in animals regardless of inoculation site. These data suggest that NAbs may be important mediators of protection from HIV-1 acquisition. Understanding development of the more rare, HIV-1-specific NAb isolated from infected individuals may provide insight on how to generate such responses with an effective vaccine.

**HIV-1-Specific CD8 T cells**

Analysis of virus-specific responses during SIV/HIV-1 infection has established the importance of CD8 T cell responses in control of virus replication. The emergence of HIV-1-specific CD8 T cells during primary infection coincides with the initial reduction of pVL from its peak by two to four logs (47). This temporal association was an early demonstration of the importance of CD8 T cell responses in controlling HIV-1 replication (12). When SIV-infected macaques were treated with an anti-CD8 antibody, control of virus replication was lost and VL increased 20- to 2500-fold within six days of antibody treatment (41, 78). It was only when CD8 T cells were repopulated that control of virus replication was recovered. The caveat to these studies was the fact that NK cells may
have also been depleted upon antibody treatment; nonetheless, cytotoxic effector cells were playing a role in limiting virus replication.

Additional evidence for the importance of CD8 T cells during HIV-1 infection lies in the appearance of escape mutations (33, 52). The virus mutates to evade immune pressure induced by HIV/SIV-specific CD8 T cells, which can lead to altered antigen processing or loss of HLA class I binding, thus enabling the virus to evade host immunity. Escape from the immunodominant B*27-KK10 (Gag p24, KRWILGLNK) CD8 T cell response is an example of significant loss of viral control as a result of mutation within a CD8 T cell epitope (35). Substitution of the arginine (R) residue at position 2 was associated with rapid disease progression. Interestingly, while the conversion at position 2 of this epitope enables evasion of the host immune response, it comes at a fitness cost to the virus. Additional studies have demonstrated that the virus induces compensatory mutations in an attempt to restore fitness (79).

**CD8 T cell Effector Function**

Conditions of T cell priming impact the development of downstream CD8 T cell effector function. CD8 T cell stimulation is initiated upon TCR ligation with antigenic peptide bound to an MHC class I molecule (69, 70). However, TCR engagement alone is inadequate; activation of naïve T cells is dependent upon receipt of a second stimulation signal—CD28 on the T cell binds B7 molecules on the antigen presenting cell. If either of these signals is inefficient, the engaged CTL will fail to be activated.

Parameters of CD8 T cell effector function upon antigen stimulation include proliferation, production of cytokines (interferon-γ, interleukin-2, tumor necrosis factor-
α), production of chemokines (macrophage inflammatory protein-1 α, RANTES), and the release of effector molecules involved in target cell lysis (CD107a, perforin, granzymes). Production of IL-2 and maintenance of proliferative capacity by CD8 T cells have been associated with HIV-1 control (39, 84). More recently, simultaneous production of these soluble factors by a single CD8 T cell, defined as a polyfunctional response, has been associated with improved disease outcome (11). Despite these findings, the mechanism and precise component(s) of the CD8 T cell response critical for long-lasting protection from HIV-1 disease are not clear, highlighting the need for improved methods to analyze CD8 T cell effector function.

**HIV-1 Vaccine Trials**

Although a number of HIV-1 vaccine candidates have been tested in phase I trials, very few have been promising enough to advance onto phase III studies. The first phase III trial, in which a gp120 subunit vaccine was tested, failed to prevent HIV-1 infection or delay disease progression (26). The task of designing a vaccine to induce humoral immunity to the HIV-1 envelope has proven difficult, leading to efforts to develop a vaccine primarily based on cell-mediated immunity. The phase IIb Step trial was the first to evaluate a T cell-based HIV-1 vaccine. While the trivalent MRKAd5 construct used in the Step study was highly immunogenic as analyzed by IFN-γ ELISpot assay, these responses did not equate with protection from infection or decreased viral load (14, 60). Most recently, the RV144 trial held in Thailand demonstrated limited efficacy—the vaccine was found to reduce the risk of HIV-1 acquisition by 31% within a low-risk population (76). Continued efforts at the basic and clinical levels of HIV-1 research are
vital to the development of innovative vaccine concepts, including novel targets, improved vaccine vectors, and new methods of inducing immune responses able to mediate durable protection in the face of HIV-1 disease.

**Rationale**

The challenges in designing an efficacious HIV-1 vaccine are many. Most importantly, the immune correlates of protection have not been precisely defined. Initial studies sought to induce antibodies to the HIV-1 envelope proteins. Unfortunately, no vaccine product tested thus far has been successful in inducing broadly neutralizing antibodies to HIV, a sharp contrast to pathogens for which FDA-approved vaccines exist. While antibodies are likely the determinant factor for prevention of infection, it is clear from the studies described above that CD8 T cells are essential for HIV-1 control after infection is established. If prevention of HIV-1 infection cannot be attained with a vaccine, it is possible that an effective therapeutic vaccine will induce immune responses able to reduce viremia and/or delay AIDS onset. For this latter reason, significant efforts have been placed into the development of a T cell based vaccine.

The goal of this dissertation was to develop and analyze a novel CD8 T cell assay that would better recapitulate *in vivo* control of HIV-1 replication mediated by these cells. The studies presented here contribute to the knowledge of HIV-1-specific CD8 T cell function and provide insights on how to best evaluate these responses.
EFFECTOR FUNCTION OF HIV-1-SPECIFIC CD8 T CELLS

A Review of Methods

CD8 T lymphocytes are primary immune effectors involved in the elimination of tumor transformed and pathogen infected cells (44). The ability to recognize antigen processed via the proteasome degradation pathway and induce cytolysis of a target cell make this T cell subset especially important in the face of viral infection (44).

The signaling cascade which initiates the CD8 T cell effector response begins with interaction of the T cell receptor (TCR) and a major histocompatibility complex (MHC) class I molecule to which an 8-11 amino acid long peptide is bound (42). Recognition of foreign antigen, and subsequent activation of a naïve CD8 T cell, initiates a program of differentiation and clonal expansion. Upon successful clearance of pathogen, the immune response is brought to a close by extensive cell contraction, with the establishment of a pool of memory CD8 T cells (4, 20, 40). The effectiveness of this program is determined by a number of factors, including but not limited to the nature of the invading pathogen, the efficiency of signals transmitted during CD8 T cell priming, and CD4 T cell help (39, 43).

Analysis of antigen-specific responses in HIV-1 infected controllers and SIV infected rhesus macaques has established the importance of CD8 T cell responses in control of disease (6, 15, 16, 22, 35). However, the precise components of a protective HIV-1-specific CD8 T cell response are not well understood. Identification of the nature of CD8 T cell response needed to restrict HIV-1 replication has been the subject of
intense research for many years, with the hope of applying this knowledge to the development of a vaccine capable of inducing protective HIV-1-specific CD8 T cells.

Appropriate CD8 T cell activation induces a number of downstream responses—clonal expansion, cytokine and chemokine production, cytotoxic effector molecule mobilization, and the initiation of gene regulation programs to establish pools of antigen-specific memory cells. Here we review assays most commonly used to evaluate CD8 T cell effector function.

\[ 51\text{Cr} \text{Chromium release assay} \]

Analysis of CD8 T cell targeting and lysis of infected cells provides a direct measure of the ability to eliminate an invading pathogen. The \( ^{51}\text{Cr} \) chromium release assay has been the “gold standard” method used to evaluate cytotoxic T lymphocyte (CTL) killing activity (8). Target cells are labeled with radioactive \( ^{51}\text{Cr} \) and treated with the appropriate antigen by methods such as peptide pulsing or infection with recombinant vectors that express the antigen of interest. The effector and target cells are co-cultured at multiple effector:target (E:T) ratios for a minimum of four hours. Culture supernatants are then analyzed for detection of \( ^{51}\text{Cr} \), which is released upon lysis of target cells and CTL killing is quantified as percent lysis. The quantity of \( ^{51}\text{Cr} \) detected in the supernatant reflects CTL killing efficiency. While, there may be some release of chromium that is not mediated by CTLs, use of the appropriate controls allows these background levels to be subtracted.

Calculating percent lysis:
Early analysis of HIV-1-specific CD8 T cell responses using the $^{51}$chromium release assay was done with bulk PBMCs derived from infected individuals (31, 38). These studies demonstrated a number of important truths about the HIV-1-specific immune response—HIV-1-specific CTLs could be detected in PBMCs taken from seropositive individuals, these cells had lytic function, and they showed specificity for multiple viral proteins. However, these studies were not quantitative and thus were somewhat limited. Methods were adapted to analyze CTLs in a semi-quantitative manner, including in vitro stimulation of PBMCs with anti-CD3 to establish HIV-1-specific CTL lines (9) and limiting dilution analysis to generate CTL clones. Another caveat to early studies of HIV-1-specific cell lysis was the type of cells used as targets. Epstein-Barr virus (EBV) transformed B lymphoblastoid cell lines (BLCLs) were often used as target cells, which is less relevant to in vivo conditions. As BLCLs cannot be directly infected with HIV-1, use of these relied largely on exogenous addition of HIV-1 peptides, which fails to account for the impact of antigen processing on CD8 T cell function, or recombinant viral vectors expressing HIV-1 genes (31, 38). Yang and colleagues slightly modified the assay to be more reflective of natural HIV-1 disease by using virus-infected CD4 T cells as targets, albeit these were immortalized cell lines (41). An important drawback to the chromium release assay is the use, and necessary but tedious, disposal of radioactive material. Over the years, a number of non-radioactive reagents have been used to monitor the fate of target cells when evaluating CD8 T cell lytic function, including those amenable to flow cytometric analysis (25, 37).

Despite the detection of strong CTL responses in HIV-1-infected individuals, the mere existence of HIV-1-specific CD8 T cells with lytic capacity was not sufficient for
protection from disease progression. It was clear that even in patients with robust, CD8 T cell-mediated lytic capacity, the majority of individuals still went on to develop AIDS. These findings made it evident that novel and comprehensive methods to analyze antigen-specific immune function would be necessary for improved understanding of the HIV-1-specific CD8 T cell response.

**Interferon-gamma (IFN-γ) ELISpot assay**

An important effector response of antigen-specific CD8 T cells is the production of soluble immunomodulatory factors including cytokines (interferon-γ, interleukin-2), chemokines (RANTES), and proteins involved in cell lysis (perforin, granzymes). Early analyses of HIV-1-specific CD8 T cell cytokine production was performed by enzyme-linked immunosorbent assay (ELISA). Adaptation of the ELISA protocol led to development of the enzyme-linked immunospot (ELISpot) assay, a highly-sensitive method to analyze single cell production of cytokines such as IFN-γ (23). This new tool expanded the study of CD8 T cells to quantitative analysis of antigen-specific function on a single cell level (2, 24). Recombinant virus expressing HIV-1 antigens or pools of overlapping HIV-1 peptides have been used to stimulate total PBMCs, allowing analysis of host immune responses even when HLA genotype is not known (13, 14).

To date, IFN-γ ELISpot remains a preferred and inexpensive method to rapidly map total HIV-1-specific T cell responses. However, its usefulness as a predictor of disease prognosis is limited. A number of cross-sectional studies analyzing virus-specific responses in patients with varying levels of disease control have failed to show that magnitude or total breadth of response, as detected by IFN-γ ELISpot, correlates with
pVL or CD4 T cell count (11). Interestingly, analysis by IFN-γ ELISpot has consistently demonstrated individuals with a lower pVL have an increased breadth of Gag-specific responses as detected by ELISpot (13, 18, 21).

**MHC class I tetramers**

Quantitative analysis of epitope-specific CD8 T cells was further enhanced with the development of MHC class I tetramers (3). These multimeric reagents are comprised of four individual biotinylated peptide-MHC class I protein complexes bound to a single avidin molecule. Each soluble tetramer can only bind CD8 T cells that express its cognate TCR. Conjugation of the avidin molecule to a fluorochrome enables analysis of epitope-specific cell populations by flow cytometry, enabling analysis of specific CD8 T cell subsets.

Tetramer staining of HIV-1-specific CD8 T cells showed that the frequency of tetramer positive cells was inversely correlated with plasma viral load (32), suggesting that the frequency of CD8 T cells detected in peripheral blood is dependent on antigen burden. To analyze effector function of tetramer positive cells, use of these reagents must be combined with other methods such as intracellular cytokine staining (ICS). Alternatively, tetramer staining identifies epitope-specific populations which can then be FACS sorted and used in functional assays.

Immune studies of virus-specific CD8 T cells in mice demonstrated not all tetramer positive cells maintain antigen-specific function. Goepfert and colleagues analyzed the HLA A*02-SL9 epitope-specific CD8 T cell response (SLYNTVATL, gag p17) in chronically infected individuals using tetramer, IFN-γ ELISpot, and ⁵¹chromium
release assay (14). SL9-specific cells were detected using each assay. Interestingly, it was observed that not all tetramer-positive cells produced IFN-\(\gamma\), whether measured by ELISpot or ICS. The use of tetramer to identify HIV-1-specific CD8 T cells should be coupled with other assays, as tetramer alone could overestimate the frequency of cells that contribute to the antiviral response.

**Measuring soluble antiviral factors by flow cytometry**

Significant advances in flow cytometry instrumentation and increased availability of fluorimetric reagents have revolutionized our ability to analyze antigen-specific immune responses. In particular, the intracellular cytokine staining (ICS) assay provides an opportunity to characterize the phenotype and effector response of antigen-specific cells. Effector cells in the ICS assay, which are typically PBMCs, but may also include isolated T cell subsets or expanded cell lines, are stimulated for six hours with selected antigens. Peptides that contain 9-15 amino acid residues in length provide appropriate stimulation of CD8 T cells. Critical for the detection of intracellular products is the addition of brefeldin A and monensin. Cytokine secretion inhibitors, brefeldin A and monensin act to inhibit intracellular transport at the endoplasmic reticulum and the Golgi complex (30, 36). Following the six hour stimulation period, cells are washed and stained with fluorochrome-conjugated antibodies against cell surface markers and fixed. After permeabilization, antibodies specific for intracellular markers of interest (such as IFN-\(\gamma\), IL-2, perforin) are used to stain the cells. Sample acquisition on a flow cytometer allows for analysis of effector responses at a single cell level.
CD8 T cell functional analysis using ICS has been reported by numerous groups, establishing a consensus regarding certain aspects of the CD8 T cell response during HIV-1 disease. First, virus-specific, IL-2 producing CD8 T cells are commonly detected in patients with non-progressive HIV-1 disease, but nonexistent in those with progressive disease (45). This IL-2 effector response has been associated with the maintenance of CD8 T cell proliferative capacity in HIV-1 controllers (19). Furthermore, it has been demonstrated that controllers have an increased frequency of antigen-specific, polyfunctional CD8 T cells, those positive for multiple effector functions including IFN-γ, IL-2, TNF-α, CD107, and MIP1β (6). Recently molecules more closely associated with cell lysis, such as perforin and granzyme B, have also been associated with HIV-1 viral control (17, 28, 29).

**Proliferation of HIV-1-specific CD8 T cells**

A responding antigen-specific CD8 T cell will undergo substantial proliferation upon stimulation with cognate antigen. It has been reported that up to 44% of total CD8 T cells targeted a single viral epitope during primary EBV infection (10). After the establishment of a memory pool, proliferative capacity is an essential function in the case of secondary infection or re-challenge (20).

Proliferation studies are performed by labeling cells of interest with a reagent that will undergo a defined change in concentration as cells undergo successive cycles of division. These reagents include tritiated thymidine [³H], bromodeoxyuridine (BrdU), Ki-67, and carboxyfluorescein diacetate succinimidyl ester (CSFE). The *in vitro* CFSE proliferation assay has become a preferred method of tracking cell division, as it is easily
monitored by flow cytometry (27), affording identification of the cell phenotype through cell surface marker analysis and even the sorting of very specific generations of antigen-specific cells. Proliferation of HIV-1-specific CD8 T cells has been consistently associated with disease control (28, 45). Using the CFSE proliferation assay to analyze virus-specific responses, Migueles and colleagues observed a significant correlation between frequencies of HIV-1-specific CFSE<sub>lo</sub> (cells having gone through cell division) CD8 T cells and plasma viral RNA levels. Additional studies of CD8 T cell proliferative capacity revealed differences based on HLA class I expression. HLA-B27 and -B57 restricted cells remained replication competent during chronic HIV-1 infection in contrast to responses restricted by other class I alleles (19); the percentage of CFSE<sub>lo</sub> cells was significantly higher within populations restricted by these protective alleles. The ability of CD8 T cells to proliferate in response to antigen is an important feature of the antiviral effector response that should be considered when designing HIV-1 vaccines.

**T cell receptor (TCR) clonotyping**

The inability to correlate the frequency of HIV-1-specific CD8 T cells with control of virus replication suggests that the quality of response rather than the quantity of cells is a determinant of CD8 T cell efficacy. Based on this realization, an effort to examine differences in effector function mediated by HIV-1-specific CD8 T cells became a significant focus of HIV research efforts.

The CD8 T cell response begins with T cell receptor (TCR) engagement. We know that factors defining the TCR–peptide-MHC interaction, including antigen abundance, duration, and affinity, will impact downstream effector functions. It is
possible that the nature of the TCR itself, determined during T cell development in the thymus, may impact the quality of CD8 T cell function, which has prompted analysis of antigen-specific CD8 T cell clonotypes. The complementarity determining region 3 (CDR3) of the TCR β-chain interacts with the peptide that is bound to an HLA class I molecule. Sequencing this region of the TCR β-chain provides an identifiable signature for a single CD8 T cell clone.

Analysis of two immunodominant SIV CD8 T cell responses by tetramer sorting and TCR β-chain sequencing revealed that polyclonal populations comprised the response to both epitopes (33). More than 3000 TCR sequences were generated from samples taken at two time points from twelve SIV-infected macaques, and remarkably, there was strong conservation of TCR β-chain usage within the two epitope-specific populations. The authors observed that CD8 T cells targeting an epitope in the Tat protein bore a TCR which contained a conserved CDR3 motif, which they believe accounted for the inability of this response to overcome an escape mutation that developed within 5 weeks of infection. Alternatively, the cells targeting a Gag epitope showed no CDR3 sequence bias, which may allow these cells to better tolerate development of escape mutations. Despite restriction by the same MHC class I molecule, CD8 T cells targeting two SIV epitopes demonstrated diverse clonotypic profiles, which was detectable within five weeks of infection.

Douek and colleagues sought to determine the diversity of HIV-1-specific CD8 T cell clonotypes during chronic infection (12). PBMCs were stimulated with HIV-1 peptides corresponding to immunodominant epitopes, and using a cytokine capture assay, IFN-γ producing CD8 T cells were sorted for TCR β-chain sequencing. HIV-1-specific
CD8 T cells with an IFN-γ effector response did not demonstrate any particular CDR3 sequence. A heterogeneous population of clonotypes was identified for the six epitopes analyzed; in half of the samples, a single clonotype dominated the effector response. Longitudinal analysis of clonal populations specific for the A*02-SL9 Gag epitope [SLYNTVATL], an immunodominant epitope during HIV-1 chronic infection, revealed changes in the frequency of clonotypes as plasma VL changed. Furthermore, there was significant conservation in the CD8 T cell clonotypes detected as A*02-SL9 variants emerged, suggesting SL9-specific clones can tolerate epitope sequence variation. These studies demonstrated that the ability of a CD8 T cell subpopulation to recognize epitope variants is impacted by the clonal profile of responding cells.

While quantification of HIV-1-specific CD8 T cells by tetramer staining and qualitative analysis of effector function by ELISpot, ICS, and TCR sequencing have provided important details regarding the effector response in individuals with and without control of HIV replication, these assays do not capture direct cytotoxic activity. The application of assays that detect CD8 T cell-mediated killing of target cells and suppression of virus replication will enable better characterization of responses necessary to limit HIV-1 replication. Furthermore, combining these different methods of CD8 T cell analysis will aid in defining the type of response that an effective HIV-1 vaccine should elicit.

**Analysis of CD8 T Cell Effector Function During Chronic HIV-1 Infection**

Long-term nonprogressors (LTNP) and elite controllers (EC) maintain control of virus replication in the absence of ART. The mechanism(s) underlying the level of
protection seen in these individuals is a subject of critical importance as the correlates of protection from HIV-1 disease have yet to be identified.

It is clear that the mere presence of HIV-1-specific CD8 T cells is insufficient to control HIV-1 replication. CD8 T cells that mediate control in the face of HIV-1 disease may have a unique quality of response that provides protection which exists amongst controllers but not progressors. When qualitative responses are analyzed, preservation of IL-2 effector function is one distinct CD8 T cell response associated with control of HIV-1 disease. To determine if IL-2 producing CD8 T cells represent unique clonal populations with distinct TCR sequences when compared to IFN-γ producing cells, we analyzed samples from ten chronically infected patients by IFN-γ ELISpot, ICS, and TCR β-chain sequencing.

Clinical characteristics for all study participants are listed in Table 1. The median pVL is 9,219 RNA copies/mL, with four individuals having a pVL less than 2,000 copies/mL. None of the patients were severely immunocompromised as CD4 T cell counts were greater than 200 cells/mm³ of blood.

HIV-1-specific responses in each subject were mapped using the IFN-γ ELISpot assay. PMBCs were stimulated with optimized, HIV-1 CD8 epitopes, which were selected based on HLA class I genotype of each participant.
Table 1

Clinical characteristics of study participants.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>plasma VL (copies/mL)</th>
<th>CD4 T cell count (cells/mm³)</th>
</tr>
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<tbody>
<tr>
<td>001*</td>
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<td>323</td>
</tr>
<tr>
<td>002*</td>
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<tr>
<td><strong>Median</strong></td>
<td><strong>9,219</strong></td>
<td><strong>503</strong></td>
</tr>
</tbody>
</table>

With the exception of patient 005, all subjects were off antiretroviral therapy. Asterisk (*) indicates samples that were analyzed by TCR β-chain sequencing.

Representative data from an IFN-γ ELISpot mapping experiment is depicted in Figure 1A and total responses for the study group is shown in Figure 1B. The immunodominant response, as detected by IFN-γ ELISpot, was selected for subsequent ICS analysis; in subjects 001, 002, and 004 the two most dominant responses were analyzed.
(A) Total PBMCs from subject 004 were stimulated with HIV-1 peptides for 20 hours. Numbers indicate automated spot count of IFN-γ producing cells. Red boxes indicate control samples—negative control was not stimulated and PHA stimulation was used as a positive control. Blue boxes indicate immunodominant responses: 1) response to B*57-KF11 [KAFSPEVIPMF] and 2) response to B*57-IW9 [ISPRTLNAW]. (B) Net virus-specific responses for the entire cohort. Horizontal bars indicate median response.
For ICS analysis, cryopreserved PBMCs were thawed and rested overnight in complete media at 37°C with DNase I (10 U/mL). The following morning, cell volume was adjusted to achieve a concentration of 5 x 10^6 cells/mL. Costimulatory monoclonal antibodies (anti-CD28 and anti-49d, 1 µg/mL), monensin (0.7 µL/mL), brefeldin A (10 µg/mL) and anti-CD107a-Alexa 680 were added to the cells. Cells were stimulated with appropriate HIV-1 peptides for 6 hours.

Following incubation, cells were washed twice and stained with violet fluorescent reactive dye to distinguish live and dead cells. Samples were then stained with surface antibodies against CD3, CD4, CD8, CD27, CD45RA, CD57, and CCR7. Antibodies against CD14 and CD19 were used to enable monocyte and B cell exclusion from flow cytometry analysis. Cells were fixed and permeabilized using a commercially available kit (Cytofix/Cytoperm). After fixation, cells were stained with antibodies against intracellular markers interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), and perforin. Cells were washed, resuspended in 1% paraformaldehyde, and stored at 4°C overnight.

To analyze clonal populations of HIV-1-specific CD8 T cells with different effector responses, a three-way sort was performed— IFNγ^+IL2^−, IFNγ^+IL2^+, and IFNγ^−IL2^+. Figure 2 illustrates the sorted populations in three subjects, 001, 002, and 006. Monofunctional, HIV-1-specific CD8 T cells producing IFN-γ were detected in 9 out of 10 subjects. While bifunctional IFNγ^+IL2^+, cells were only detected in 4 out of 10 subjects.
FIGURE 2. ICS analysis of HIV-1-specific CD8 T cell responses. PBMCs from subjects (A) 001, (B) 002, and (C) 006 were analyzed for IFN-γ and IL-2 production after stimulation with the indicated peptides. Samples have been gated on CD3⁺CD8⁺ cells. Media indicates negative control. SEB stimulation was used as positive control. Representative flow plots depicting sorted populations (IFNγ⁺, IFNγ⁺IL2⁺, IL2⁺). Numbers represent the percentage of CD8 T cells.

DNA from sorted populations was purified and used to determine the TCR-β chain CDR3 sequence. A minimum of 500 sorted cells was required to obtain adequate samples for PCR analysis. A multiplex PCR protocol was used to amplify TCR sequences from genomic DNA, requiring 23 primers specific to TCR β-chain V regions and 13 primers external to the TCR β-chain J regions. Product from the first round of
PCR was purified and used as template in the second round of PCR, which was completed using 13 primers internal to the TCR β-chain J regions. The final product, expected to be 250-300 base pairs in length, was gel purified and ligated into the pGEM-T Easy Vector. The cloning plasmid was used to transform *Escherichia coli* DH5α competent cells. Colonies were selected based on loss of β-galactosidase expression. The target gene was amplified and sequenced to obtain TCRβ chain CDR3 sequences.

Using this DNA-based PCR protocol, we were able to detect differences among HIV-1-specific CD8 T cell clonal populations based on cell function in 3 subjects (Tables 2-5). In two subjects for which both monofunctional IFN-γ⁺ and bifunctional IFN-γ⁺IL2⁺ cells were detected, CAAN and ROCH, the IL-2 producing clonal populations were a distinct subset of the larger monofunctional population. It is interesting that despite expressing identical TCRs, the induced effector responses diverge.

Recently, Beuneu and colleagues observed varying expression of IFN-γ in antigen-specific CD8 T cells primed with dendritic cells bearing different concentrations of antigen (7). In this transgenic mouse model where all CD8 T cells express the same TCR, it was suggested that the conditions of T cell priming on initial contact with peptide-loaded dendritic cells led to differences in the activation program observed in the responding CD8 T cells. It is possible that differential conditions of CD8 T cell priming would generate HIV-1-specific cells, although bearing identical TCRs, with varying effector responses.
Table 2

*B*57-KF11-specific clones derived from subjects 001 and 002.

<table>
<thead>
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<th>TCRBJ</th>
<th>Well Count</th>
</tr>
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<tbody>
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<td></td>
<td></td>
</tr>
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Table 3

*B*57-QW9-specific, IFN-γ^+^IL-2^+^ clones derived from subject 001.

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Table 4

*B*27-KK10-specific, IFN-γ^+^ clone derived from subject 006.

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A novel in vitro assay to measure CD8 T cell-mediated HIV-1 suppression

Virus-specific CD8 T cells are key mediators in controlling HIV-1 replication, exerting immune pressure that the virus attempts to evade, often at the cost of replication fitness. However, the precise mechanism(s) of CD8 T cell-mediated protection from HIV-1 disease progression remains unclear. The assays most commonly used to assess CD8 T cell function measure antigen-specific production of immunomodulatory soluble factors such as IFN-γ, IL-2, MIP-1-β, etc. While these methods reveal noticeable differences in the quality of response, they do not directly capture the ability of HIV-1-specific CD8 T cells to eliminate an infected cell or inhibit virus replication. With the outcome of the Step Study, it has become increasingly apparent that improved methods of analyzing CD8 T cell responses are necessary. Using a modified in vitro suppression assay (iVSA), we analyzed the capacity of CD8 T cells to suppress HIV-1 replication. The seven-day iVSA was optimized for the use of primary, HIV-1 infected CD4 T cells as target cells (T) and epitope-specific CD8 T cell lines as the effectors (E).

Isolation of effector CD8 T cells

Analysis of epitope-specific CD8 T cells directly ex vivo can be difficult, as frequencies of pathogen- or tumor antigen-specific cell subpopulations often fall below the limit of detection. The low yield of CD8 T cells derived ex vivo may limit the ability to analyze pathogen-specific functional responses. This is especially true of HIV-1 infected controllers that display superb control of virus replication (5). To address this issue, we utilized a protocol which involved the use of APCs to stimulate and expand T
cells in vitro. To derive HIV-1-specific CD8 T cell lines, we adapted a method using autologous monocytes to stimulate negatively isolated CD8 T cells over fourteen days.

Cells used for antigen presentation are derived by plate adherence of primary monocytes. This method of monocyte separation from total PBMCs has been well documented and surface marker analysis by flow cytometry demonstrates that the adherent cells have a monocyte phenotype (34). The adherent monocytes are subjected to gamma-irradiation and subsequently pulsed with the appropriate peptide for two hours.

The MACS CD8 T cell isolation kit was used to isolate CD8 T cells by negative selection. This method enabled purification of CD8 T cells without manipulation through binding of antibodies or beads that may non-specifically stimulate cells. Isolated CD8 T cells were plated onto the irradiated, peptide-pulsed monocytes. On day seven, CD8 T cells are restimulated. The expanded cell lines were evaluated for HIV-1-specific CD8 T cell function between days thirteen and fifteen. Using this protocol, we are able to derive epitope-specific CD8 T cells in adequate number for additional analysis of effector responses by ICS and iVSA (Figure 3).
FIGURE 3. HIV-1-specific CD8 T cell function before and after expansion. Representative flow plots of B*57-IW9-specific CD8 T cells (A) before and (B) after in vitro expansion. Cells were analyzed for degranulation (CD107a), cytokine production (IFN-γ, IL-2, TNF-α), and mobilization of perforin.

Generation of target cells used in the iVSA

Primary CD4 T cells were selected for use as target cells in the iVSA, providing conditions that are relevant to the in vivo tropism of HIV-1. PBMCs were first treated with CD8 Dynabeads to deplete CD8+ cells. The isolated CD4 T cells are then activated by 72-hour stimulation using IL-2 (50 U/mL) and phytohemaglutinin (PHA, 2μg/mL).

Target cell infection was optimized such that in vitro infection would enable quantification of HIV-1 replication over seven days and minimize virus-induced cytotoxicity. We used lab-adapted HIV-1 strains YU2, which uses the CCR5 co-receptor.
and NL4-3, a highly infectious molecular clone which utilizes the CXCR4 co-receptor (1). Activated, primary CD4 T cells were infected with HIV-1_{NL4-3} or HIV-1_{YU2} at various multiplicities of infection (MOI). Twelve hours after infection, CD4^+ target cells were plated in 96-well plates at 5000 cells/well and cultured for seven days in complete media supplemented with IL-2. Half volume of the supernatant was exchanged on days 0, 1, 2, 3, 4, 5, and 7; supernatants were stored at -80°C until time of analysis.

To analyze virus replication in the stored supernatants, we used the TZM-bl (JC53) dual-reporter cell line which expresses β-galactosidase and luciferase under the control of the HIV-1 LTR promoter. We measured luciferase expression as an indicator of virus replication levels, as luciferase production in TZM-bl cells is highly sensitive and linearly related to the quantity of infectious HIV-1 present within a sample. Depicted in Figure 4, background luciferase production in uninfected primary CD4 T cells was on the order of 1000 relative luciferase units (RLU). Both strains allowed for productive infection of primary cells, increasing luciferase production as much as 2.5 logs at peak virus replication. However, HIV-1_{YU2} replication (blue and purple lines) was not stable over seven days, the optimal duration of the iTSA. Cells infected with HIV-1_{NL4-3} at an MOI of 0.1 or 0.01 (Figure 4, red and orange lines) demonstrated a two-log increase in virus replication within 36 hours of infection. Such high viral concentrations could negatively impact cell viability and lead to inappropriate calculations of CD8 T cell mediated suppression. Target cells used in all subsequent iTSA experiments were infected with HIV-1_{NL4-3} at an MOI of 0.001, enabling productive infection of cells with minimal virus-induced cytotoxicity.
FIGURE 4. Optimization of target cell infection. Activated CD4 T cells were infected with HIV-1 strain NL4.3 (red and orange lines) or strain YU2 (green, blue, and purple lines) at various MOIs. Cells were placed in culture for seven days and supernatant was pulled on days 0, 1, 2, 3, 4, 5, and 7. Samples were analyzed for HIV-1 replication using the TZM-bl reporter cell line. Luciferase expression is quantified in relative luciferase units (RLU). Black line indicates background luciferase expression.

**in vitro suppression assay (iVSA)**

Epitope-specific CD8 T cell lines and HIV-1 infected CD4 T cells, generated as described above, were used in the iVSA to analyze the ability of CD8 T cells to suppress HIV-1 replication. To ensure that antiviral CD8 T cell function would be quantified adequately, despite potential differences in effector cell potency, we performed the iVSA over a broad range of E:T ratios—0:1, 0.2:1, 0.5:1, 1:1, 5:1, 10:1. Each epitope-specific cell line was tested for suppression against autologous and non-autologous target cells.

On day -1, one-half of the activated CD4 T cell culture was left uninfected, while the other half was infected with HIV-1NL4-3 and left overnight at 37°C. On day 0, target cells were plated in round-bottom 96-well plates at 5000 cells/well and the appropriate
number of effector cells for each experimental condition was added. All conditions were plated in duplicate. The samples remained in culture for seven days and supernatant was collected as stated above. HIV-1 replication was analyzed using the TZM-bl reporter cell line. Luciferase expression was read on an automated luminometer and percent suppression of virus replication was calculated as \[\frac{1 - (\text{RLU of sample with E})}{(\text{RLU of sample without E})} \times 100\].

The in vitro suppression assay is a novel, quantitative method of analyzing suppression of HIV-1 replication as mediated by antigen-specific CD8 T cells. An alternative to the $^{51}$chromium release assay, direct HIV-1-specific CD8 T cell antiviral function can be quantified without the use of radioactive reagents. The assay is extremely sensitive, able to detect inhibition of virus replication by effector cells at an E:T ratio as low as 0.2:1. The experimental set-up does not require large numbers of cells; analysis of one antigen-specific CD8 T cell line against autologous and non-autologous targets at six E:T ratios in duplicate, requires $0.5 \times 10^6$ effector cells and $0.2 \times 10^6$ effector cells. With the use of epitope-specific CD8 T cells, one can analyze antiviral efficacy of CD8 T cells derived from the same individual but targeting different regions of HIV-1.

Importantly, the iVSA provides an improved method of analyzing HIV-1 vaccine induced CD8 T cell responses. Results from the Step Study demonstrate that the assessment of vaccine efficacy based solely upon methods such as IFN-γ ELISpot assay is no longer sufficient. The ultimate goal of a CD8 T cell response in face of infection is clearance of the invading pathogen, thus quantitative methods to evaluate inhibition of virus replication would be useful tools in the development and evaluation of an HIV-1 vaccine.
REFERENCES


INTERLEUKIN-2 PRODUCTION BY POLYFUNCTIONAL HIV-1-SPECIFIC CD8 T-CELLS IS ASSOCIATED WITH ENHANCED VIRAL SUPPRESSION

by

OLUSIMIDELE T. AKINSIKU, ANJU BANSAL, STEFFANIE SABBAJ, SONYA L. HEATH, PAUL A. GOEPFERT

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ABSTRACT

**Background:** Assays to measure the induction of HIV-1-specific CD8 T-cell responses often rely on measurements of indirect effector function such as chemokine and cytokine production, which may not reflect direct elimination of an invading pathogen. Assessment of the functional ability of CD8 T-cells to suppress HIV-1 replication has been viewed as a surrogate marker of an effectual immune response. To further investigate this, we measured the capacity of virus-specific CD8 T-cells to inhibit HIV-1 replication in an *in vitro* suppression assay (iVSA).

**Methods:** We expanded 15 epitope-specific CD8 T-cell lines from PBMCs of chronically HIV-1 infected progressors (*n*=5) and controllers (*n*=4) who were not on antiretroviral therapy. Cell lines were tested for their ability to produce effector molecules (CD107a, IL-2, IFN-γ, TNF-α, perforin) and suppress virus replication in autologous CD4 T-cells.

**Results:** CD8 T-cell lines from both progressors and controllers had largely similar effector function profiles as determined by intracellular cytokine staining. In contrast, we observed that CD8 T-cell lines derived from controllers show enhanced virus suppression when compared to progressors. Virus suppression was mediated in an MHC-dependent manner and found to correlate with a polyfunctional, IL-2⁺ CD8 T-cell response.

**Conclusions:** Using a sensitive iVSA, we demonstrate that CD8 T-cell mediated suppression of HIV-1 replication is a marker of HIV-1 control. Suppressive capacity was found to correlate with polyfunctional, IL-2 production. Assessment of CD8 T-cell mediated suppression may be an important tool to evaluate vaccine-induced responses.

**Key Words:** HIV-1; CD8 T-cells; virus suppression; IL-2; polyfunctional; vaccines
INTRODUCTION

Immune correlates of protection for human immunodeficiency virus-1 (HIV-1) infection are poorly defined, presenting a significant challenge to the development of an effective vaccine (49). The difficulty in defining these correlates relates to the fact that, without antiretroviral therapy (ART), more than 99% of HIV-1 infected individuals develop AIDS, as they lack an antiviral response that affords long-term protection from disease progression (30, 43). Failure of the HIV-1-specific immune response is attributed to on-going depletion of CD4 T-cell populations (10, 20), an established HIV-1 reservoir in latently infected cells (13, 23), and virus escape from the host response (9, 26, 51). However, evidence of durable control exists among individuals identified as long-term nonprogressors (LTNP) or elite controllers (EC), who maintain low plasma viral loads (pVL) for many years without the use of ART (14, 19, 47). Hence, understanding the mechanisms that underlie delayed disease progression amongst controllers will aid in the identification of correlates of protection and design of a therapeutic HIV-1 vaccine.

The critical role CD8 T-cells play in controlling virus replication has been documented in several studies of HIV and SIV infection (8, 25, 31, 38, 40, 56). Furthermore, the strong association between certain MHC class I molecules and delayed disease progression suggests CD8 T-cells contribute to viral control. (35) CD8 T-cells that are polyfunctional (7) and target multiple Gag epitopes (18, 21, 22, 36) correlate with improved disease outcome, suggesting that the quality of response is an important determinant of their ability to restrict HIV-1 replication.

The Step Study, the first trial to evaluate a T-cell based HIV-1 vaccine, was closed when interim analysis revealed a possible enhanced risk of infection in vaccine
recipients (12). While the trivalent MRKAd5 construct used in this trial was highly immunogenic as analyzed by IFN-γ ELISPOT assays, this effector response did not equate with protection from infection or decreased viral load upon seroconversion (45, 52). These results may be explained by a number of factors including poor induction of CD8 T-cells and a limited quality of response. The outcome of the Step Study underscores the need for improved methods to evaluate vaccine-induced T-cell responses (46).

Several groups have developed quantitative assays to measure inhibition of HIV-1 and SIV replication (15, 17, 24, 48, 58, 59, 64). Some studies have shown an association between enhanced antiviral efficacy and Gag specificity (15, 33). Recently, analysis of HLA-B*2705-restricted responses indicated that inhibition of HIV-1 replication was related to the kinetics of infected target cell recognition by epitope-specific CD8 T-cells (50). Despite these reports, the CD8 T-cell phenotype associated with enhanced control of virus replication remains unclear. Based on previous reports (7, 65), we hypothesized that IL-2 production would identify a population of HIV-1-specific CD8 T cells with enhanced suppressive capacity and this population would be increased amongst controllers. To address this question, we sought to quantitatively assess CD8 T-cell-mediated suppression of HIV-1 replication using a modified in vitro suppression assay (iVSA).
METHODS

Study participants

Samples were collected from HIV-1 infected controllers \((n=4)\) and progressors \((n=5)\) enrolled at the University of Alabama at Birmingham (UAB) 1917 Clinic. Progressors had a pVL greater than 2,000 copies/mL and controllers were defined as having a pVL less than 2,000 copies/mL (Table, Supplemental Digital Content 1). CD4 T-cell counts were determined as previously described. Plasma HIV-1 RNA levels were measured using Amplicor Ultra Sensitive HIV-1 Monitor assay (Version 1.5; Roche Diagnostic Systems, Indianapolis, IN). All patients were off ART for at least six months at the evaluated time points. Genomic DNA extracted from PBMCs was used for PCR-based genotyping of HLA class I alleles (60). Informed consent was obtained from all participants and the UAB Institutional Review Board approved the study.

IFN-\(\gamma\) ELISPOT Assay

Participants were screened for HIV-1-specific responses based on IFN-\(\gamma\) production. The ELISPOT assay was performed as previously described (6). Peptides were selected based on optimized CD8 epitopes predicted by each subject’s HLA class I genotype and described in the LANL HIV Molecular Immunology Database (www.hiv.lanl.gov/content/immunology/index.html). A positive response was defined as values twice background (unstimulated cells) and greater than 55 SFC/10^6 PBMCs.
Expansion of CD8 T-cell lines

To generate antigen-specific CD8 T-cell lines, immunodominant responses detected by ELISPOT were expanded *in vitro*. PBMCs were resuspended in serum-free media, and plated at 1.2x10^6 cells/well. Non-adherent cells were removed after two-hour incubation at 37°C, 5% CO₂. Adherent cells, previously shown to be monocytes (11, 55), were irradiated (33 gray), pulsed with HIV-1 peptide (10 μg/mL) for two hours, and washed to remove excess peptide. Autologous CD8 T-cells were negatively isolated using the MACS CD8⁺ T cell isolation kit (Miltenyi Biotec, Gladbach, Germany), resuspended in complete media supplemented with IL-7 (25 ng/mL), and plated onto the peptide-pulsed monocytes at 0.5x10^6 cells/well. Fresh IL-2 (50 U/mL) was added to cell cultures every 2-3 days. On day 7, CD8 T-cells were restimulated with peptide-pulsed monocytes. Expanded CD8 T-cell lines were tested for HIV-1-specific function on day 14 or 15.

Intracellular Cytokine Staining (ICS) Assay

ICS was performed as previously described (5). Approximately 0.5-1x10^6 cells were incubated with appropriate HIV-1 peptide (10 μg/mL) for six hours in the presence of anti-CD107a. Cells were labeled with LIVE/DEAD fluorescent reactive dye (Invitrogen, Carlsbad, CA) and stained with antibodies against CD3, CD4, and CD8. Cells were then fixed, permeabilized (FIX & PERM, Invitrogen, Carlsbad, CA), and stained with antibodies for intracellular markers IFN-γ, TNF-a, IL-2, and perforin. A minimum of 100,000 events were acquired on an LSRII (BD Immunocytometry Systems, San Jose, CA) and data analyzed using FlowJo software (v7.6.1, TreeStar, Ashland, OR). Responses greater than 0.02% and twice the background response (negative control) were
considered positive. Boolean gating was used to generate polyfunctional subsets.

Analysis and presentation of distributions was performed using SPICE version 5.1, downloaded from <http://exon.niaid.nih.gov/spice> (53).

**Antibodies:** CD3-Pacific blue, CD8-PerCP-Cy5.5, CD107a-FITC, IFN-γ-Alexa 700, IL-2-APC, TNF-α-PE-Cy7 or PerCP-Cy5.5 (all from BD Biosciences, San Diego, CA), CD4-Qdot 605, CD8-Qdot 655 (both from Invitrogen, Carlsbad, CA), anti-perforin-PE (Cell Sciences, Canton, MA)

**in vitro Suppression Assay (iVSA)**

HIV-1-specific CD8 T-cell lines were used as effector (E) cells in the iVSA. To generate target cells (T), PBMCs were depleted of CD8+ cells using CD8 Dynabeads (Invitrogen, Carlsbad, CA). Enriched CD4+ cells were activated with IL-2 (50 U/mL) and PHA (2 μg/mL) for 72 hours. Cell infection with HIV-1NL4-3 was optimized at a multiplicity of infection (MOI) of 0.001, minimizing virus-induced cytotoxicity. Effector cells were cocultured with autologous and non-autologous targets at multiple E:T ratios (range 0:1 to 5:1) in a 96-well plate for seven days. Supernatant was collected on days 0, 1, 3, 5, 7 and stored at -80°C until analysis. The TZM-bl reporter cell line was used for analysis of HIV-1 replication, in which luciferase expression is highly sensitive and linearly related to the quantity of infectious HIV-1 (62). Luciferase was quantified on a luminometer (Microplate Luminometer, Applied Biosystems, Foster City, CA). All cell lines tested in the iVSA were run in duplicate. CD8 T-cell mediated suppression was quantified as percent suppression on the day of maximum HIV-1 replication.

Percent suppression = \[1 - (\text{RLU of sample with E}) / (\text{RLU of sample without E})\] * 100
**Statistical Analysis**

Clinical markers and T-cell functional responses between progressors and controllers were compared using the nonparametric Mann-Whitney test. Spearman rank correlation coefficient was calculated to analyze the relationship between suppression (E:T, 1:1) and functional/clinical parameters. Statistical analyses were performed with Prism software (GraphPad Software, La Jolla, CA). Comparison of distributions generated with SPICE was performed using a partial permutation test as described (53).
RESULTS

Identification and expansion of immunodominant HIV-1-specific responses

To determine if there is a signature phenotype for CD8 T-cells capable of virus suppression, we analyzed effector function in controllers with superior HIV-1 control, off ART, compared to individuals with progressive disease. Immunodominant CD8 T-cell responses are major determinants of CTL escape and such responses could be important mediators of virus suppression (4, 32). We, therefore, identified immunodominant responses in each subject using the IFN-γ ELISPOT assay. IFN-γ production by virus-specific CD8 T-cells is often the last function detected before cells are fully exhausted, making it a reliable marker to identify HIV-1-specific responses during chronic infection (37, 57). A positive HIV-1 response to at least one 9-11mer was detected in all individuals (range, 1-13 positive responses), with the magnitude ranging from 55 to 1438 SFC/10^6 PBMC in progressors and 55 to 4083 SFC/10^6 PBMCs in controllers (Supplemental Digital Content 2). Despite a significant difference in burden of disease as evidenced by pVL (p=0.02), there was no difference in the magnitude of response between progressors and controllers. Interestingly, the lowest ELISPOT responses were detected in an EC, C1655, who has maintained undetectable pVL for over twenty years, highlighting the limited utility of ELISPOT measurements as a correlate of an efficacious HIV-1-specific response.

Due to low frequencies of HIV-1-specific CD8 T-cells in controllers such as C1655 (3), the potentially important information gleaned from ex vivo functional analysis of these cells is infrequently attempted. We therefore used a fourteen-day stimulation protocol to expand epitope-specific CD8 T-cells from cryopreserved PBMCs.
Immunodominant responses detected by ELISPOT (Supplemental Digital Content 2, open symbols) were selected for expansion. Of fifteen CD8 T-cell lines, nine targeted epitopes restricted by HLA class I alleles associated with delayed disease progression (Table 1). Consistent with the dominance of Gag-targeting amongst controllers (1, 21), all lines obtained from this group were specific to epitopes within p24.

Table 1. Epitope-specific CD8 T-cell lines used in the in vitro suppression assay.

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<sup>a</sup>Underlined epitopes represent those restricted by HLA class I alleles associated with delayed disease progression.  
<sup>b</sup>Numbers in parentheses indicate epitope alignment to the HIV-1<sub>HXB2</sub> reference strain.
Phenotypic analysis of HIV-1-specific CD8 T-cells

While expansion of epitope-specific CD8 T-cells is frequently performed, few studies have analyzed functional changes that occur after in vitro expansion. To establish the utility in analyzing expanded HIV-1-specific CD8 T-cells, we used ICS to measure multiple effector functions and compared responses both ex vivo (Figure 1A) and in vitro (Figure 1B). We did not detect a significant difference between the median ex vivo or in vitro responses in progressors compared to that of controllers for any of the functions analyzed. As expected, in vitro expansion increased the frequency of antigen-specific CD8 T-cells (CD107a, IFN-γ, TNF-α, perforin, Figure 1C). In fact, in some ECs, this was the only method of detecting HIV-1-specific effector function. Interestingly, enhanced perforin mobilization was detected after expansion in progressors and controllers despite the fact that the former had few perforin-producing cells when analyzed ex vivo. Thus, while the frequency of epitope-specific responses increased after in vitro expansion, there was no preferential expansion of CD8 T-cells within either group.
Figure 1. ICS analysis of multiple effector functions elicited by *ex vivo* and *in vitro* expanded CD8 T-cells. PBMCs from controllers and progressors were stimulated with HIV-1 peptides and analyzed by ICS for production of CD107a, IFN-γ, IL-2, TNF-α, and perforin. Representative flow cytometry plots depict (A) *ex vivo* and (B) *in vitro* expanded CD8 T-cell responses in subject C5168. Top and bottom panels represent the unstimulated (negative control) and B27-KK10 stimulated response respectively. Lymphocytes were gated by forward and side scatter, viable cells, CD3+ cells, and then CD8+ cells. Numbers indicate the percent of CD8 T-cells positive for the indicated function. (C) Total *ex vivo* and *in vitro* CD8 T-cell responses detected in HIV-1 infected progressors (*ex vivo*, n=6; *in vitro*, n=9; open symbols) and controllers (*ex vivo*, n=6; *in vitro*, n=6; closed symbols); analysis of *ex vivo* immune response was not completed for subject P5766 due to limited sample availability. Each dot represents a single epitope-specific response. Horizontal bars indicate median response. Significant differences (*p*≤0.05) between *ex vivo* and *in vitro* response are indicated by asterisks.
Polyfunctional responses are a hallmark of non-progressive HIV-1 disease (7, 34), we therefore analyzed these responses \textit{ex vivo} and noted that controllers had an increased frequency of epitope-specific CD8 T-cells with three or more functions compared to progressors (Figure 2A). After \textit{in vitro} expansion, however, these differences disappeared as cell lines derived from progressors were also polyfunctional (Figure 2B). This improved function was particularly prominent with perforin producing cells (orange arcs in Figure 2A, 2B), especially those that also expressed IFN-\(\gamma\) and CD107a (Figure 2D). There was no significant difference in the median frequency of IL-2\(^+\) cells between the two groups (Figure 1C). IL-2\(^+\) CD8 T-cells tended to be monofunctional in progressors (black arcs border gray slices, Figures 2A, 2B), although this finding was not significant \((p=0.145)\). Amongst controllers, black arcs border the yellow, blue, and red pie slices, indicating IL-2 is being produced by cells capable of three or more functions. Figures 2C and 2D depict 31 unique combinations of CD8 T-cell responses detected \textit{ex vivo} and after \textit{in vitro} expansion. Analysis of these subsets revealed that polyfunctional CD8 T-cells which produce IL-2 tended to be enriched in controllers after expansion \((p=0.066)\).
Figure 2. Polyfunctionality of *ex vivo* and *in vitro* expanded HIV-1 specific CD8 T-cell responses. Data generated by ICS was analyzed using SPICE software for coincident production of IL-2, perforin, IFN-γ, CD107a, and TNF-α by epitope-specific CD8 T-cells. Pie charts denote the proportion of CD8 T cells producing 1 (gray), 2 (purple), 3 (yellow), 4 (blue), or 5 (red) functions. (A) *Ex vivo* and (B) *in vitro* expanded CD8 T-cell responses were averaged within each group. Arcs identify cell populations that are positive for IL-2 (black arc), perforin (orange), and IFN-γ (green), and CD107a (maroon). Bar graphs depict the relative frequency of responses measured (C) *ex vivo* and (D) after *in vitro* expansion. Responses for all 31 possible subsets are shown for both groups—progressors (pink bars) and controllers (dark blue bars). Plus signs denote a positive response for the effector function listed to the left—2 indicates IL-2, P indicates perforin, G indicates IFN-γ, C indicates CD107a, and T indicates TNF-α.
Epitope-specific CD8 T-cell lines derived from controllers demonstrate increased HIV-1 suppression

Despite similar cytokine profiles among the expanded CD8 T-cell lines, we investigated whether these cells varied in their ability to suppress HIV-1 replication. CD8 T-cell line P7, specific for B57-IW9, was derived from progressor P2824 and tested in the iVSA. Autologous, HIV-1 infected CD4 T-cells cultured without effector cells reached maximal HIV-1 replication on day 3, a two-log increase above day 0 (Figure 3A). As CD8 T-cells were added at increasing concentrations, we observed a slight diminution in HIV-1 replication as quantified by luciferase expression. At an E:T ratio of 1:1, cell line P7 demonstrated 54.5% suppression of virus replication. Analysis of CD8 T-cells derived from controllers showed a significantly different pattern of suppression. As the cell line C6 (B57-TF11-specific; derived from controller C1655) was added at increasing concentrations, we observed a dose-dependent decrease in HIV-1 replication (Figure 3C), with substantial suppression at the lowest concentration of effector cells (97.2%, 0.2:1) and complete virus suppression (99%) at a ratio of 1:1. When cell lines C6 and P7 were cultured with non-autologous, infected targets (Figure 3B, 3D), there was no inhibition of virus replication, indicating an MHC class I dependent mechanism of suppression.

Total analysis of HIV-1-specific CD8 T-cell lines revealed that controllers effectively suppress virus replication compared to progressors (Figure 3E). The median percent suppression in progressors was 73.7% at an E:T ratio of 0.2:1, 76.0% at 0.5:1, compared to 92.9% and 98.8%, respectively in controllers. When the total analysis was adjusted by removing outlier data points (P6, P7, P9), we still detect significant
differences in suppressive capacity, with controllers exhibiting enhanced virus suppression (0.2:1, \( p = 0.017 \); 0.5:1, \( p = 0.004 \), \( p = 0.0087 \)). At the highest effector cell concentration (E:T, 5:1), the difference in suppression of autologous and non-autologous targets was no longer distinct, suggesting non-specific targeting of infected cells (Figure 3B, 3D).
Figure 3. Enhanced HIV-1 suppression mediated by epitope-specific CD8 T-cells derived from controllers. CD4 T-cells (targets) were infected with HIV-1NL4-3 and cocultured with expanded CD8 T-cell lines (effectors) at multiple E:T ratios for seven days. Supernatant from days 0, 1, 3, 5, and 7 were analyzed for production of luciferase using the TZM-bl reporter cell line. Background luciferase production in uninfected primary cells was approximately 1000 relative luciferase units (RLU). All experiments were run in duplicate. (A-D) Representative iVSA data is shown for a CD8 T-cell line derived from a progressor (cell line P7 in A, B) and a controller (cell line C6 in C, D). Targets cells used were autologous (left panels) or non-autologous (right panels). (E) Antiviral suppression by CD8 T-cell lines derived from progressors (P, n=9) compared to lines derived from controllers (C, n=6) at indicated E:T ratios. Each cell line is denoted by a unique symbol. Black bars indicate median percent suppression. Mann-Whitney test was used to compare the median percent suppression between groups at each E:T ratio. NS, not significant.
Polyfunctional CD8 T-cells that maintain IL-2 production are associated with HIV-1 suppressive capacity

We sought to identify a more readily measured correlate of antiviral function. No single marker, when analyzed ex vivo or after in vitro expansion, was associated with enhanced virus suppression (Table 2). This was true even for perforin, regardless of its production with other effector molecules. Polyfunctionality, without an IL-2 response, did not correlate with CD8 T-cell mediated suppression of HIV-1 replication. Rather, CD8 T-cell lines that were polyfunctional and positive for IL-2 production, as measured after expansion, were associated with enhanced in vitro virus suppression (r=0.56; p=0.03, Table 2). The same function measured ex vivo tended to correlate with suppression, although it did not reach significance (r=0.51; p=0.09).

Table 2. Correlation between magnitude of epitope-specific response and HIV-1 percent suppression at E:T of 1:1.

<table>
<thead>
<tr>
<th></th>
<th>Ex vivo response</th>
<th>In vitro response (Expanded lines)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spearman coefficient (r)</td>
<td>P value</td>
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<tr>
<td>CD107a+</td>
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<tr>
<td>IFN-γ+</td>
<td>0.08</td>
<td>0.80</td>
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<tr>
<td>IL-2+</td>
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<td>TNF-α+</td>
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<td>Perforin+</td>
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<td>Polyfunctional (≥3 functions)</td>
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<td>0.40</td>
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<tr>
<td>Polyfunctional + IL-2 production</td>
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DISCUSSIONS

The qualitative features of virus-specific CD8 T-cells that contribute to protection from HIV-1 disease have not been clearly defined. In this study, we quantified the ability of epitope-specific CD8 T-cells to suppress HIV-1 replication, which may represent a more direct marker of antiviral effector function (46, 63). When tested in the iVSA, CD8 T-cell lines derived from HIV-1 infected controllers showed significantly increased suppressive capacity compared to those from progressors. CD8 T-cells were tested against autologous CD4 T-cells, revealing a dose-dependent decrease in virus replication as effectors were added at increasing concentrations. As others have shown, our data provides further evidence that controllers maintain CD8 T-cell populations with potent anti-viral function. At high E:T ratios, virus suppression between the two groups was indistinguishable. As previously observed, this decreased sensitivity at high E:T ratios is likely due to non-specific targeting when CD8 T-cells are in excess (15, 50, 64).

Furthermore, increased suppressive capacity may reflect the highly avid CD8 T-cell responses often detected in those with superior viral control (2). Thus, progressors may require more CD8 T-cells to attain similar protection due to low avidity populations. Enhanced suppressive capacity is an identifiable marker of an effective HIV-1 specific CD8 T-cell response and is one plausible mechanism by which controllers delay disease progression.

For this study, expanded CD8 T-cell lines underwent two rounds of in vitro stimulation with peptide-pulsed, autologous monocytes. This protocol provides a method of expanding populations of low-frequency, antigen-specific cells, thereby permitting evaluation of CD8 T-cells that would otherwise not be analyzed ex vivo, but may play an
important role in the host immune response. This is particularly important in view of the fact that many HIV-1 infected individuals with excellent viral control off ART have low to undetectable frequencies of HIV-specific CD8 T-cells (3). A potential caveat of this protocol is that we have enriched for epitope-specific CD8 T-cells with increased survival capacity.

While several groups have demonstrated that HIV-1-specific CD8 T-cells obtained from controllers are more efficient at viral suppression, a phenotypic profile of the CD8 T-cell capable of enhanced HIV-1 suppression has yet to be well defined (15, 50, 58, 59)

Results from a recent study suggested that antiviral function is dependent on antigen specificity, having observed increased HIV-1 inhibition by Gag-specific CD8 T-cells compared to Env-specific cells (15). Our study did not address Env-specific CD8 T-cells; however, Gag-specificity was not a universal predictor of HIV-1 suppressive capacity. In fact, Gag-specific CD8 T-cells derived from progressors lacked suppressive function. While suppressive capacity may depend upon which epitopes are targeted, a larger sample size will be needed to address this question.

Production of soluble effector molecules may be an important predictor of antiviral efficacy as polyfunctional HIV-1-specific CD8 T-cells are more frequently observed among controllers (7). Comparison of ex vivo CD8 T-cell responses between progressors and controllers did not reveal a significant difference in production or mobilization of any individual function (CD107a, IFN-γ, TNF-α, IL-2, perforin). This was also true for responses detected after in vitro expansion in both groups. As previously reported (50), we observed that expanded HIV-1-specific CD8 T-cells had an
enhanced functional phenotype when compared to *ex vivo* responses, with increased production of CD107a, IFN-γ, TNF-α, and perforin. Polyfunctional, IL-2 producing CD8 T-cells were present in controllers after expansion, in contrast to progressors in whom IL-2 producing CD8 T-cells were predominantly monofunctional. CD8 T-cell lines able to produce IL-2 in combination with at least two other effector molecules exhibited increased virus suppression (*r*=0.56; *p*=0.03). The same function, measured *ex vivo*, trended towards an association with virus suppression, which may be related to the relatively small sample size and limited detection of IL-2 producing CD8 T-cells.

Earlier studies have associated IL-2 production by HIV-1-specific CD8 T-cells with viral control (27, 65). More recently, polyfunctional CD8 T-cell responses were associated with increased suppressive capacity (33); however it remains unclear if IL-2 production, polyfunctionality, or both are involved in viral control or if these phenotypes are the result of controlled virus replication. While our association of virus suppression with polyfunctional, IL-2 production likely represents a means to identify CD8 T-cells that have matured in the context of viral control, it may indicate that IL-2 is involved in this control. One potential mechanism is that IL-2 producing CD8 T-cells have a better proliferative capacity (28, 41), and these may transition to an effector phenotype thus providing a stable population of cells able to maintain control of HIV-1 replication *in vivo*.

Other groups have been able to correlate degranulation and mobilization of perforin and granzymes with enhanced suppression *in vitro* (48). In contrast, we found that expanded CD8 T-cells obtained from progressors have no difficulty increasing perforin production after cell expansion. These observed differences might be due, in
part, to the fact that previous studies used a perforin antibody recognizing pre-formed perforin (clone δG9), but not newly synthesized perforin. We utilized a perforin antibody (clone D48), able to detect de novo synthesis of the protein (29). Incomplete staining with the anti-perforin, clone δG9 antibody would make it difficult to completely quantify upregulation of perforin after stimulation. Furthermore, our method of expansion, using autologous monocytes compared to bulk PBMCs for antigen presentation is significantly different from that of other groups, and it has been demonstrated that the type of antigen presenting cell used for stimulation can impact functional development of effector CD8 T-cells (39, 54).

Studies in SIV-infected rhesus macaques have yielded varying results, unable to clearly define the CD8 T-cell phenotype capable of restricting virus replication. Suppression of SIV replication by CD8 T-cells from infected macaques in earlier studies showed associations with host level of viral control (17) and CD8 T-cell epitope specificity (42), while a more recent study could not identify a correlate of SIV suppression despite measuring a number of immune parameters (61). However, vaccine-induced responses in rhesus macaques immunized with a DNA prime/Ad5 boost vaccine demonstrated that CD8 T-cells were able to suppress SIV replication in vitro, which trended toward both a lower peak VL during acute infection and a lower VL setpoint (44). These inconsistencies may relate to differences in which effector cells were derived. Yet, we report, as others have consistently observed, that HIV-1-specific CD8 T-cells from controllers have an enhanced ability to inhibit virus replication when compared to CD8 T-cells from patients who lack control, despite varying methods used to isolate effector cells (15, 16, 58, 59).
CD8 T-cell recognition and elimination of infected cells is a critical component of the host immune response to viral infection. However, this effector function is not routinely analyzed in pre-clinical studies of candidate HIV-1 vaccines. We demonstrate that increased *in vitro* suppression of HIV-1 replication is a hallmark of non-progressive disease. This enhanced suppression was associated with a polyfunctional, IL-2+ CD8 T-cell response, which may have implications for analysis of vaccine-induced responses. While these studies were completed in the context of chronic HIV-1 infection, and may not translate to protection mediated by vaccine-induced responses, our data suggests that quantification of additional effector functions, especially virus suppression, should be incorporated to evaluate HIV-1 vaccines in a comprehensive manner.
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Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus.

Nat Med 3:205-211.


27. Harari, A., C. Cellera, F. Enders, J. Köstler, L. Codarri, G. Tapia, O. Boyman, E. Castro, S. Gaudieri, I. James, M. John, R. Wagner, S. Mallal,


### SUPPLEMENTAL DIGITAL CONTENT 1

Clinical and immunologic characteristics of study participants.

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<thead>
<tr>
<th>Patient ID</th>
<th>Plasma VL (copies/mL)</th>
<th>CD4 count (cells/mm$^3$)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>A1/A2</td>
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</tr>
<tr>
<td>P4470</td>
<td>53,220</td>
<td>602</td>
<td>0201/0301</td>
</tr>
<tr>
<td>P5766</td>
<td>4,675</td>
<td>505</td>
<td>0201/1101</td>
</tr>
<tr>
<td>P1918</td>
<td>192,800</td>
<td>349</td>
<td>0201/0301</td>
</tr>
<tr>
<td>P2824</td>
<td>54,100</td>
<td>877</td>
<td>3001/3002</td>
</tr>
<tr>
<td>P2883</td>
<td>32,657</td>
<td>717</td>
<td>3014/8001</td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td><strong>53,220$^‡$</strong></td>
<td><strong>602</strong></td>
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<tr>
<td><strong>CONTROLLERS</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C6602</td>
<td>&lt;50</td>
<td>776</td>
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<tr>
<td>C5168</td>
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$^‡p=0.02$

$^a$HLA class I alleles associated with delayed HIV-1 disease progression are underlined.
Mapping HIV-1-specific responses to optimized CD8 T-cell epitopes. Total PBMCs from HIV-1 infected progressors and controllers were stimulated with epitopes that correspond to the HLA class I genotype of each subject in an IFN-γ ELISPOT assay. Each point indicates a response to a single HIV-1 epitope. Responses subsequently used for CD8 T-cell line expansions are indicated by open circles. Horizontal bars indicate median response within a subject. Dotted line denotes the lower limit for a positive response, 55 SFC/10⁶ PBMCs.
CD8 T CELL-MEDIATED SUPPRESSION DURING PRIMARY HIV-1 INFECTION

by

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SONYA L. HEATH, PAUL A. GOEPFERT

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Format adapted for dissertation
Introduction

While the infected host mounts a robust HIV-1-specific immune response, the majority of untreated individuals eventually lose total immune function and progress to AIDS. Adherence to antiretroviral therapy (ART) limits virus replication and delays disease progression, but will not eliminate the virus. Thus, without a cure (“permanent remission of a disease in the absence of therapy”(3)) for HIV-1 disease, the ultimate goal in controlling the epidemic is to prevent infection. While a number of methods including needle-exchange programs and prevention of mother-to-child transmission are indispensable tools in curbing the spread of virus, the availability of such programs on a consistent basis is still limited in some regions (9). A preventive HIV-1 vaccine would be the key strategy in stopping new infections. To achieve this, understanding the innate and adaptive mechanisms which underlie durable, immune-mediated protection from infection and during disease is critical.

Recent analysis of the earliest HIV-1-specific T cell responses has demonstrated, in a more direct manner, that CD8 T cell effector function plays a key role in reducing viremia during primary infection (5). HIV-1-specific CD8 T cells characterized during primary infection were associated with higher functional avidity (7) and a polyfunctional response (8). However, few studies have analyzed CD8 T cell-mediated virus inhibition during primary infection. Chun and colleagues examined bulk CD8 T cell antiviral activity in four individuals during acute infection, observing strong suppression of virus replication (2). The suppressive capacity of CD8 T cells in these individuals was
comparable to that detected in long-term nonprogressors (LTNPs). The enhanced CD8 T cell function observed in these individuals may have been a result of early administration of ART (within 4 months of acute HIV symptoms), or may demonstrate a preservation of function in the absence of advanced disease. Nonetheless, it is intriguing that CD8 T cell-mediated restriction of HIV-1 replication was observed and warrants further investigation.

Using a modified in vitro suppression assay (iVSA), we analyzed the ability of epitope-specific CD8 T cells to suppress HIV-1 replication in autologous CD4 T cells during primary infection. We hypothesized that CD8 T cells from earlier time points would exhibit enhanced suppressive capacity when compared to responses from a more advanced stage of disease.

Results and Discussion

To investigate CD8 T cell function during early HIV-1 infection, before the administration of ART, we assessed in vitro suppression of virus replication mediated by epitope-specific cell lines. Study participants included four individuals identified during primary HIV-1 infection (Table 1). Based upon results of diagnostic assays, patients were classified according to Feibig stages (4). At the earliest time point, plasma VL ranged from 686 – 4,750,000 copies/mL and CD4 T cell counts ranged from 164 – 726 cells/mm$^3$. 

Table 1

Clinical characteristics of study participants identified during primary HIV-1 infection

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<tr>
<th>Patient ID</th>
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<th>Fiebig stage at first time point</th>
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<td>ER7218</td>
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<td>4,750,000</td>
<td>164</td>
<td>n/a</td>
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<tr>
<td>WI7268</td>
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<td>V</td>
<td>609,000</td>
<td>242</td>
<td>113,000</td>
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<tr>
<td>ST7281</td>
<td>Positive</td>
<td>V</td>
<td>686</td>
<td>726</td>
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A 14-day stimulation protocol was used to expand epitope-specific CD8 T cells derived from cryopreserved peripheral blood mononuclear cells (PBMCs). Monocytes, isolated by plate adherence, were irradiated and pulsed with the appropriate HIV-1 peptide. Negatively-isolated, autologous CD8 T cells were cultured with the freshly prepared monocytes in the presence of IL-2 and IL-7 for seven days. CD8 T cells were re-stimulated on day 7 and tested for antigen-specific function on day 14 or day 15. Samples used to prepare monocytes, CD8 T cells, and CD4 T cells used as targets in experiment 1 were taken from the same time point.

We expanded four epitope-specific CD8 T cell lines (Table 2) and tested the HIV-1 suppressive capacity of these cells. Target cells were generated by infecting activated CD4 T cells with the lab adapted HIV-1_{NL4.3} strain at an MOI of 0.001. Effector (E) and target (T) cells were co-cultured at various E:T ratios for seven days. Supernatant was pulled on days 0, 1, 2, 3, 5, and 7 and later analyzed using the TZM-bl cell line, which expresses luciferase under control of the HIV-1 promoter.
Table 2

*CD8 T cell lines derived during primary HIV-1 infection*

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<tr>
<th>Patient ID</th>
<th>Line ID</th>
<th>MHC class I Restriction-Epitope</th>
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<td>ST7281</td>
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<td>ETINEEAAEW</td>
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<td></td>
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<td>B07-TL10</td>
<td>Nef (128-137)</td>
<td>TPGPGVRYPL</td>
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<td>AC003</td>
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<td>Gag p17 (20-28)</td>
<td>RLRPGGKKK</td>
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<td>AC004</td>
<td>A03-RK9</td>
<td>Gag p17 (20-28)</td>
<td>RLRPGGKKK</td>
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</table>

Results from experiment 1 indicated there was poor infection of target cells when CD4 T cells were derived from samples taken at the earliest time point (Figure 1). This may be attributed to pro-inflammatory conditions that exist during HIV-1 infection, especially when virus replication is at a peak. To generate infected target cells to be used for subsequent iVSA experiments, CD4 T cells were isolated from a time point after the initiation of ART, when pVL had been reduced (Table 3).

Table 3

*Time point of samples used to generate effector and target cells in experiment #2*

<table>
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<th>CD4 T cells, Targets</th>
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<tr>
<td><strong>EXPERIMENT #2</strong></td>
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FIGURE 1. Analysis of CD8 T cell suppression during primary HIV-1 infection, Experiment #1. The seven-day in vitro suppression assay was used to assess effector function of CD8 T cell lines AC001 (A) and AC003 (B). HIV-1 infected target cells were cultured with effector cells for seven days at multiple E:T ratios. HIV-1 replication was measured using the TZM-bl reporter cell line. The dashed blue line represents samples to which no effector cells were added (E:T of 0:1).

When CD8 T cell lines expanded during experiment 2 were tested in the iVSA, we detected a differential capacity to suppress virus replication in autologous CD4 T cells (Figure 2). The strongest inhibition of virus replication was mediated by cell line AC004, expanded from patient WI7268, at the latter time point (Fig. 2C). Although maximum inhibition of virus replication was not observed at the early time point, there was some evidence of suppression. At the latter time point, the difference in suppression mediated by line AC004 at 0.5:1 and 1:1 suggests there may be a quantitative threshold that must be overcome to restrict HIV-1 replication. Interestingly, this data is in agreement with early studies demonstrating a reduction of viremia as CD8 T cells expand to detectable levels (1, 6). Prior studies analyzed HIV-1 suppression at a single E:T ratio (2) which, as evidenced here, may not capture the full dynamics of CD8 T cell suppressive capacity.
FIGURE 2. Analysis of CD8 T cell suppression during primary HIV-1 infection, Experiment #2. Cell lines (A) AC003 and (B) AC004 were analyzed using the iVSA. Expanded CD8 T cells were co-cultured with infected, autologous CD4 T cells at the indicated E:T ratios for seven days. Supernatants from days 0, 1, 2, 3, 5, and 7 were analyzed for luciferase production using TZM-blow reporter cells. Cell line AC004 was tested at an early (left) and late (right) time point.

To determine if a marker of CD8 T cell function could predict suppressive capacity during primary infection, the cell lines expanded in experiment #2 were assessed by intracellular cytokine staining (ICS); we measured cytokine production (IFN-γ, IL-2, TNF-α), degranulation (CD107a), and lytic capacity (perforin). Table 4 lists the percent of the CD8 T cell response (background subtracted) that was positive for the indicated effector marker. Functional responses were detected in each of the expanded cell lines. Cell line AC003 displayed robust antigen-specific responses, positive for three out of five functions, while the frequency of responses in AC004 was minimal at the earliest time point. Interestingly, there was a substantial increase in the frequency of antigen-specific CD8 T cells when cell line AC004 was analyzed at the latter time point.
Table 4

*CD8 T cell functional responses as measured by ICS, Experiment #2*

<table>
<thead>
<tr>
<th>Function</th>
<th>AC003</th>
<th>AC004, early (Percent of CD8 T cell response*)</th>
<th>AC004, late</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD107α⁺</td>
<td>1.55</td>
<td>--</td>
<td>4.72</td>
</tr>
<tr>
<td>IFN-γ⁺</td>
<td>1.52</td>
<td>0.36</td>
<td>4.79</td>
</tr>
<tr>
<td>IL-2⁺</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TNF-α⁺</td>
<td>1.06</td>
<td>--</td>
<td>1.64</td>
</tr>
<tr>
<td>Perforin⁺IFN-γ⁺</td>
<td>--</td>
<td>0.07</td>
<td>0.654</td>
</tr>
</tbody>
</table>

*Listed responses were twice above background

Using Boolean analysis, we measured polyfunctional responses in the expanded cell lines (Figure 3). As demonstrated in prior work (8), polyfunctional CD8 T cells were detected during primary HIV-1 infection (yellow, dark blue, and red pie slices). Analysis of the RK9-specific response in participant WI7268 revealed an enhanced ability of HIV-1-specific CD8 T cells to proliferate as time progressed. While no obvious differences in virus suppression were noted at the early vs late timepoint, these results do not take into account the actual number of epitope-specific effectors used in the assay.
FIGURE 3. Polyfunctional response of expanded CD8 T cell lines. Flow cytometry data collected from cell lines expanded during experiment 2 was analyzed using SPICE. Pies depict the fraction of cells producing 1 (gray), 2 (light blue), 3 (yellow), 4 (dark blue), or 5 (red) functions. The arcs bordering the pies represent the effector markers analyzed—CD107a (green), IFN-γ (orange), IL-2 (black), perforin (maroon), TNF-α (purple).

To calculate a more accurate analysis of CD8 T cell-mediated suppression, we estimated the number of effectors needed to suppress at least 90% of virus replication based on IFN-γ production. Although expansion of cell line AC004 at the early time point was less efficient, fewer numbers of these cells were needed to achieve 90% suppression when compared to cells generated at the late time point (effective E:T ratio for ≥90% suppression, early 1:4382 vs late 1:276).

While these studies are limited, they raise interesting questions regarding CD8 T cell responses during primary HIV-1 infection. Analysis of cell line AC004 demonstrates changes in CD8 T cell effector function over time, which seemingly improved when analyzed by ICS and the iVSA (early vs late). Even proliferative capacity seemed to be enhanced as the frequency of epitope-specific cells increased at the late time point. We may have missed critical changes in development of the HIV-1-specific CD8 T cell response as this patient was identified during Fiebig stage V. It would be necessary to
extend these analyses and determine what changes in cell phenotype and function may occur during the early stages of chronic disease. Participant ER7128, however, was identified at Fiebig stage II/III and we observed that the majority of responses in cell line AC003 were polyfunctional (Figure 3A). Longitudinal analyses would provide additional insight regarding the dynamics of CD8 T cell effector function at peak viremia and once set point is established. We intend to complete analyses of additional CD8 T cell lines, using ICS and the iVSA, derived from individuals in our primary HIV-1 infection cohort.

Characterizing the nature of HIV-1-specific immune responses during acute infection is challenging, given that the majority of patients present well into chronic disease. However, increased screening of high-risk populations is helping to identify infected individuals, enable early treatment, and provide an opportunity to analyze immune function during the earliest phases of HIV-1 disease.
REFERENCES


CONCLUSIONS

A significant challenge to the development of an efficacious HIV-1 vaccine remains the inability to define precise correlates of long-lasting immune protection. Natural evidence of spontaneous control of HIV-1 lies in a small population of infected individuals identified as long-term nonprogressors (LTNP) and elite controllers (EC) (21, 66). LTNP and ECs develop HIV-1-specific immune responses able to restrict virus replication, in the case of ECs to undetectable levels, without the use of ART. It is believed that this level of immune control, at least in part, is due to CD8 T cell responses. However, which component of the CD8 T cell response needed to achieve this level of control has not been defined. The work presented in this thesis evaluates epitope-specific CD8 T cell effector function during acute and chronic HIV-1 infection to determine the quality of response associated with viral control. We demonstrate that in vitro suppression of HIV-1 replication mediated by CD8 T cells is a correlate of delayed disease progression (3). Combining ICS analysis with the iVSA, we observe that increased suppressive capacity is associated with a polyfunctional CD8 T cell response that includes IL-2 production.

In order to define the quality of HIV-1-specific CD8 T cell responses needed for control of virus replication, we analyzed CD8 T cell function using IFN-γ ELISpot, intracellular cytokine staining (ICS), clonotyping by TCRβ chain sequencing, and the
*in vitro* suppression assay (iVSA). We first compared epitope-specific responses in individuals who are unable to control virus replication, progressors, and individuals who demonstrate spontaneous control of virus replication without the use of ART, controllers.

To define HIV-1-specific responses in our study participants, we used the rapid and sensitive IFN-γ ELISpot assay to map HIV-1-specific responses. As has been previously reported, we did not detect a correlation between the magnitude of response and a measure of clinical status, either pVL or CD4 T cell count. Although IFN-γ production is an indicator of an HIV-1-specific response, the frequency of cells producing this cytokine is not a determinant of antiviral efficacy. The IFN-γ ELISpot assay can also be limiting when analyzing responses in ECs as low frequency T cell responses are often not detected directly *ex vivo*. ELISpot analysis, while a sensitive and useful tool, may underestimate antigen-specific responses that play a role in restricting virus replication but are at the limit of detection when analyzed *ex vivo*.

To determine whether TCR usage impacts the quality of response in HIV-1-specific effector CD8 T cells, we sorted stimulated cells based on production of IFN-γ and IL-2. TCRβ chain sequencing in four individuals revealed that CD8 T cell populations with different functional responses contained different clonal profiles. We observed that IFN-γ producing cells were polyclonal, while cells positive for both IFN-γ and IL-2 were comprised of a subset of those clones. Further analysis will be required to determine whether we identified cells that express the same TCRβ chain and have lost (IFN-γ⁺IL-2⁺ → IFN-γ⁺) or gained effector function (IFN-γ⁺ → IFN-γ⁺IL-2⁺). It is also possible that differences in signals received during CD8 T cell priming would enable two cells bearing the same TCR to develop different functional capacities. Evidence
generated using transgenic mouse models suggest the latter may be true. Clonotype analysis in an infected controller, BRST, revealed a single clonotype dominated the B*27-KK10 CD8 T cell response. As previously described, the B*27-KK10 response is an immunodominant response highly associated with delayed disease progression (35, 71). We were surprised to see a limited clonal profile in B*27-KK10-specific CD8 T cells, but our data parallels the work of Almeida and colleagues where the KK10-specific population was dominated by a single TCR (4). It has been suggested that a lack of clonal diversity negatively impacts the CD8 T cell response in that there is less tolerability of epitope variation, increasing the likelihood of HIV-1 evading the immune response via escape mutations. Longitudinal analysis of the CD8 T cell clonotypes in subject BRST will help to address this question.

We also examined CD8 T cell function using the ICS assay, both ex vivo and after in vitro expansion. Using polychromatic flow cytometry, immunodominant CD8 T cell epitopes identified by ELISpot were used to stimulate effector cells and mobilization of CD107a and perforin, as well as production of IFN-γ, IL-2, and TNF-α was quantified. HIV-1-specific CD8 T cell responses displayed functional heterogeneity when analyzed by ICS. IL-2 producing cells were primarily detected in subjects with pVLs lower than 2000 copies/mL.

We sought to evaluate whether CD8 T cells derived from HIV-1 infected controllers have an enhanced ability to suppress virus replication when compared to progressors. To generate adequate numbers of epitope-specific cells, we adapted an expansion protocol to establish CD8 T cell lines from cryopreserved PBMCs of HIV-1-infected individuals. This method allows one to expand and evaluate low frequency cell
populations that might not otherwise be observed in an *ex vivo* assay. In fact, it was only after *in vitro* expansion, could we generate enough cells for additional functional analyses in two of our controllers, AL6602 and CO1655.

We demonstrated that CD8 T cells derived from HIV-1 controllers show enhanced *in vitro* suppression of HIV-1 replication when compared to progressors (median percent suppression, \( p = 0.003 \)) (3). Our findings are in line with work from Saez-Cirion *et al* and Chen *et al* in which individuals able to spontaneously control virus replication have an increased ability to inhibit virus (17, 81). Enhanced suppression of virus replication is one plausible mechanism by which controllers are able to delay disease progression.

Having observed a significant difference in antiviral efficacy of CD8 T cells, we combined iVSA and ICS analysis to identify a marker that might serve as a predictor of HIV-1 suppressive capacity. Interestingly, polyfunctionality by itself was not a predictor of HIV-1 suppressive capacity. We found that IL-2 production by polyfunctional CD8 T cell lines correlated with HIV-1 suppressive capacity (\( r = 0.59, p = 0.02 \)). Previous reports have indicated that maintenance of proliferative capacity and IL-2 production are unique features of HIV-1-specific CD8 T cells in controllers (84). During persistent infections, the CD8 T cell IL-2 response is typically lost early as cells become exhausted. Progressors, who have higher levels of viremia, maintain CD8 T cells that have likely lost the ability to produce IL-2. It is possible that IL-2 serves as a surrogate marker for CD8 T cell proliferative capacity, and it is these cells that are better able to respond in the iVSA.

Surprisingly, we did not detect a correlation between perforin production and suppression of virus replication. In fact, upon CD8 T cell expansion, the frequency of
perforin producing cells increased in both progressors and controllers. This data is in contrast to reports of poor perforin production in patients with progressive disease (65) and may be due to differences in CD8 T cell expansion methods or the monoclonal antibodies used to detect perforin.

As described in previous studies, the initial HIV-1-specific CD8 T cell response drives the decrease in viremia observed during primary infection (12, 32, 47). However, studies addressing the quality of CD8 T cell response at the earliest stage of HIV-1 infection have been limiting, particularly the analysis of virus suppression (18). We analyzed CD8 T cell function as measured using ICS and the iVSA in a group of patients identified during primary HIV-1 infection. We observed that the ability of CD8 T cells to expand in vitro improved as time progressed. Interestingly, suppressive capacity was measured at greater than 90% at early and late time points in patient WI7628. When production of functional markers was analyzed, the frequency of antigen-specific CD8 T cells increased over time; we also detected increased polyfunctional responses. So while CD8 T cell expansion was limited at the earliest time points, these cells were able to suppress virus replication. These studies are limited in number, thus additional studies will be necessary to better understand the development of CD8 T cell effector function during primary HIV-1 infection.

An effective HIV-1 vaccine will likely need to induce effective responses from both arms of the adaptive immune system, humoral and cell-mediated. The challenge to this has been the difficulty in identifying what defines protective immunity in the face of HIV-1 infection. In this thesis, we evaluated the quality of HIV-1-specific CD8 T cells that we may better understand which components of the response are associated with
protection from disease. We describe an adapted *in vitro* suppression assay (iVSA) as a method to evaluate the ability of CD8 T cells to suppress HIV-1 replication. Using this assay, we observed that *in vitro* HIV-1 suppressive capacity is a correlate of delayed disease progression.


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isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science **224**:500-503.


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with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 68:4650-4655.


APPENDIX

IRB APPROVAL FORMS
## Protection of Human Subjects

### Assurance Identification/IRB Certification/Declaration of Exemption (Common Rule)

Privacy: Research activities involving human subjects may not be conducted or supported by the Department and Agencies adopting the Common Rule (55 FR 34200, June 18, 1990), unless the activities are exempt, approved or reviewed in accordance with the Common Rule. See section 101(b) of the Common Rule for exemptions; institutions submitting applications or proposals for supported research shall notify the institutional review board (IRB) of the exemption status. The IRB shall review the application or proposal and submit its recommendations to the Department or Agency in accordance with the Common Rule.

### 1. Request Type
- [ ] Original
- [ ] Continuation
- [ ] Exemption
- [ ] Other

### 2. Type of Mechanism
- [ ] Grant
- [ ] Contract
- [ ] Fellowship
- [ ] Cooperative Agreement
- [ ] Other

### 3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.

### 4. Title of Application or Activity

HIV-1 Suppression by MHC Class I Restricted CD8+ T Cells

### 5. Name of Principal Investigator, Program Director, Fellow, or Other

GOEPFERT, PAUL A.

### 6. Assurance Status of this Project (Respond to one of the following):

- [X] This Assurance, on file with Department of Health and Human Services, covers this activity.
  - Assurance Identification No: RWA000005880
  - the expiration date: 09/29/2013
  - IRB Registration No.: IRB00000196

- [ ] This Assurance, on file with [agency/dept.]
  - Assurance No.: ____________________________
  - the expiration date: ________________________
  - IRB Registration/Identification No.: ________
  - (if applicable)

- [ ] No assurance has been filed for this institution. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

### 7. Certification of IRB Review (Respond to one of the following: If you have an Assurance on file)

- [X] This activity has been reviewed and approved by the IRB in accordance with the Common Rule and any other governing regulations.
  - [ ] Full IRB Review on [date of IRB meeting] or [X] Expedited Review on [date] ________
  - [ ] if less than one year approval, provide expiration date

- [ ] This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the Common Rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

### 8. Comments

Protocol subject to Annual continuing review.

HIV-1 Suppression by MHC Class I Restricted CD8+ T Cells

**Title X0701100B**

**Comment**

IRB Approval Issued:

- 1/2 - 5 - 10

**Date**

**10. Name and Address of Institution**

University of Alabama at Birmingham
701 20th Street South
Birmingham, AL 35294

**11. Phone No. (with area code)**

(205) 934-7000

**12. Fax No. (with area code)**

(205) 934-1301

**13. Email**

smoore@uab.edu

**14. Name of Official**

Marilyn Moore, M.A.

**15. Title**

Vice Chair, IRB

**16. Signature**

[Signature]

Sponsored by IRB

[Public reporting burden for this collection of information is estimated to average less than an hour per response. An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number. Comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, should be directed to OMB Reports Clearance Officer, Room 314-B, Independent Avenue, S.W., Washington, DC 20503. Do not return the completed form to this address.]

[End of Form]
Project Revision/Amendment Form

(Protect TYPE: In MS Word, highlight the shaded, underlined box and replace with your text; double-click checkboxes to check/uncheck.)

- Federal regulations require IRB approval before implementing proposed changes.
- Change means any change, in content or form, to the protocol, consent form, or any supportive materials (such as the Investigator's Brochure, questionnaires, surveys, advertisements, etc.).
- Complete this form and attach the changed research documents.

Today's Date: 01/27/09

1. Contact Information
Principal Investigator's Name: Paul Gorgorti, M.D. Blazer ID: paulig E-mail: paulig@uab.edu
Contact Person's Name: Michael P. Cooney Blazer ID: mcconey E-mail: mcconey@uab.edu
Telephone: 5-8607 Fax: 55824
Campus Address: CCB 334A

2. Protocol Identification
Protocol Title: HIV-1 Suppression by MHC class I restricted CD8 T Cells
IRB Protocol Number: 090116008

Current Status of Project (check only one):
- [X] Currently in Progress (Number of participants entered: 64)
- [ ] Study has not yet begun (No participants entered)
- [ ] Closed to participant enrollment (remains active)—
  Number of participants on therapy/intervention:
  Number of participants in long-term follow-up only:
- [ ] Closed to participant enrollment (data analysis only)—
  Total number of participants enrolled:

This submission changes the status of this study in the following manner (check all that apply):
- [ ] Protocol Revision
- [ ] Protocol Amendment
- [ ] Study Closed to participant entry
- [ ] Study Closure
- [ ] Other, (specify) __________
- [X] Revised Consent Form
- [ ] Addendum (new) consent form
- [ ] Enrollment temporarily suspended by sponsor
- [X] Change in protocol personnel

3. Reason for change
Briefly describe, and explain the reason for, the change. If normal, healthy controls are included, describe in detail how this change will affect those participants.
Include a copy of the protocol and any other documents affected by this change (e.g., consent form, questionnaire) with all the changes highlighted.
Please add Olivemidele (Simi) Abisogun as Sub-Investigator to this protocol. I am attaching the certificate for her 2008 IRB training for your records.

4. Does this change revise or add a genetic or storage of samples component?
- [X] Yes  [ ] No
If yes, please see the Guidebook to assist you in revising or preparing your submission, or call the IRB office at 934-7399.

5. Does the change affect subject participation (e.g., procedures, risks, costs, location of services, etc.)?
- [X] Yes  [ ] No
If yes, Fiscal Approval Process (FAP)-designated units complete a FAP submission and send to fap@uab.edu. For more on the UAB FAP, see www.uab.edu/ohr.

6. Does the change affect the consent document(s)?
- [X] Yes  [ ] No
If yes, briefly discuss the changes.
Include the revised consent document with the changes highlighted.
Will any participants need to be reconsented as a result of the changes?
- [ ] Yes  [X] No
If yes, when will participants be reconsented? __________

Signature of Principal Investigator: ______________________ Date: 2/2/09

224 - PRA_Form_CCT_08.doc
10/15/08
MEMORANDUM

DATE: January 27, 2009

TO: Institutional Review Board for Human Use
    University of Alabama at Birmingham

FROM: Paul A. Goepfert, M.D.
      Alabama Vaccine Research Clinic at UAB

RE: HIV-1 Suppression by MHC Class I Restricted CD8 T-Cells

Principal Investigator: Paul A. Goepfert, M.D.
IRB Protocol #: X 070116008

Ms. Olusimide (Simi) Akinsiku is a graduate student in the Microbiology department. Ms. Akinsiku joined our group in September 2006. She is currently working on her thesis project, entitled “Protective Immune Mechanisms Mediated by CD8^+ T cells during HIV-1 Infection.”

In completing her thesis work, Ms. Akinsiku has been investigating the functional profile of virus-specific CD8^+ T cells derived from individuals infected with HIV-1. Her thesis work began in April 2007 and involves testing peripheral blood mononuclear cells (PBMCs) in various assays that have been optimized in my laboratory. This work is consistent with the experimental goals stated in IRB Protocol No. X070116008, titled “HIV-1 Suppression by MHC Class I Restricted CD8 T-Cells.” As she is continuing the experiments related to Protocol No. X070116008, a project revision amendment form has been submitted so that she may be added as a sub-investigator.
Investigator's Progress Report

☐ Continuing Review
☐ Final Report (When all study activities including data analysis)
☐ Expedited
☐ For—OR—☐ Convened

NOTE: Not following the format shown below or ignoring or deleting questions will result in deferral of the protocol for IRB review.

Today's Date (MM/DD/YYYY): 12/01/10

1. Name of Principal Investigator (First, Middle, Last): Paul A. Goepfert, MD
   Email Address: paulg@uab.edu
   Campus Address: Department: Medicine Building: CCB
   Room: 328 A UAB Zip: 2050
   Name of Contact Person: Michael F. Cooney Title/Role: Clinical Trials Specialist
   Phone: 5-58007 Fax: 5-5824 Email Address: mcooney@uab.edu

2. IRB Protocol Number: X 070116008

Protocol Title: HIV-1 Suppression by MHC class I restricted CD8 T Cells
   Study Sponsor: National Institute of Health (NIH) / National Institute of Allergy and Infections Diseases (NIAID)
   OGCA Tracking # or Link #: T0608230001 / 000286275

3. Briefly describe the purpose of the study (2-3 sentences in non-technical, lay language).
   To (1) determine the relative potency of HIV-1-specific CD8 T cells clones in killing and suppressing viral replication in vitro and (2) determine the ex vivo functional phenotypes of HIV-1-specific CD8 T cells associated with the corresponding in vitro killing and suppression capacities of CD8 T cell clones.

4. Starting Date of Project: 02/02/2007 Date of Last IRB Approval: 12/29/09

5. Individuals Screened and Entered

   For projects conducted at multiple institutions, the numbers stated should be for participants enrolled by the UAB Investigator only.

   a. Number of individuals screened for entry into study since the start of the project? 68
   b. Number of individuals entered into the study since the start of the project? 68
   c. Number of individuals entered into the study since the last IRB review? 4
   d. Complete the age, sex, and racial/ethnic composition grid below:

<table>
<thead>
<tr>
<th>Racial/Ethnic Composition</th>
<th>Individuals Screened (Should reflect # in 5.a.)</th>
<th>Individuals Entered (Should reflect # in 5.b.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
</tbody>
</table>
   | Caucasian                 | 26-52 | 22 | 25-29 | 5
   | African American          | 20-62 | 27 | 33-56 | 13
   | Native American           | 0 | 0 | 0 | 0
   | Asian                     | 40 | 1 | 0 | 0
   | Hispanic                  | 0 | 0 | 0 | 0
   | Other                     | 0 | 0 | 0 | 0
   | Caucasian                 | 26-52 | 22 | 25-29 | 5
   | African American          | 20-62 | 27 | 33-56 | 13
   | Native American           | 0 | 0 | 0 | 0
   | Asian                     | 40 | 1 | 0 | 0
   | Hispanic                  | 0 | 0 | 0 | 0
   | Other                     | 0 | 0 | 0 | 0
6. For each investigator and staff member involved in the design, conduct and reporting of the research answer the questions below:

The following definitions are used for item #6:

**Immediate family** means spouse or a dependent of the employee. **Dependent** is any person, regardless of his or her legal residence or domicile, who receives 50% or more of his or her support from the public official or public employee or his or her spouse or who resided with the public official or public employee for more than 180 days during the reporting period.

**Financial Interest Related to the Research** means financial interest in the sponsor, product or service being tested, or competitor of the sponsor.

Have each investigator and staff member involved in the design, conduct and reporting of the research answer the questions below:

(Repeat this section for each individual)

**Name: Paul Goepfert, PI**
Do you or your immediate family have any of the following? (Check all that apply)

- ☐ An ownership interest, stock options, or other equity interest related to the research of any value.
- ☐ Compensation related to the research unless it meets two tests:
  - Less than $10,000 in the past year when aggregated for the immediate family.
  - Amount will not be affected by the outcome of the research.
- ☐ Proprietary interest related to the research including, but not limited to, a patent, trademark, copyright, or licensing agreement.
- ☐ Board of executive relationship related to the research, regardless of compensation.

**Name: Geeta Daata, Ph.D., Sub-Investigator**
Do you or your immediate family have any of the following? (Check all that apply)

- ☐ An ownership interest, stock options, or other equity interest related to the research of any value.
- ☐ Compensation related to the research unless it meets two tests:
  - Less than $10,000 in the past year when aggregated for the immediate family.
  - Amount will not be affected by the outcome of the research.
- ☐ Proprietary interest related to the research including, but not limited to, a patent, trademark, copyright, or licensing agreement.
- ☐ Board of executive relationship related to the research, regardless of compensation.

**Name: Sonya Heath, Sub-Investigator**
Do you or your immediate family have any of the following? (Check all that apply)

- ☐ An ownership interest, stock options, or other equity interest related to the research of any value.
- ☐ Compensation related to the research unless it meets two tests:
  - Less than $10,000 in the past year when aggregated for the immediate family.
  - Amount will not be affected by the outcome of the research.
- ☐ Proprietary interest related to the research including, but not limited to, a patent, trademark, copyright, or licensing agreement.
- ☐ Board of executive relationship related to the research, regardless of compensation.
Name: Olusimidele (Simi) Akinsiku, Sub-Investigator

Do you or your immediate family have any of the following? (Check all that apply)

☐ An ownership interest, stock options, or other equity interest related to the research of any value.

☐ Compensation related to the research unless it meets two tests:
  - Less than $10,000 in the past year when aggregated for the immediate family.
  - Amount will not be affected by the outcome of the research.

☐ Proprietary interest related to the research including, but not limited to, a patent, trademark, copyright, or licensing agreement.

☐ Board of executive relationship related to the research, regardless of compensation.

If you checked any of the above, a financial interest disclosure has to be submitted to or currently on file with the CIRB and the completed CIRB Evaluation has to be available before the IRB will conduct its continuing review.

7. Since the last IRB review, have you received any of the following types of information?
   a. Multi-center trial reports? ☐Yes ☑No
      If yes, attach a copy of any multi-center trial reports not previously forwarded to the IRB, and summarize those reports here:____

   b. Data and safety monitoring board reports? ☐Yes ☑No
      If yes, attach a copy of any data and safety monitoring board reports not previously forwarded to the IRB, and summarize those reports here:____

   c. Interim findings? ☐Yes ☑No
      If yes, state both the positive and negative results received to date:____

   d. Published literature? ☐Yes ☑No
      If yes, attach a copy and summarize the published findings here:

   e. Any other relevant information regarding this research, especially information about risks associated with the research? ☐Yes ☑No
      If yes, attach a copy if applicable, and summarize this information here:

   f. Could any of the information described above relate to the participants’ willingness to continue participating? ☐Yes ☑No
      If yes, describe here whether and how this information will be provided to participants:____

8. Since the last IRB review, have any of the following occurred?
   a. Have participants experienced any harms (expected or unexpected)? ☐Yes ☑No
      If yes, attach Problem Summary Sheet, and briefly describe here the harms (serious and/or non-serious) experienced by participants:____

   b. Have there been any unanticipated problems involving risks to participants or others?
Principal Investigator Signature: [Signature]  Date: 12/11/10

Paul A. Goepfert, M.D.

Attach to this Investigator's Progress Report a copy of the current consent form, if applicable. If your research involves children/minors under the age of 19 years; the PI must provide a memo that confirms the previously assigned CRL # or reassigns the CRL and give the reasons the CRL has changed.

If your study involves Gene Therapy, attach to this Investigator's Progress Report a copy of the current consent form, if applicable, and a signed copy of a memo from your Project Review Panel addressing the following questions:
1. Has the Panel's assessment of the risk-benefit ratio of this project changed? ☐ Yes ☐ No
   If yes, please explain.
2. Did the Panel have any recommendations regarding the protocol or the consent form? ☐ Yes ☐ No
   If yes, please explain.
3. If the study includes children/minors under the age of 19 years, provide a memo the previously assigned CRL # and have the Panel either confirm it or reassign it. If the panel chooses to reassign the CRL, they must give the reasons the CRL has changed.