REGULATION AND FUNCTION OF INTERLEUKIN-21-PRODUCING T CELLS AND IMMUNE-MEDIATED CONTROL OF HIV-1 INFECTION

by

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CD8 T cells play a critical role in controlling intracellular pathogens, particularly viruses. However, during persistent viral infections, such as human immunodeficiency virus-1 (HIV-1) infection, virus-specific CD8 T cells become increasingly impaired by poorly understood mechanisms. Massive CD4 T cell depletion is a hallmark of HIV-1 infection and is associated with CD8 T cell dysfunction and ineffective viral containment. CD4 T cells provide critical helper signals to CD8 T cells, especially during uncontrolled viral infections. Mouse models of chronic viral infection implicate interleukin-21 (IL-21), produced primarily by CD4 T cells, as a vital factor necessary for the maintenance of fully functional CD8 T cells and viral containment. The studies presented in this dissertation have investigated the role of IL-21 as a mediator of CD4 T cell help during HIV-1 infection. Our results demonstrate that both CD4 and CD8 T cells produce IL-21 in response to HIV-1, with the latter cell type more closely associated with viral control. IL-21-producing CD4 T cells, compared to those producing other cytokines, were the best indicator of functional CD8 T cells. Interestingly, virus-specific CD8 T cells were not identified outside the context of HIV-1 infection. We therefore hypothesized that under conditions of reduced CD4 T cell help, as seen in HIV-1 infection, CD8 T cells producing IL-21 may partially compensate for this loss. Upon polyclonal stimulation, IL-21-producing CD8 T cells exhibited some characteristics of helper T cells, including impaired production of perforin and granzyme B as well as CD40 ligand upregulation; however, we ob-
served limited expression of the chemokine receptor CXCR5, suggesting a functional profile distinct from the T follicular helper subset. Our studies reveal that CD8 T cell induction of IL-21 competency is independent of CD4 T cell loss but instead contingent upon the extent of peripheral CD8 T cell activation. These findings suggest that IL-21 production is remarkably limited to CD4 T cells during the steady state, and CD8 T cells producing IL-21 can either mediate viral control or moderate inflammation depending on whether these cells act in a specific or nonspecific manner, respectively.

Keywords: Interleukin-21, HIV-1 infection, CD4 T cell help, CD8 T cells
DEDICATION

I would like to dedicate this dissertation to my family – first to my parents, Daniel and Gail Williams, as well as my sister, Kendra, who have been extremely supportive throughout my education. Thank you for your encouragement and your prayers, especially during my graduate school years. To my extended family, I hope I have made you proud, as I am the first member of our family to have pursued and completed a doctorate degree.
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<td>anti-retroviral therapy</td>
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<td>Blimp-1</td>
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<td>CD</td>
<td>Cluster of differentiation; cluster of designation</td>
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<td>CMV</td>
<td>cytomegalovirus</td>
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<td>CXCR5</td>
<td>C-X-C chemokine receptor type 5</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>EC</td>
<td>elite controller</td>
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<td>GALT</td>
<td>gut-associated lymphoid tissues</td>
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<td>γc</td>
<td>common γ chain</td>
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<td>HIV</td>
<td>human immunodeficiency virus</td>
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<td>HLA</td>
<td>human leukocyte antigen</td>
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<td>Inducible T cell co-stimulator</td>
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<td>idiopathic CD4 lymphocytopenia</td>
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<td>IL-21</td>
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<td>IL-21R</td>
<td>interleukin-21 receptor</td>
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<tr>
<td>JAK</td>
<td>Janus tyrosine kinase</td>
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<tr>
<td>LAG-3</td>
<td>lymphocyte activated gene-3</td>
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<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
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<td>Lef1</td>
<td>lymphoid enhancer-binding factor 1</td>
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<td>LTR</td>
<td>long terminal repeat</td>
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<td>MAP</td>
<td>mitogen-activated protein</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>mL</td>
<td>milliliter</td>
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<td>NFAT</td>
<td>Nuclear factor of activated T cell</td>
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<td>NK</td>
<td>natural killer</td>
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<td>P</td>
<td>progressor</td>
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<td>peripheral blood mononuclear cell</td>
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<td>PD-1</td>
<td>Programmed death-1</td>
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<td>PDBu</td>
<td>Phorbol 12, 13-dibutyrate</td>
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<td>PI3</td>
<td>phosphoinositide 3</td>
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<td>PIC</td>
<td>pre-integration complex</td>
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<td>PHI</td>
<td>primary HIV-1 infection</td>
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<tr>
<td>SIV</td>
<td>simian immunodeficiency virus</td>
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<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<td>TCR</td>
<td>T cell receptor</td>
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<td>Tcf7</td>
<td>transcription factor 7</td>
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<td>T helper 17</td>
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<td>T cell immunoglobulin and mucin domain-containing protein-3</td>
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<td>Tfh</td>
<td>T follicular helper</td>
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<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
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<td>VC</td>
<td>viremic controller</td>
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INTRODUCTION

The Urgent Need to Better Understand HIV-1 Pathogenesis

Nearly thirty years since its description as the causative agent of acquired immunodeficiency syndrome (AIDS), human immunodeficiency virus-1 (HIV-1) has continued unabated and remains the number one infectious cause of death in the world (11, 53, 64). By the end of 2010, the World Health Organization estimated that the prevalence and annual mortality exceeds 34.2 and 1.8 million, respectively, with more than 2.7 million persons becoming newly infected in that year alone (189). Despite remarkable strides in the development of pharmacologic strategies to thwart its propagation, anti-retroviral therapy (ART) can only control but cannot fully eradicate HIV-1, the spread of which continues upon treatment interruption (42, 63, 216). However, in the developing world, access to such therapy is hampered in part due to the extreme financial burden and lack of adequate healthcare infrastructure (96). When available, ART significantly reduces HIV-1-associated morbidity and mortality but is associated with adverse side effects and the potential for emergence of drug-resistant strains (13, 32, 99, 125, 148, 198). Clearly, the best solution to curb this global pandemic would be the development of a safe and effective HIV-1 vaccine that could either ideally completely block new infections or at least reduce viral loads and clinical disease progression if infection occurred. The latter could conceivably diminish HIV-1 transmission rates and might have a substantial impact on a population level.
A formidable barrier in the development of successful therapeutic and preventative measures against HIV-1 lies within properties of the virus itself, including its high mutation rate that allows for the emergence of antigenic escape variants, sophisticated mechanisms to avoid immune surveillance, and ability to establish latent reservoirs of infected lymphocytes (112, 116, 209). Foremost among these, is the fact that its preferred target is CD4 T cells, which are central participants in orchestrating the immune response against viruses (44). Overcoming these impediments will require further understanding of the nature of protective humoral and cellular immune responses and how to best elicit such responses to restrict HIV-1 replication. Moreover, results from the recent, modestly protective RV144 vaccine trial have boosted optimism that a candidate is within reach (165, 170).

Intriguingly, this vaccine regimen induced robust HIV-1-specific CD4 T cell responses in recipients, suggesting an important contribution of this cellular subset to durable control of HIV-1 replication and possibly in protection against HIV-1 disease progression (38). Furthermore, a growing body of evidence implicates CD4 T cells in providing critical helper signals that govern the generation and maintenance of CD8 T cells, which kill virally-infected cells (128, 183, 197). Therefore, a major goal is to comprehensively define the specific qualities of antiviral CD4 and CD8 T cell responses required to contain HIV-1. In this regard, I have sought to investigate the role of interleukin-21 (IL-21), a cytokine produced primarily by CD4 T cells, in impacting effective anti-HIV-1 CD8 T cell responses and viral control.
The Origins of Human Immunodeficiency Virus and Challenges of Subtype Diversity

The viruses that cause AIDS in humans, HIV-1 and HIV-2, are primate lentiviruses of the family Retroviridae, closely related to simian immunodeficiency viruses (SIV) that naturally infect more than twenty different species of primates from sub-Saharan Africa (78, 178). Both evolutionary distinct viruses have emerged from zoonotic, cross-species transmission from primates in Africa to humans (66, 67). It is thought that this transfer occurred through human exposure to infected primate blood or mucosal secretions during hunting, butchering, or consumption of contaminated ‘bushmeat’ (154). HIV-2 is most closely related to SIVsm, which is found at high prevalence in sooty mangabey monkeys in West Africa (28, 87). By contrast, HIV-1 is most closely related to strains of SIVcpz, isolated from chimpanzees that inhabit Western and Central Africa (66, 172). Phylogenetic analyses indicate that the global strains of HIV-1 fall into three main groups – group major (M), outlier (O), and nonmajor and nonoutlier (N), each resulting from independent cross-species transmission events in the early 20th century (66, 78). Recently discovered in 2009 and confirmed in 2011, group P has a reported similarity with gorilla SIV and has only been identified in two individuals of Cameroonian descent (159, 192).

Responsible for the majority of HIV-1 cases worldwide, group M has been further subdivided into nine recognized subtypes or clades and sub-subtypes based on sequence divergence, as denoted with letters A-K. Clades or phylogenetically-linked strains are also linked epidemiologically or geographically, with clade A common to West Africa,
clade B dominant in the Americas, and clade C found primarily in sub-Saharan Africa (84).

HIV-2 is largely restricted to Western Africa and is comprised of eight distinct lineages, likely arising from independent host transfers from sooty mangabeys (67). These subtypes have been termed A-H. Compared to HIV-1, HIV-2 is associated with lower viral loads and reduced transmission rates, with virtually no mother-to-child transmissions (161). Most individuals diagnosed with HIV-2 do not progress to AIDS, although the individuals who do, have symptoms similar to those seen in HIV-1-infected individuals (171).

The extraordinary genetic diversity of HIV-1 is attributable to several factors. First, its reverse transcriptase is highly error prone, lacking the ability to confirm that the DNA transcript makes an accurate copy of the RNA code. This results in an extremely rapid mutation rate of $3.4 \times 10^{-5}$ mutations per base pair per replication cycle and the generation of $10^{10}$-$10^{12}$ new virions each day (155). HIV-1 is also marked by a high degree of recombination that occurs when an individual is infected with more than one strain of the virus, resulting in numerous circulating forms and the continued emergence of new variants or quasispecies within the host (186). In fact, viruses within the same clade can differ by as much as 20% whereas up to 35% variation is seen between subtypes (84). Remarkably, within a single individual, the amount of HIV-1 diversity can exceed that found during the entire global influenza epidemic, which requires production of a new vaccine each year (199). Thus, developing a single vaccine candidate that can cope with this rapid genetic diversification presents a considerable
challenge. Moreover, vaccine-mediated protection will have to rely on the capacity of immune responses to efficiently target highly heterologous viral strains.

**HIV-1 Genome Organization**

The HIV-1 genome is comprised of nine open reading frames of structural and nonstructural proteins. There are three major structural proteins common to all retroviruses: *gag* (group-specific antigen), *pol* (polymerase), and *env* (envelope protein) (Figure 1). Making up the core of the virion, *gag* encodes a polyprotein precursor, Pr55, which is cleaved into the matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p7), and p6. A frameshift mutation during Pr55 translation is necessary for Pol synthesis to occur. Pol encodes the viral enzymes protease, reverse transcriptase (RT), and integrase (IN) which are cleaved from Pr160<sub>GagPol</sub> by protease; these enzymes are encapsulated within the capsid core along with two identical strands of genomic length RNA. Also synthesized as a polyprotein precursor (gp160), Env is processed by a cellular protease during trafficking to the cell surface, resulting in the envelope proteins gp120 (surface component) and gp41 (transmembrane component). This gp120/gp41 complex is arranged into a functional trimer and plays an important role in fusing the viral and host lipid bilayers during virus entry into target cells. HIV-1 also encodes a number of regulatory proteins including Tat and Rev, which are responsible for transcription and transport of viral RNAs from the nucleus to the cytoplasm, respectively. Vpu, Vif, Vpr, and Nef are accessory proteins that were once thought to be unnecessary for virus replication, but recent studies suggest otherwise (124). The entire genomic 9.2
Figure 1. HIV-1 genome and structure. (A) The HIV-1 genome is composed of nine open reading frames containing fifteen proteins. Protein coding regions are shown as grey boxes; polyprotein-domain junctions are depicted as solid vertical lines. Genes start and end sites are numbered according to the NL4-3 lab-adapted strain. CA, capsid; IN, integrase; MA, matrix; NC, nucleocapsid; PR, protease; RT, reverse transcriptase. (B) Structure of the mature HIV-1 virion. The viral envelope is composed of a lipid bilayer containing the surface and transmembrane glycoproteins, gp120 and gp41, respectively. Within the envelope, the bullet shaped inner core or capsid contains two single-stranded genomic length RNA copies along with the viral enzymes RT, IN, and PR.

Note: (A) from “Architecture and secondary structure of an entire HIV-1 RNA genome” by Watts et al., *Nature*, 460, p. 712. Copyright 2009 Nature Publishing Group. Reprinted with permission. (B) Adapted from the National Institute of Allergy and Infectious Diseases.
kb sequence is flanked by 5’ and 3’ long terminal repeat (LTR) units containing the viral promoter and various regulatory elements needed for gene expression (56, 57).

HIV-1 transcripts also contain a number of alternative reading frames (ARF), both in the sense and antisense directions, which have the potential to encode proteins of unknown function. It was recently discovered that cryptic epitopes, derived from ARF, are frequently targeted in both acute and chronic HIV-1 infection, expanding the repertoire of epitopes recognized by the host immune system (9, 15).

**HIV-1 Replication Cycle**

HIV-1 preferentially infects activated CD4 T cells, but macrophages, which also bear the CD4 receptor, can become productively infected but usually with less cytopathic effects. Moreover, HIV-1 establishes a reservoir of latently infected, non-dividing cells carrying integrated proviral DNA which can become activated upon antigen recognition or as bystanders in the proinflammatory milieu (31). In fact, it is estimated that a single HIV-1-infected individual with no detectable viremia can harbor up to $10^7$ latently infected cells, mostly memory CD4 T cells (30). This selective targeting of the CD4 T cell compartment for destruction has catastrophic consequences on the immune system, as this subset is absolutely critical for coordinating the adaptive immune response by providing help to B cells and CD8 T cells.

The HIV-1 lifecycle is typical of other retroviruses and can be divided into two major phases: the early phase which is characterized from cell binding to integration of the viral cDNA into the host genome and a late phase beginning with expression of viral genes through release of mature virions (142). Through a high affinity interaction, the
envelope surface component gp120 binds to the CD4 molecule and engages a chemokine co-receptor, either CCR5 or CXCR4, located on the surface of the host cell to be infected (Figure 2). This leads to conformational changes in the envelope glycoprotein, thereby allowing gp120 to dissociate from gp41 and the subsequent transition of gp41 into its fusogenic format. Upon fusion of the viral and host plasma membranes, the viral core is then released into the cytoplasm, uncoated, and converted to the reverse transcription complex (RTC). This complex contains the viral genomic RNA, the MA, NC, RT, IN, and Vpr. RT then catalyzes conversion of the RNA genomes into double-stranded DNA. Afterwards, the viral DNA is transported to the nucleus as part of the pre-integration complex (PIC). IN catalyzes the insertion of the linear DNA into the host cell chromosome; this integrated DNA is now referred to as the provirus. Within the nucleus, viral transcripts are expressed, and a set of multiply spliced RNAs, encoding Tat, Rev, and Nef, move from the nucleus to the cytoplasm for translation and packaging. These viral proteins act to enhance HIV-1 transcription and facilitate moving the Gag and Gag-Pol polyproteins to the cell membrane to initiate viral particle assembly. The core particle is then assembled from the genomic RNA and transcripts encoding the remaining structural, enzymatic, and accessory proteins. Progeny virions subsequently bud from the surface of the host cell, mature, and are now ready to efficiently infect the next cell (56, 57, 142).

Since 1987, several classes of antiviral drugs have been developed, each targeting different stages of the HIV-1 lifecycle. These include inhibitors of entry/fusion, reverse transcription (nucleoside and nucleotide analogues and non-nucleoside inhibitors), integration, and maturation (protease) (160). While ART for the chronic suppression of
Figure 2. HIV-1 replication cycle. HIV-1 entry is mediated by the binding of the viral envelope protein, gp120, to the host cell surface receptor, CD4 and a co-receptor, either CCR5 or CXCR4. After fusion of the HIV-1 virion to the host cell, the HIV-1 core is released into the cytoplasm. Viral RNA is reverse transcribed into a DNA copy by the viral enzyme reverse transcriptase. The viral cDNA is then transported across the nucleus and integrates into the host cell DNA. The new viral RNA transcripts are transported to the cytoplasm and used as genomic RNA to make viral proteins on host cell machinery. Proteins assemble at the host cell plasma membrane and initiate budding of progeny viruses. Finally, new viruses undergo maturation to become infectious virions.

HIV-1 replication has been the major accomplishment of HIV/AIDS medicine, ongoing viremia can still be detected at levels of 1 to 50 copies per milliliter (mL) in the majority of patients (55). Therefore, there is a great need for continued research efforts to achieve a cure for HIV-1 so that ART is no longer a lifelong necessity.

**HIV-1 Immunopathogenesis**

HIV-1 immunopathogenesis may be viewed in the context of two distinct phases of the illness: the acute infection occurs after the first few days of mucosal exposure in which the virus preferentially infects and massively destroys the CD4 memory T cell compartment, and the chronic phase in which the crippled immune system slowly succumbs and viral replication rebounds (43, 193). Ultimately, ART-untreated individuals die from AIDS-related opportunistic infections within eight to ten years.

Studies in SIV-infected macaques have provided clues as to how transmission occurs, yet our understanding of these events during HIV-1 infection remains less clear. Our knowledge of the early immune response to HIV-1 has recently expanded with the advent of new technologies to identify patients within the first few weeks of infection. Exposure to HIV-1 occurs primarily by sexual transmission through the lower genital tract or rectal mucosa. Surprisingly, transmission is a rare event that usually requires multiple exposures, although success of infection is enhanced in the presence of co-existing genital infections (65, 77). Interestingly, through single genome amplification techniques, it has been discovered that approximately 80% of heterosexual, mucosally transmitted HIV-1 infections result from a single-transmitted or founder virus that gives rise to all the viral quasispecies within an infected individual (108).
By an unknown mechanism, HIV-1 crosses through the mucosa to the epithelial cell barrier where it makes contact with resident T cells and dendritic cells (DC). The time between infection and the detection of plasma viral RNA is approximately 10 days (eclipse phase). After the eclipse phase, the virus migrates to draining lymph nodes to encounter its targets of choice, activated CD4^+CCR5^+ T cells, thereby allowing further infection and dissemination to other lymphoid tissues including the gut-associated lymphoid tissue (GALT) (24). This induces an acute illness (either symptomatic or asymptomatic) that is associated with an exponentially high viremia, usually more than one million RNA copies per mL of blood, peaking between 21-28 days (34, 85). Coincident with this high viral load is the sharp decline of peripheral blood and GALT CD4 T cells and establishment of a pool of latently infected CD4 T cells (54). Subsequent development of an HIV-1-specific immune response induces a 100-1000-fold fall in the plasma viral load over the next 10-12 weeks to a more stable level known as the viral load set point, a strong predictor of disease outcome (88, 114, 131, 173). At the same time, peripheral blood CD4 counts partially rise to near normal levels, but this does not occur in the GALT where 80% of CD4 T cells are already destroyed (45, 129, 194).

A generally asymptomatic chronic infection ensues marked by a state of persistent immune activation. As a result of continuous antigenic stimulation, immune cells are subject to increased cell turnover, increased frequencies of cells with an activated phenotype, increased pro-inflammatory cytokine production, and functionally exhausted immune responses (83, 117). This has deleterious consequences on the immune systems of HIV-1-infected individuals, leading to drainage of the memory T cell compartment, damage to lymphoid tissue, and generation of targets for the virus itself, which drives
further replication and CD4 T cell depletion (75, 174). Recent studies have shown that translocation of microbial products into the blood through the damaged gut epithelium is a cause of immune activation in chronically HIV-1-infected individuals (23). Importantly, the degree of immune activation is a better predictor of HIV-1 disease progression than plasma viral load (39, 71). Thus, understanding the events that lead to mucosal damage and immune cell depletion will be critical for devising novel therapies to decrease hyperimmune activation and slow HIV-1 disease progression.

**Cellular Immune Responses during Successfully Cleared Viral Infections**

A major goal of HIV-1 vaccine design is to elicit antiviral CD8 cytotoxic T lymphocytes to control HIV-1 replication and CD4 T cells that can help induce and maintain CD8 T cell and B cell responses. It would therefore be prudent to define the attributes of antiviral CD4 and CD8 T cells which successfully eradicate acute viral infections to determine the types of responses that should be elicited in an effective HIV-1 vaccine.

CD8 T cells are critical for the elimination of intracellular pathogens such as viruses and bacteria, in addition to tumor transformed cells (202). Upon T cell receptor (TCR) recognition of antigen presenting cells bearing peptide/major histocompatibility complex (MHC) class I complexes, CD8 T cells undergo a program of clonal expansion and differentiation that allows for the acquisition of effector functionality (102). During this proliferative period, changes in gene expression and activation lead to antiviral cytokine and chemokine production as well as mobilization of cytolytic effector molecules such as perforin and granzymes; this results in a pool of CD8 T cells capable
of elaborating an array of functions necessary for cytolysis of infected target cells. Following viral clearance, the majority of activated CD8 T cells are subjected to a contraction and death phase, thereby leaving a fraction of memory cells capable of rapidly responding upon pathogen rechallenge (8, 103). The potency of this antiviral response is largely dependent upon the nature of the invading pathogen and receipt of adequate co-stimulatory signals during priming and CD4 T cell help (204, 214).

CD8 T cells require CD4 T cells for the generation of optimal CD8 T cell responses. Although CD8 T cells can be primed in the absence of CD4 T cells, the secondary expansion upon antigen re-encounter is inefficient under such circumstances (97, 128, 183, 207). CD4 T cells exert helper activities through several mechanisms. Indirect help to CD8 T cells is facilitated by CD40 ligand/CD40 interactions between CD4 T cells and antigen presenting cells, such as DC, to prime the CD8 T cell response (14, 168, 176). Alternatively, CD8 T cells can receive help directly through CD40 expression (21). Furthermore, CD4 T cells produce supportive cytokines, such as IL-2, which can directly act on CD8 T cells, thereby aiding in the initial expansion of the response and memory cell differentiation (7, 208). Thus, developing an in-depth understanding of the factors that regulate the induction and maintenance of antiviral T cell responses is essential to developing rational strategies to combat chronic viral infections, such as HIV-1, for which the current treatment and prevention methods are inadequate.
Role of CD8 T Cells in Control of HIV-1 Replication

The induction of a robust CD8 T cell response is vital to the purging of HIV-1-infected target cells. Seminal observations in simian immunodeficiency virus (SIV)-infection models have provided cues that CD8 T cells are necessary to control HIV-1 replication. First, in vivo transient CD8 T cell depletion dramatically increases viral load (98, 132, 175). Second, the emergence of SIV-specific CD8 T cells in acute infection leads to reduced viral replication and slowed disease progression (98, 175). In line with these observations, the precipitous fall in HIV-1 viral load after acute infection coincides with the emergence of HIV-1-specific CD8 T cell responses (20, 114). Third, viral immune escape from dominant CD8 T cell responses is associated with the loss of immune control (73). Finally, the role of CD8 T cells in controlling the virus is further emphasized by the strong influence of certain human leukocyte antigen (HLA) class I molecules in determining viral load set point and ultimately the rate of progression to AIDS (4, 27, 46, 68, 82, 106, 109, 110, 134, 138, 185). Taken together, these data provide unequivocal evidence that CD8 T cells are vital to HIV-1 control; however, the mechanism of this control remains unclear.

Disruption of T cell Homeostasis by Chronic HIV-1 Infection

Unlike acute infection, resolution of chronic viral infections, such as HIV-1, is associated with viral containment rather than eradication. Ongoing antigenemia induces profound functional defects in both CD4 T and CD8 T cells, and virus-specific cells fail to differentiate into memory cells. This loss of function is hierarchical, as proliferative potential, cytotoxicity, and IL-2 production are lost early, whereas TNF-α and IFN-γ are
compromised later (203). As HIV-1 infection progresses, viral control is lost and functional exhaustion ensues (17, 113).

The underlying mechanisms for T cell dysfunction were first recognized in mouse models of chronic infection, including lymphocytic choriomeningitis virus (LCMV), which results in defects similar to those seen in HIV-1 (72, 74, 76, 113, 118, 163, 164, 177, 188, 190, 191). From these studies, the molecular signatures of exhausted T cells are being elucidated. Sustained co-expression of multiple T cell inhibitory receptors including programmed death-1 (PD-1), T cell immunoglobulin and mucin domain-containing protein-3 (TIM-3), and lymphocyte activated gene-3 (LAG-3) is associated with T cell non-responsiveness (10, 18, 37, 58, 100, 107, 157, 158, 187). In addition, exhausted T cells downregulate cytokine receptors necessary for homeostasis, including IL-7 and IL-15 cytokine networks (101, 202). Notably, T cell exhaustion is exacerbated under conditions of high levels of chronic antigenic stimulation and reduced CD4 T cell help (61, 62, 128, 203).

**Protective T cell Responses are Polyfunctional**

Remarkably, fully functional CD4 and CD8 T cells are preserved in a cohort of rare HIV-1-infected individuals who spontaneously control viremia without ART (17, 105). Representing less than 1% of all infected individuals, elite controllers (EC) can remain clinically and immunologically stable for over 25 years without ART administration; this subset of individuals maintain viral loads below the limits of detection by standard assays (<50 copies HIV RNA/mL) and usually exhibit minimal rates of CD4 T cell decline over time (40). Therefore, EC represent an area of intense
investigation to elucidate the immune factors that differentiate these individuals from those with progressive disease (25, 141, 151, 179).

The frequency, breadth of epitope recognition, and functional quality of HIV-1-specific CD8 T cells likely contribute to the control seen in EC (1, 16, 47, 69, 123, 127). Increasing data indicate that the quality rather than the quantity of virus-specific CD8 T cell responses is a better correlate of immune protection. Numerous reports have shown that the functional profile of progressors (P) is largely skewed to an IFN-γ-dominated response (49, 50, 79). Conversely, the quality of the response in EC is varied from an IFN-γ-driven response to more balanced IL-2-producing response as demonstrated by a significantly elevated fraction of cells making IL-2 or other measures of CD8 T cell function (133, 162). Polyfunctional CD8 T cell responses inversely correlate with viral load, implying that the ability to secrete multiple antiviral molecules, especially IL-2, is associated with protective immunity and slowed disease progression (17, 49, 156, 162). The question of why EC maintain HIV-1-specific CD8 T cell responses with improved functionality remains to be fully understood, although this may partly be explained by the ability of EC to maintain cognate CD4 T cell help (105).

Lack of CD4 T cell Immunity may contribute to CD8 T cell Functional Exhaustion

While it is clear that HIV-1-specific CD8 T cells are critical mediators in controlling HIV-1, their sole presence, even in large numbers, fails to fully clear infection. It has been suggested that virus-specific CD8 T cells persist in an exhausted state due to a lack of CD4 T cell help. Since both antibody and CD8 T cell effector responses are coordinated and regulated by CD4 T cells, the involvement of these latter
cells in anti-HIV-1 immunity is almost implicit. Given that profound CD4 T cell loss is a hallmark of both primary and chronic HIV-1 infection, it is probable that a deficit of these cells is partly responsible for the failure of CD8 cells to fully control infection.

Several mouse models and human studies of chronic viral infection demonstrate that impaired CD4 T cell help influences the generation and maintenance of memory CD8 T cells (2, 12, 26, 41, 52, 70, 80, 111, 115, 121, 126, 128, 135, 166, 182, 197, 200). In the chronic infection model of LCMV, CD4-deficient mice exhibit severe functional exhaustion, and the physical deletion of IL-2, TNF-α, IFN-γ-producing cells is discernible by 3 months post-infection; this consequently leads to viral persistence (12, 128, 197, 214). In the absence of virus-specific CD4 T cells, exhausted HIV-1-specific CD8 T cells are characterized by a lack of effector function, including inability to secrete IFN-γ (72, 180). Robust HIV-1-specific CD4 T cell proliferative responses are preferentially found in EC and inversely correlate with viral load (169). Furthermore, a strong correlation exists between HIV-1-specific CD8 T cell precursor frequencies and HIV-1-specific proliferative T helper responses (104).

HIV-1-specific CD4 T cells co-expressing three antiviral cytokines, IL-2, TNF-α, and IFN-γ, are found in EC and but not P (105). The qualitative impairment of CD4 T cell function occurs very early in the course of infection prior to quantitative decreases in peripheral CD4 T cell numbers (139, 140). Thus, fully functional CD4 T cells may be required for the programming of potent polyfunctional, memory CD8 T cell responses.

Collectively, both CD4 T helper and CD8 T cell responses are critical determinants of the clinical sequelae associated with HIV-1 infection. Therefore, a major goal is to further understand the relevant immune responses that correlate with protective
immunity, which may provide insights into vaccine design. To further dissect the nature of CD4 T cell help during HIV-1 infection, the present studies have evaluated whether the helper factor, IL-21, produced primarily by CD4 T cells, contributes to efficacy of CD8 T cell functionality and viral containment.

**IL-21: a CD4 T Cell-derived Co-stimulatory Cytokine and Regulator of CD8 T cell Function**

Discovered in 2000, IL-21 is a four α-helical bundle type I cytokine belonging to the common \( \gamma (\gamma_c) \) chain receptor family that includes IL-2, IL-4, IL-7, IL-9, and IL-15. Produced primarily by CD4 T cells of the T helper 17 (Th17) and T follicular helper (T\( _{FH} \)) lineages, IL-21 is also made by natural killer (NK) T cells (33, 130, 145, 153, 181). However, it has been recently demonstrated that CD8 T cells are capable of IL-21 secretion (144). IL-21 signals through the IL-21 receptor (IL-21R) and has pleiotropic effects on a range of lymphoid lineages, including T cells, B cells, NK cells, DC, and macrophages (Figure 3) (6, 22, 120, 130, 145, 153). IL-21 enhances differentiation of B cells into plasma cells, proliferation of lymphoid cells synergistically in combination with IL-6, IL-7, or IL-15, and cytotoxicity and IFN-\( \gamma \) production by NK cells and CD8 T cells. Collectively, IL-21 has been demonstrated to promote activation and differentiation of innate and adaptive immune responses but has inhibitory effects on antigen presentation by DC, and can be pro-apoptotic for B cells and NK cells. Therefore, it is of prime interest to understand the complex regulation of IL-21, especially as it relates to influencing immunity to viral infections.
Figure 3. IL-21 is a pleiotropic cytokine with broad actions on innate and adaptive immune responses.

Regulation of IL-21/IL-21 Receptor Expression and Signal Transduction

In humans, the *IL21* gene is encoded on chromosome 4 and is adjacent to the *IL2* gene. IL-21 is a 131 amino acid length protein that is produced primarily by CD4 T cells and a subset of NKT cells upon calcium-dependent TCR activation. Nuclear factor of activated T cells (NFAT) binding sites in the IL-21 promoter region are responsible for regulating IL-21 transcription.

The human IL-21R is a 538 amino acid protein located on the chromosome 16, immediately downstream from the IL-4Rα but is more closely related to IL-2Rβ. In addition to its constitutive expression on lymphoid cells, the IL-21R is also expressed by fibroblasts, epithelial cells, and keratinocytes, consistent with the broad actions of IL-21. IL-21 signals through the IL-21R chain, that is coupled with the γc (CD132) and together comprises the cytokine receptor complex (181). Engagement of IL-21 with its receptor complex turns on the JAK/STAT pathway by phosphorylating Janus tyrosine kinase 1 (JAK1) and JAK3, primarily activating downstream signal transducer and activator of transcription (STAT) 3 and to a lesser extent STAT1 and STAT5. IL-21 can also activate the phosphoinositide 3 (PI3)-kinase /Akt and mitogen-activated protein (MAP) kinase pathways. Activation of these signaling pathways controls induction of transcriptional networks that confer cell lineage commitment and fate. Transcription factors controlled by IL-21 include B cell lymphoma-6 (Bcl-6), transcription factor 7 (Tcf7) and lymphoid enhancer-binding factor (Lef1), which are important for maintaining proliferation of cells that are less differentiated. In addition, IL-21 controls expression of Maf and B-lymphocyte induced maturation protein 1 (Blimp-1), thereby influencing terminal differentiation and effector functions (210).
**IL-21 Levels Influence Disease Pathology**

In addition to its role in lymphocyte development and function, it has become increasingly apparent that IL-21 plays a critical role in a variety of disease settings including autoimmunity, tumors, and infections. The ability of IL-21 to enhance NK cell- and CD8 T cell-mediated killing of tumors in mice has been translated to human clinical trials with results showing tumor regression in patients with metastatic melanoma, metastatic renal cell carcinoma, and relapsed/refractory indolent non-Hodgkin’s lymphoma (35, 36, 81, 201). However, augmenting immune responses with IL-21 administration could pose a considerable challenge as IL-21 is known for its critical involvement in the initiation and progression of inflammatory reactions where self-reactive immune cells or antibodies cause tissue damage. Indeed, elevated levels of IL-21 have been found in autoimmune diseases including psoriasis, type I diabetes, inflammatory bowel syndrome, and systemic lupus erythematosus. Taken together, these studies suggest that the level of IL-21 is a critical determinant of disease pathology (119).

**IL-21 and Antiviral CD8 T cell Immunity**

Most notably, IL-21 has been referred to as a “third signal” (in addition to MHC class I engagement and co-stimulation) for differentiation, expansion, and phenotype of bulk and antigen-specific CD8 T cells (33, 51, 120, 130, 143, 146, 147, 153, 181, 196). Indeed, transgenic mice engineered to overexpress IL-21 have a massive accumulation of the memory CD8 T cell pool with a concomitant with a reduction in naïve T cell numbers, suggesting an important role for this cytokine in driving memory formation (3). IL-21 has also been shown augment the ability of CD8 T cells to respond to co-
stimulatory ligands by sustaining CD28 expression on activated bulk and antigen-specific CD8 T cells (5, 122). Moreover, human CD8 T cells activated in the presence of IL-21 accumulate cytotoxic molecules perforin, granzyme B, and granulysin, thereby increasing their cytotoxic activity (89, 152). Interestingly, the acquisition of this highly cytotoxic function is associated with a memory phenotype, characterized by expression of CCR7 and CD27 in humans and CD44 and CD62L in mice (3, 86, 152).

Recent studies demonstrate a role for IL-21 in viral infections (90, 215). IL-21-deficient mice chronically infected with LCMV are unable to induce potent polyfunctional CD8 T cell responses (as seen by the absence of dual IFN-γ/IL-2-producing cells) and thus are unable to clear virus (48, 60, 211). Notably, T cell exhaustion was confirmed by increased expression of CD43 and PD-1 on virus-specific CD8 T cells (211). The requirements for IL-21 are less stringent following acute infections as mice deficient in the IL-21R exhibit intact responses to acute pathogens; however, in the absence of IL-21, these CD8 T cells have a reduced capacity to attain the polyfunctional trait of IL-2 production (48, 60, 184, 212). Based on these findings, it is clear that IL-21 is essential for the induction of polyfunctional responses and control of chronic viral infections.

**A Role for IL-21 in HIV-1 Infection**

Early *in vitro* studies in bulk CD8 T cells from HIV-1-infected patients showed a role for IL-21 in upregulating perforin expression in memory CD8 T cells, protecting CD8 T cells from undergoing apoptosis, and restoring cytotoxicity in combination with IL-15. On the other hand, IL-21 did not seem to augment proliferation of CD8 T cells or
increase degranulation (205). One other group has explored IL-21 levels in the serum of HIV-1 infected patients. IL-21 (as quantified by ELISA) in these patients on ART was significantly reduced compared to seronegative controls ($P=0.0087$) (93). Moreover, IL-21 has been used as an adjuvant in mice vaccinated with an env-expressing vaccinia virus, and induced strong anti-HIV-1 CD8 T cell responses and resisted viral challenge (19). Thus, the use of IL-21 as a therapeutic agent to boost HIV-1-specific CD8 T cell responses holds great potential.

**Concluding Remarks, Project Aims, and Rationale**

Successful antiviral immunity requires the elaboration of potent CD4 and CD8 T cell responses which act cooperatively to control the infection. It has been proposed that progressive loss of CD4 T cells in HIV-1 infection is associated with CD8 T cell dysfunction, but the mechanisms involved are not well understood. Recent studies in the murine model of LCMV indicate that CD4 T cell production of IL-21 is required for the maintenance of CD8 T cell function in chronic but not resolving viral infections. In light of the altered IL-21 production in HIV-1 infection and its proposed importance in the generation of effective virus-specific CD8 T cell responses, we hypothesize that the loss of polyfunctional CD8 T cells in chronic infection is a consequence of the functional abrogation of IL-21-producing HIV-1-specific CD4 T cells. The goal of this dissertation was to elucidate the quantitative and qualitative requirements of the latter cells in contributing to the generation of protective, long-lived HIV-1-specific CD8 T cell responses and viral control. The studies presented here contribute to the knowledge of the molecular mechanisms accounting for CD4 and CD8 T cell dysregulation in HIV-1
immunopathogenesis, thus providing new insights into the types of responses that should be elicited in an effective HIV-1 vaccine.
INTERLEUKIN-21-PRODUCING HIV-1-SPECIFIC CD8 T CELLS ARE PREFERENTIALLY SEEN IN ELITE CONTROLLERS

by

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ABSTRACT

A hallmark of human immunodeficiency virus type 1 (HIV-1) pathogenesis is the rapid loss of CD4 T cells leading to generalized immune dysfunction, including an exhausted CD8 T cell phenotype. Understanding the necessary factors that govern the functional quality and protective potential of antiviral T cell responses would facilitate rational vaccine design and improve therapeutic strategies to combat persistent infections. Mouse models of chronic viral infection demonstrate that interleukin-21 (IL-21), produced primarily by CD4 T cells, is required for the generation and maintenance of functionally competent CD8 T cells and viral containment. We reasoned that preserved IL-21 production during HIV-1 infection would be associated with enhanced CD8 T cell function, allowing improved viral control. Here we analyzed the ability of CD4 and CD8 T cells to produce several cytokines in addition to IL-21 ex vivo following stimulation with overlapping HIV-1 peptides. Both CD4 and CD8 T cells were able to produce IL-21 in response to HIV-1 infection, with the latter cell type more closely associated with viral control. Furthermore, IL-21-producing HIV-1-specific CD4 T cells (compared to those producing other cytokines) were the best indicator of functional CD8 T cells. Our results demonstrate that HIV-1-specific IL-21-producing CD8 T cells are induced following primary infection and enriched in elite controllers, suggesting a critical role for these cells in the maintenance of viremia control.

INTRODUCTION

Despite induction of ample virus-specific CD8 T cell responses, ongoing antigenemia precludes human immunodeficiency virus type 1 (HIV-1) containment in the
majority of patients. A hallmark of patients with primary HIV-1 infection (PHI) is the massive depletion of CD4 memory T cells (3, 24). This virus-induced destruction continues into chronic infection and is accompanied by the functional abrogation of robust HIV-1-specific CD4 T cell responses (19, 40). The impaired capacity of virus-specific CD4 T cells to provide cognate help to CD8 T cells compromises the protective immune responses necessary to control chronic viral infection (19). As such, HIV-1-specific CD8 T cells gradually become less functional and persist in an exhausted state, unable to elicit potent polyfunctional effector traits or effectively eradicate infected targets. Remarkably, polyfunctional CD8 T cells, capable of cytokine secretion, proliferation, and degranulation are maintained in HIV-1 controllers, chronically infected patients who spontaneously control viremia in the absence of antiretroviral therapy (ART) (1). Still, virus-specific T cell populations in these patients are considerably heterogeneous, and attributes that confer protection remain elusive. Understanding the necessary factors that regulate the induction, functional quality, and longevity of such responses is imperative for the rational design of preventative and therapeutic interventions against HIV-1.

The underlying mechanisms for T cell dysfunction were first recognized in mouse models of chronic viral infection, including lymphocytic choriomeningitis virus (LCMV), which results in defects similar to those seen in HIV-1 infection. From these studies, the molecular signatures of exhausted T cells are being elucidated. Alterations in expression of costimulatory common gamma-chain (γc) cytokines, such as interleukin-2 (IL-2), IL-7, and IL-15 are a common feature of many viral infections and have been shown to correlate with T cell non-responsiveness (18, 33). Notably, T cell exhaustion is
exacerbated in conditions of chronic antigenic stimulation and reduced CD4 T cell help, as seen in progressive HIV-1 infection (12, 13, 22, 34).

Several lines of evidence suggest that early CD4 T cell-mediated CD8 T cell priming events are crucial for programming vigorous effector and memory CD8 T cell responses. CD4 T cells are principal producers of IL-21, a γc cytokine referred to as a “third signal” (in addition to major histocompatibility complex class I [MHC-I] engagement and costimulation) for the differentiation, expansion, and phenotype of antigen-specific CD8 T cells (5, 30). IL-21/IL-21 receptor (IL-21R)-deficient hosts infected with LCMV exhibit exhausted CD8 T cells unable to clear virus, thereby resembling CD4 T cell-deficient hosts (9, 11, 37, 38). Strikingly, IL-21 administration reverses CD8 T cell exhaustion and reduces viral titers (37).

Early in vitro studies of bulk CD8 T cells from HIV-1 infected patients suggested a role for IL-21 in upregulating perforin expression in memory CD8 T cells, protecting CD8 T cells from undergoing apoptosis, and restoring cytotoxicity, the latter requiring IL-15. In contrast, IL-21 was not necessary to augment proliferation or increase degranulation (35). Definitive links to protective immunity came from subsequent studies showing enhanced suppression of HIV-1 replication following IL-21 exposure to CD8 T cells (6). In vivo evidence of the importance of IL-21 is demonstrated by reduced serum levels in HIV-1-infected patients and the finding that IL-21 production from total CD4 T cells correlates with disease progression (16, 17). However, the only study that comprehensively analyzed CD4 T cell responses by intracellular cytokine staining (ICS) demonstrated no major differences in HIV-1-specific IL-21 production when comparing chronically infected patients with various levels of viral control (39). These discordant
results highlight the importance of further studies examining HIV-1-specific CD4 and CD8 T cells in well-characterized cohorts.

Here we present a comprehensive analysis demonstrating that HIV-1 controllers and primary infected patients have qualitatively superior HIV-1-specific T cells capable of IL-21 secretion, which is associated with enhanced CD8 T cell functionality and viral containment. Using peripheral blood mononuclear cells (PBMC) from primary and chronically infected patients, we directly assess several attributes of HIV-1-specific IL-21-producing CD4 and CD8 T cells *ex vivo*, including the functional profile, frequency, and protein targeting of these populations. These data clearly indicate a relationship between the aforementioned parameters and the ability to curtail the infection, providing additional insights into complexity and qualitative requirements of immune protection against HIV-1 disease progression, thus aiding in vaccine design.

**MATERIALS AND METHODS**

**Study Subjects.** Forty-three HIV-1 infected patients were recruited from the University of Alabama at Birmingham (UAB) Adult AIDS 1917 clinic after informed consent and UAB institutional review board approval were obtained. PBMC were isolated from anticoagulated peripheral blood by Histopaque density gradient centrifugation and cryopreserved for later use. Subjects were stratified according to immune control of virus and categorized as follows: primary (*n* = 17) based on identification less than 6 months after infection (Fiebig stages II to VI); elite controllers (EC; *n* = 7), based on spontaneous control of viremia without ART initiation and undetectable viral loads (VL) (<50 RNA copies/ml plasma); viremic controllers (VC; *n* = 8), based on VL of >50 but <2000 RNA
copies/ml in the absence of ART; and progressors (P; n = 11), based on VL of > 10,000 RNA copies/ml in the absence of ART. All patients with chronic infection were not on ART, while 11/17 with primary infection began ART 2 to 4 weeks prior to sample collection.

**Viral load and CD4 T cell count determination.** HIV-1 RNA levels were quantified in plasma samples using the Amplicor ultrasensitive HIV-1 monitor assay, in accordance with the manufacturer’s protocol (Roche Diagnostics). The absolute CD4 T cell count was measured by flow cytometry using the flow count method and analyzed on a FACScan/FACSort instrument using MultiSET software (BD Biosciences).

**Peptides.** Synthetic peptides (15-mers overlapping by 11 amino acids; clade B consensus sequence; courtesy NIH AIDS Research and Reference Reagent Program) spanning the entire HIV-1 proteome were used to enumerate antigen-specific CD4 and CD8 T cell responses. Lyophilized peptides were reconstituted at 50 µg/ml in dimethyl sulfoxide or distilled water. Five pools were prepared – one corresponding to each HIV-1 antigen (Gag, Pol, Env, and Nef and accessory proteins Rev, Tat, Vif, Vpr, and Vpu were combined to form the final pool) – and used at final concentrations of 2 µg/ml. Optimized HLA class I-restricted epitopes (New England Peptide) were alternatively used to confirm CD8 T cell specificity.

**Intracellular cytokine staining assay.** PBMC were thawed, washed twice (in RPMI medium supplemented with 10% human AB sera), and resuspended at 2 x 10⁶ cells/ml.
Cells were then pulsed with the appropriate peptide pool (2 µg/ml) in the presence of costimulatory monoclonal antibodies (anti-CD28 and anti-CD49d; each at 1µg/ml), Benzonase (50 U/ml; Novagen), GolgiStop (10 µg/ml; BD Biosciences), GolgiPlug (10 µg/ml; BD Biosciences), and anti-CD4-Qdot 655 (BD Biosciences). An unstimulated and positive control (phorbol 12,13-dibutyrate [PDBu] and ionomycin [25ng/ml and 500 ng/ml, respectively; Sigma-Aldrich]) were included in each assay. Following a 5-h incubation period at 37°C in 5% humidified CO₂, cells were harvested and washed twice with phosphate-buffered saline (PBS) containing 5% fetal bovine serum (FBS) prior to labeling with a fluorescent LIVE/DEAD fixable dead cell dye (Molecular Probes, Invitrogen). Fluorochrome-conjugated monoclonal antibodies anti-CD3-Pacific Blue, CD8-Qdot 605, and CD45RO-allophycocyanin (APC) (BD Biosciences) were used for surface labeling. Following fixation and permeabilization with Caltag Fix and Perm reagents, cells were washed and phenotypically identified by staining with monoclonal antibodies against intracellular markers IL21-phycoerythrin (PE) (eBioscience), gamma interferon (IFNγ)-Alexa Fluor 700 (BD Biosciences), IL-2-fluorescein isothiocyanate (FITC) (BD Biosciences), IL-17-peridinin chlorophyll protein (PerCP)-Cy5.5 (eBioscience) and tumor necrosis factor alpha (TNFα) PE-Cy7 (BD Biosciences). Following staining, cells were washed, fixed in 2% paraformaldehyde (Sigma-Aldrich), and analyzed on an LSRII flow cytometer within 24 hours (BD Biosciences).

**Flow cytometric analysis.** At least 100,000 CD3⁺ events were acquired from each sample. Data analysis was performed using FlowJo version 8.1.1 software (Tree Star). CD3⁺ CD4⁺ or CD3⁺ CD8⁺ lymphocytes were gated based on forward and side scatter
properties after the exclusion of dead cells and doublets. Gates were set relative to the media control and subsequently applied to all samples per individual analyzed. Contemporaneous assays were performed using PBMC from HIV-1 uninfected individuals ($n=5$) to derive strict criteria for a positive IL-21 response. A positive response was determined to be (i) any value greater than or equal to the mean plus three standard deviations for all HIV-1-uninfected samples, as determined relative to medium control values, (ii) twice the medium control value for each individual, and (iii) greater than 0.05. A Boolean gating strategy was employed, and polyfunctionality was determined using PESTLE and SPICE software (courtesy of Mario Roederer, Vaccine Research Center, NIH).

**Quantification of IL21 and IFNG mRNA transcripts in CD4 and CD8 T cells.** CD4 and CD8 T cells from cryopreserved PBMC were obtained by negative selection (CD4$^+$ and CD8$^+$ T cell isolation kits, Miltenyi Biotec) and cocultured in RPMI medium containing 10% FBS with HIV-1 peptide-pulsed monocytes (stimulated with Gag, Env, Nef, or accessory peptide pools or HLA-A*03-, B*07-, and -B*57-restricted [QVPLPPMTYK {Nef}, HPVHAGPIA {Gag}, and KAFSPEVIPMF {Gag}, respectively] CD8 T cell epitopes) for 6 h. Total RNA was extracted from cell pellets using the AllPrep DNA/RNA/Protein minikit (Qiagen). mRNA transcripts (500 ng) were converted to cDNA with the RT$^2$ First Strand kit (SABiosciences) before being tested in real-time, quantitative PCR (custom RT$^2$ PCR Array, cat. no. CAPH09425; SABiosciences). The abundance of IL21 and IFNG mRNA was normalized to that of the housekeeping gene, beta-2 microglobulin ($\beta 2M$), after 40 cycle reactions in an ABI 7500
fast system (Applied Biosystems). Upregulation of IL21 and IFNG mRNA expression was determined against cells cultured in RPMI medium-10%FBS alone using the ΔΔC_T method, where C_T is the cycle threshold for SYBR green-based detection of gene-specific PCR amplicons (SABiosciences).

**Statistical analysis.** Nonparametric Mann-Whitney, Spearman rank correlation, and Fisher’s exact tests were performed using Graph Pad Prism software version 5.0. All tests were two tailed, and P values <0.05 were considered significant.

**RESULTS**

**Induction of antigen-specific IL-21 T cells during HIV-1 infection.**

Previous studies examining IL-21 in humans have shown its selective expression by CD45RO⁺ but not CD45RO⁻ T cells (4, 26). To assess HIV-1-specific T cell IL-21 production, we stimulated patient PBMC with overlapping peptide pools (HIV-1 clade B consensus sequence Gag, Pol, Env, Nef, and accessory proteins), followed by intracellular cytokine staining for IL-21, IFN-γ, IL-2, IL-17, and TNF-α. Using multiparametric flow cytometry, IL-21 was readily detected in the CD45RO⁺ CD4 T cell compartment (Fig. 1A and B), and a subset of responding cells displayed co-production of IFN-γ and, to a lesser extent IL-2 and TNF-α (Fig. 1C). However, virus-specific T cells coexpressing IL-21 and IL-17 or IL-17 alone were not observed, as previously reported (2, 11).

IL-21 production by polyclonal CD8 T cells from psoriasis patients and LCMV-infected mice has been demonstrated (11, 27). We therefore analyzed whether HIV-1-specific CD8 T cells were cellular sources of IL-21, responsible for sustained CD8 T cell
Figure 1. HIV-1-specific IL-21 T cell responses are detected in human peripheral blood. Representative flow cytometric analysis of intracellular staining for IL-21, IFN-γ, IL-2, TNF-α, and IL-17 production by HIV-1-specific CD4 and CD8 T cells. PBMC from an elite controller were stimulated for 5 h ex vivo with a peptide pool spanning Gag (15-mers overlapping by 11 amino acids; consensus B sequence) in the presence of anti-CD28, CD49d, GolgiStop, and GolgiPlug (A) Cells were gated based on light scatter properties, followed by dead cell exclusion (fluorescent dye), and gating for singlets and CD3+CD4+ or CD3+CD8+ T lymphocytes. SSC, side scatter; FSC-A, forward scatter area; FSC-H, forward scatter height. (B and D) Elaboration of IL-21-producing Gag-specific or PDBu-ionomycin-activated CD4 (B) or CD8 (D) T cells in the CD45RO- and CD45RO+ compartments after staining for IL-21 or the isotype control (post-cell permeabilization). (C and E) Frequencies of IL-21+ Gag or Nef-specific CD45RO+ CD4 (C) or Gag or Nef-specific CD8 (E) T cells that coproduced IFN-γ, IL-2, TNF-α, or IL-17.
functionality. Indeed, a modest but consistently reproducible population of IL-21-producing CD8 T cells, capable of IFN-γ production, was discernible after antigenic stimulation (Fig. 1D and E). Similar to CD4 T cells, IL-21-producing CD8 T cells did not co-express IL-17.

**Differential IL-21 secretion from HIV-1-specific T cells across an HIV-1 cohort.**

Given the data supporting the obligatory role of IL-21-producing T cells in immune-mediated viral containment, we reasoned that these cells represent at least one contributor to the differences in HIV-1 control observed among patients. Having established that CD45RO surface expression consistently marked IL-21+ T cells, we examined polyclonal and HIV-1-specific CD45RO+ T cell IL-21 production across HIV-1 cohorts consisting of primary HIV-1 infection (PHI), chronic with undetectable or low viremia (elite controllers [EC] and viremic controllers [VC], respectively), and chronic with uncontrolled viremia (progressors [P]) (Table 1). In marked contrast to uninfected persons, the proportion of polyclonal CD4 and CD8 T cells producing IL-21 in response to PDBu / ionomycin was substantially greater in HIV-1 patients, and this coincided with the degree of viral persistence (Fig. 2A and B). Notably, these responses were significantly increased in all cohorts except EC compared to those of seronegative subjects (Fig. 2B). A comparative analysis of HIV-1-specific responses revealed similar CD4 T cell IL-21 production across the cohorts (Fig. 2C). Furthermore, neither the frequency of individuals demonstrating responses to at least one peptide pool nor the total number of positive responses distinguished the patient groups (data not shown). In contrast, EC and VC maintained significantly higher percentages of HIV-1-specific IL-
21-producing CD8 T cells than P, with PHI displaying appreciable response heterogeneity albeit an overall lower median response (EC: 0.035 [interquartile range {IQR}, 0.006 to 0.078]; VC, 0.024 [IQR, 0.014 to 0.048]; P, 0.013 [IQR 0.004 to 0.024]; PHI, 0.009 [IQR, 0 to 0.044]) (Fig. 2D). This decreased fraction of IL-21-competent CD8 T cells in P was paralleled by a 3- to 4-fold reduction in frequencies of positive responses in these subjects (P=0.001 and 0.008 versus EC and VC, respectively) (data not shown). As expected, HIV-1-specific IL-21-producing T cells were not seen in seronegative controls (Fig. 2C and D). Therefore, the magnitude and frequency of the HIV-1-specific IL-21 CD8 T cell response is a better correlate of viral control than the IL-21 CD4 T helper response. However, among the progressor cohort, the magnitude of CD8 T cells producing IL-21 did not correlate with viral load or CD4 T cell count, suggesting that a threshold level of this cytokine may be needed for enhanced viral control (data not shown).

Table 1. Clinical characteristics of study participants

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Number of Patients</th>
<th>Median plasma VL*</th>
<th>Plasma VL (range)</th>
<th>Median CD4 T Cell Count**</th>
<th>CD4 T Cell Count (range)</th>
<th>ART</th>
</tr>
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<tbody>
<tr>
<td>Primary (PHI)</td>
<td>17</td>
<td>10,841</td>
<td>1,531-15,300,000</td>
<td>521</td>
<td>164-964</td>
<td>yes***</td>
</tr>
<tr>
<td>Elite Controllers (EC)</td>
<td>7</td>
<td>&lt;50</td>
<td>&lt;50</td>
<td>769</td>
<td>368-1741</td>
<td>no</td>
</tr>
<tr>
<td>Viremic Controllers (VC)</td>
<td>8</td>
<td>236</td>
<td>63-847</td>
<td>985</td>
<td>722-1620</td>
<td>no</td>
</tr>
<tr>
<td>Progressors (P)</td>
<td>11</td>
<td>86,900</td>
<td>18,501-484,600</td>
<td>514</td>
<td>334-874</td>
<td>no</td>
</tr>
</tbody>
</table>

*Viral load (VL) in HIV-1 RNA copies/ml at the time of sample collection; **in cells/µl; ***11/17 were on antiretroviral therapy (ART) for 2-4 weeks

To further define an optimal antiviral IL-21 response, we evaluated the extent to which viral control correlated with IL-21 responses directed against different viral proteins. Despite similar protein targeting by CD8 T cells, as measured by IFN-γ
production (data not shown), Gag- and Pol-specific IL-21-producing CD8 T cells were completely ablated in P, concomitant with their exclusive presence in PHI and persistence in EC and VC (Fig. 2F). In contrast, no protein-specific differences in IL-21 production by CD4 T cells were apparent (Fig. 2E). These results are consistent with increasing evidence demonstrating that preferentially targeting Gag, and possibly Pol, is strongly associated with improved clinical markers of disease progression (8, 14, 21, 23) and suggest that CD8 T cell IL-21 responses directed towards these proteins may contribute to the maintenance of viremia control.
Figure 2. Magnitude and responder frequency of IL-21-producing HIV-1-specific CD4 and CD8 T cells. PBMC obtained from primary HIV-1 infection (PHI), elite controllers (EC), viremic controllers (VC), and progressors (P) were stimulated with HIV-1 consensus B peptide pools (15-mers overlapping by 11 amino acids) spanning the entirety of the expressed proteome (pooled to represent Gag, Pol, Env, Nef, and the remaining accessory proteins). The magnitudes of CD45RO<sup>+</sup> CD4 (A) and CD8 (B) T cells producing IL-21 are shown, with horizontal bars indicating median values. The percentages of patients with HIV-1-specific CD45RO<sup>+</sup> CD4 (C) and (D) CD8 T cells producing IL-21 in response to peptides from the indicated proteins are shown. Statistical comparisons were made using the two-tailed Fischer’s exact test. *, P = 0.03 and **, P = 0.006 (compared to results for P). #, P < 0.0001 compared to results for each group of HIV-1 seropositive patients by the Mann-Whitney U test.
HIV-1-specific *IL21* mRNA analysis mirrors protein expression by ICS.

To further elucidate the specificity of IL-21 CD8 T cell secretion, we stimulated PBMC *ex vivo* with optimized HLA class I-restricted CD8 T cell epitopes. Notably, when CD4 T cells were depleted prior to stimulation (by negative selection [>99% purity] (data not shown), mRNA analysis of isolated CD8 T cell populations demonstrated *IL21* and *IFNG* upregulation in response to HLA-A*03-, -B*07-, and -B*57-restricted epitopes (QVPLPPMTYK {Nef}, HPVHAGPIA {Gag}, and KAFSPEVIPMF {Gag}, respectively), further validating the foregoing results (Fig. 3A).

To confirm the observed differences in CD8 T cell IL-21 production across the cohorts (Fig. 2D), we next compared the transcriptional profiles of a subset controllers and progressors either exhibiting or lacking these responses, respectively (as measured by flow cytometry). Following HIV-1 peptide pool stimulation, quantitative PCR analysis of purified CD8 T cell fractions revealed that controllers uniformly upregulated *IL21* mRNA (4/4 responses), whereas none of the progressors substantially amplified transcripts (0/3 responses; *P* = 0.03; Fig. 3B). Moreover, the frequency of CD8 T cells producing IL-21, as assessed by flow cytometry, directly correlated with the abundance of *IL21* transcripts (*P* = 0.003, correlation coefficient [r] = 0.96).
Figure 3. **IL21 mRNA expression by HIV-1-specific CD8 T cells.** CD8 T cells from controllers (n = 3) and progressors (n = 3) were negatively selected from PBMC by magnetic bead isolation (99% purity). T cells were cocultured with HIV-1 peptide-pulsed monocytes [stimulated with HLA class I A*03-, B*07-, or B*57-restricted epitopes [QVPLPPMTYK {Nef}, HPVHAGPIA {Gag}, or KAFSPEVIPMF {Gag}, respectively] (A) or HIV-1 peptide pools (comprising either Gag, Env, Nef, or accessory proteins) (B) and activated in the presence anti-CD28 and anti-CD49d for 6 h. The abundance of IL21 and IFNG mRNA was determined by real-time quantitative PCR and normalized to the housekeeping gene, beta-2 microglobulin (β2M). Upregulation of IL21 and IFNG mRNA expression was determined using the ΔΔC_T method, where data are expressed as fold changes relative to cells cultured in RPMI-1640 alone. Numbers above bars indicate the fraction of stimulations which resulted in IL21 fold change values of >2. *, 4/4 responses in controllers versus 0/3 responses in progressors; P=0.03 by Fisher’s exact test. Results are reported as median values, with error bars demonstrating the interquartile ranges, and are representative of at least two experiments. Comparison of the magnitudes of IL21 fold changes; P=0.06 by Mann-Whitney U test.
**IL-21-producing CD4 T cells are more multifunctional in elite controllers.**

Given the role of polyfunctional T cells in viral control, we speculated that IL-21 production in conjunction with other cytokines may be an important determinant of protective immunity (1, 20). To examine the relationship between percentage of polyfunctional IL-21-producing T cells and viral load, a Boolean gating strategy was employed for HIV-1-specific CD4 and CD8 T cells, yielding 15 unique response patterns comprising every combination of the four potential measurements (IL-21, IFN-γ, IL-2, and TNF-α). Despite the lack of association between the magnitude of IL-21 CD4 T cell responses and viral control, we observed distinct differences in functional quality of HIV-1-specific CD4 T cells that produced IL-21 (Fig. 4A). Specifically, EC and VC maintained significantly greater proportions of multifunctional cells capable of simultaneously expressing two or more functions, whereas a largely monofunctional phenotype was observed in PHI and P (Fig. 4A). Overall, EC exhibited a superior functional profile comprised of greater than 20% of responding cells being positive for three or four functions compared to less than 5%, as seen in the other cohorts ($P < 0.03$) (Fig. 4A). Conversely, while the functionality of antiviral IL-21-producing CD8 T cells was not significantly enhanced in EC compared to that in VC and P, it was significantly higher than that in PHI (Fig. 4B).
Figure 4. IL-21-producing CD4 T cells are more functional in elite controllers. CD45RO+ CD4 (A) and CD8 (B) T cells that produced IL-21 were analyzed for their ability to coproduce IFN-γ, IL-2, or TNF-α using SPICE. The magnitudes of virus-specific mono- and multifunctional CD4 and CD8 T cells producing at least IL-21 from PHI, EC, VC, and P cohorts were assessed. Horizontal bars indicate median percentages of IL-21+ T cells. Statistical comparisons were made using the two-tailed non-parametric Mann-Whitney U test. **, greater 3 or 4 functions; P < 0.03 versus PHI, VC, and P cohorts.

Association of CD4 T cell IL-21 production with CD8 T cell functionality.

We hypothesized that the functional impairment of IL-21-producing CD4 T cells compromised CD8 T cell functional quality (i.e., IL-2 production) and memory potential, similar to those of IL-21-deficient mice (37, 38). Therefore, we assessed whether the total magnitude of HIV-1-specific IL-21 production from CD4 T cells correlated with the total magnitude of virus-specific CD8 T cell IL-2 secretion. When all patients were grouped together, we found that IL-21 modestly correlated with the percentages of CD8
T cells expressing IL-2 \((r=0.54, P=0.0003)\) (Fig. 5A). This correlation remained significant even when the data were limited to CD4 T cells that produced IL-21 in the absence of the other cytokines \((r=0.37, P=0.01)\) (Fig. 5B). However, the numbers of samples studied was too small to discern whether polyfunctional IL-21-producing CD4 T cells were better correlates of IL-2\(^{+}\) CD8 T cells compared with their monofunctional counterparts. Despite demonstrating a modest relationship between total IL-2\(^{+}\) CD4 and IL-2\(^{+}\) CD8 T cells \((r=0.45, P=0.003)\) (Fig. 5C), this association was no longer significant when examining the fraction of CD4 T cells that produced IL-2 without co-expression of IL-21 \((r=0.21, P=0.18)\) (Fig. 5D), suggesting that IL-2 and IL-21 act cooperatively to induce protective, polyfunctional CD8 T cell responses. Interestingly, when we restricted our analysis to the stratified cohorts, PHI sustained these associations, although trends were apparent in EC (Table 2, compare the \(r\) values). Hence, these findings further solidify that the link between IL-21-mediated T helper function and subsequent acquisition of CD8 T cell multifunctional traits is established in primary infection but rapidly lost in chronic, progressive infection.

### Table 2. Association of HIV-1-specific CD4 T cell cytokine production with CD8 T cell IL-2 production.

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>PHI</th>
<th>EC</th>
<th>VC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(p)</td>
<td>(r)</td>
<td>(p)</td>
<td>(r)</td>
</tr>
<tr>
<td>CD4 IL-21 total</td>
<td>0.0003</td>
<td>0.54</td>
<td>0.01</td>
<td>0.63</td>
</tr>
<tr>
<td>CD4 IL-21 alone</td>
<td>0.01</td>
<td>0.37</td>
<td>0.03</td>
<td>0.54</td>
</tr>
<tr>
<td>CD4 IL-21 without IL-2</td>
<td>0.02</td>
<td>0.37</td>
<td>0.03</td>
<td>0.53</td>
</tr>
<tr>
<td>CD4 IL-2 total</td>
<td>0.01</td>
<td>0.45</td>
<td>0.04</td>
<td>0.52</td>
</tr>
<tr>
<td>CD4 IL-2 alone</td>
<td>0.29</td>
<td>0.17</td>
<td>0.21</td>
<td>0.33</td>
</tr>
<tr>
<td>CD4 IL-2 without IL-21</td>
<td>0.18</td>
<td>0.21</td>
<td>0.14</td>
<td>0.38</td>
</tr>
</tbody>
</table>

All CD4 T cell cytokine production was compared to total CD8 T cell IL-2 production.  
\(P\) and \(r\) values were calculated using Spearman rank correlation test.
Figure 5. IL-21-producing CD4 T cells predict functional CD8 T cells. Correlation between the magnitude of HIV-1-specific CD4 T cell cytokine production (summed across all peptide pools) with CD8 T cell IL-2 production. CD45RO^+ CD4 T cells expressing IL-21 in combination with other cytokines (A), IL-21 alone (B), IL-2 in combination with other cytokines (C), or IL-2 without co-expression of IL-21 (D) were plotted against the corresponding proportion of HIV-1-specific IL-2^+ CD45RO^+ CD8 T cells. P and r values were calculated using the Spearman rank correlation test.
DISCUSSION

Collectively, these data extend our understanding of the regulation of IL-21-producing antiviral T cell responses during primary and chronic HIV-1 infection. Previous reports of mice chronically infected with LCMV have demonstrated that virus-specific CD8 T cell responses elicited in the absence of IL-21 are functionally inferior, lacking the necessary attributes to fully clear virus-infected cells (9, 11, 37, 38). Indeed, in different cohorts of HIV-1-infected patients with distinct viral loads, we confirm that antigen-experienced CD4 T cells are a major cellular source of IL-21 and further define a previously unappreciated role for HIV-1-specific CD8 T cells – IL-21 secretion capacity, which is not detectable in the majority of patients with persistent viremia. Notably, IL-21-producing HIV-1-specific CD8 T cells responding against Gag and Pol epitopes were absent in progressors, while those producing IFN-γ and even IL-2 to these proteins were still present. Whereas the overall magnitude and frequency of CD4 T cell-derived HIV-1-specific IL-21 production did not correlate with viral load, elaboration of a polyfunctional IL-21 response reliably predicted HIV-1 containment. This poor induction of IL-21 by CD4 T cells seemed to translate into an inability of CD8 T cells to effectively produce IL-2, a parameter consistently linked with improved control of virus (42). Thus, our findings are consistent with the essential role that IL-21 plays in containing murine viral infections.

Several studies have now convincingly demonstrated that IL-21 impacts HIV-1 disease chronicity, albeit with somewhat paradoxical findings (6, 16, 17, 39). It has been proposed that the stepwise and progressive impairment of IL-21 in HIV-1, from serum and ionomycin-activated CD4 T cells, occurs with disease progression, despite the lack of
association with viral load (6, 16). This view is in contrast to studies by our group and others showing the sequential elevation of polyclonal IL-21-producing CD4 T cells into chronic infection, such that levels exceed those found in seronegative controls (39). This heightened IL-21 production was common to all patients with the exception of a few EC, a phenomenon reminiscent of the chronic immune activation prominently associated with this disease (28, 39). Indeed, this interpretation is consistent with the involvement of IL-21 in inflammatory bowel disease, triggering aberrant immune responses directed towards microbial constituents, thereby potentiating inflammation and damage to the mucosal barrier (25, 36).

While not demonstrated in this analysis, prior studies revealed the rapid loss of HIV-1-specific IL-21+ CD4 T cells following primary infection (6, 39). The basis for this apparent discrepancy is not clear but does not exclude the possibility that differences in PHI cohort characteristics (time of sample collection from date of infection, etc.) are a likely culprit. Nevertheless, the two studies that comprehensively analyzed IL-21 directly by flow cytometry were unable to show significant differences in the induction of these responses in long-term nonprogressors versus chronically infected patients with uncontrolled viremia, suggesting that compromised IL-21 secretion likely reflects early insults to the CD4 T cell compartment (39). The notion that HIV-1 uniformly disables CD4 T cell function following primary infection, regardless of controller or progressor classification, is further supported by a number of studies that argue against the assumption that elite controllers are exempt from a spectrum of immune alterations, namely immune activation. The fact that the memory CD4 T cell pool is already depleted prior to the chronic phase, compounded by the limited reconstitution of the memory CD4
but not CD8 T cell pool, could provide an explanation as to why the CD4 fraction is
damaged while the CD8 pool is left relatively intact (7). In line with these observations,
levels of activated HIV-1-specific CD38^+HLA-DR^+ CD4 T cells are similar among elite
controllers and noncontrollers whereas only elite controllers maintain lower levels of
activated CD8 T cells (28). Thus, our findings are consistent with the foregoing
observations whereby CD4 T cell function appears similar while CD8 T cell function is
enhanced in the EC cohort.

Seminal studies established IL-21 as a principal product of CD4 T cells.
However, increasing evidence supports that IL-21 production is not exclusively restricted
to CD4 T cells. *IL21* mRNA expression in CD4-depleted splenocytes of LCMV-infected
mice suggested that natural killer T cells, B cells, macrophages, and dendritic cells are
cellular sources (9). In accordance with the present findings, polyclonal CD8 T cell IL-21
production has been demonstrated in mice infected with LCMV via mRNA and
protein expression, albeit at reduced levels compared to those of CD4 T cells (11).
Furthermore, the latter cells have been implicated in the pathogenesis of psoriasis and
immune thrombocytopenia in humans, suggesting a biological ability rather than a virus-
specific modification (27, 41). Although it remains unresolved which cells are directly
responsible for mediating viral control, our data favors the participation of CD4 and CD8
T cell subsets during HIV-1 infection. Thus, it is tempting to speculate that CD8 T cells
from HIV-1 controllers are endowed with IL-21 secretion capabilities to self-sustain CD8
T cell homeostasis (11). Indeed, activation of purified CD8 T cells differentiated in the
presence of anti-CD3/CD28 and IL-21 results in robust *IL21* mRNA expression (15).
A connecting hypothesis among the reports describing antigen-specific IL-21 is that this cytokine is a critical player in enhancing the functional quality and protective potential of CD8 T cell responses. It has been suggested that since IL-2 is rapidly abolished early in infection, IL-21 may compensate for the lack of IL-2 helper function during chronic infection (9). It is noteworthy that HIV-1-specific IL-21, but not IL-2 produced alone, from CD4 T cells correlates with CD8 T cell IL-2 secretion only during primary and possibly controlled chronic infection. From these data, it appears that although IL-21 responses are present in progressors, they fail to effectively program CD8 T cells to produce IL-2. It is plausible that the phenotypic alterations induced by HIV-1, including collapse of lymph node architecture, could potentially disrupt CD4/CD8 T cell interactions and prevent crucial CD4 T cell-mediated costimulatory signaling required for optimal CD8 T cell function (31, 32). Moreover, the preferential preservation of lymph node architecture in long-term nonprogressors further supports this idea (10, 29). We propose that IL-21, along with IL-2 and other helper factors, act cooperatively to regulate CD8 T cell functional competence, which may be a consequence of the tightly clustered genomic organization of IL-21 and IL-2 (30).

While the studies presented here do not demonstrate causality, they nonetheless reveal a unique quality in elite controllers that may explain their superior viral control, namely, the ability to elicit IL-21-producing CD8 T cells. Furthermore, we provide evidence that HIV-1-specific CD8 T cells producing IL-21 are a better correlate of HIV-1 control than their CD4 T cell counterparts. These findings represent a key step towards improving our understanding of the composition of optimal HIV-1-specific T cell
repertoires, which is directly relevant to strategies aiming to enhance vaccine efficacy or restore fatigued T cells.

ACKNOWLEDGEMENTS

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L.D.W., S.S., A.B., and S.L.H. contributed to the design of the experiments, analyses of T cell responses, and manuscript preparation. W.S. and J.T. provided expertise in performing mRNA analysis. A.J.Z. contributed to the conception and design of experiments as well as preparation of the manuscript. P.A.G. supervised the entire project, designed and coordinated experiments, and contributed to manuscript preparation.
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IMMUNE ACTIVATION DRIVES CD8 T CELL IL-21 PRODUCTION
IN HIV-1-INFECTED INDIVIDUALS

by

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Format adapted for dissertation
ABSTRACT

We have previously demonstrated that interleukin-21 (IL-21), produced primarily by CD4 T cells, is also produced by CD8 T cells from individuals who spontaneously control HIV-1 replication. Our current work demonstrates that we do not observe IL-21-producing CD8 T cells in other viral infections. As such, we hypothesized that under conditions of reduced CD4 T cell help, as seen in HIV-1 infection, CD8 T cells producing IL-21 may partially compensate for this loss. Here, we analyzed the phenotype of IL-21-producing CD8 T cells for functions generally attributed to CD4 T cells and compared them to cells producing IFN-γ in HIV-1-infected individuals. Upon polyclonal stimulation with PDBu/ionomycin, IL-21-producing CD8 T cells exhibited some characteristics of helper T cells such as impaired production of perforin and granzyme B and CD40 ligand upregulation; however, we also observed limited expression of the chemokine receptor CXCR5, suggesting a functional profile distinct from that of the T follicular helper subset. We further show the absence of IL-21⁺ CD8 T cells in persons with idiopathic CD4 lymphocytopenia, suggesting that induction of IL-21 competent CD8 T cells in HIV-1-infected individuals is independent of CD4 T cell loss. Instead, elevated IL-21 production by CD8 T cells was associated with high levels of activated CD38⁺HLA-DR⁺ CD8 T cells that could persist into chronic infection despite ART-induced CD4 T cell rebound. Thus, it is likely that these cells emerge as a consequence of excessive chronic immune activation, rather than CD4 lymphopenia-induced HIV-1 disease progression.
INTRODUCTION

T cell dysfunction is a salient feature of chronic viral infections and prevents optimal viral control (34). In particular, infection with human immunodeficiency virus type 1 (HIV-1) results in the sustained depletion of memory CD4 T cells accompanied by persistently high levels of virus replication and defective virus-specific cellular and humoral immune responses (3, 12, 20). A growing body of evidence also suggests that systemic immune activation plays a central role in driving CD4 T cell depletion and ultimately progression towards AIDS (8).

Development of effective immunity against chronic viral infections requires the coordinated action of distinct cell subsets, which function to dampen productive viral replication. In particular, CD4 T cells have been implicated in provision of help to B cells and CD8 T cells through direct cell-to-cell interaction or secretion of soluble factors, leading to the improved potency and quality of antiviral immune responses (17, 19). Since its discovery as a principal product of CD4 T cells, interleukin-21 (IL-21) has emerged as a key helper factor required for control of chronic viral infections, owing to its ability to influence the initiation and maintenance of B cell high affinity antibody production and CD8 T cell cytolytic activity (37). Mice deficient in IL-21 exhibit functionally exhausted CD8 T cells upon chronic infection with lymphocytic choriomeningitis virus (LCMV) and are thus unable to mediate viral clearance (9, 10, 38, 39). Similarly, compromised IL-21 production has been noted in the context of human chronic viral infections, particularly during human immunodeficiency virus-1 (HIV-1) infection, thus providing a plausible explanation for dysregulated immune responses and
lack of viral containment (4, 14, 35, 40). Nevertheless, the effects of IL-21 are pleiotropic, and its precise roles during HIV-1 infection have not been fully elucidated.

It is now recognized that a diversity of innate and adaptive immune cells produce IL-21, including natural killer T cells, B cells, macrophages, and dendritic cells, albeit at reduced levels compared to CD4 T cells (5, 9). An increasing number of reports have suggested that cells bearing the glycoprotein CD8 are permissive to IL-21 secretion, and readily detectable, expanded bulk populations of these cells have been described in the settings of HIV-1 infection and autoimmune inflammation, the latter found specifically in psoriasis, immune thrombocytopenia, and systemic lupus erythematosus (7, 22, 26, 35, 41). Indeed, we have previously shown that HIV-1-specific IL-21 production is not limited to CD4 T cells and have found an enriched fraction of HIV-1-specific IL-21-producing CD8 T cells in HIV-1 elite controllers, those subjects able to spontaneously control viremia in the absence anti-retroviral therapy (ART) (35). The basis for this apparent division of IL-21 competency between CD4 and CD8 T cell subsets is unclear, but it is reasonable to speculate that irrespective of cellular source, the principal and requisite role of IL-21 during HIV-1 infection is to exert helper function.

The notion that CD4− T cells may co-opt CD4 T helper function is supported by a number of studies in rodent and non-human primate models of infection in which the CD4 gene has been disrupted or CD4 T cells have been depleted, respectively. Early work revealed the existence of CD4−CD8− T cells in CD4−/− mice that are capable of adopting characteristics of T helper cells in response to Leishmania major, LCMV, and vesicular stomatitis virus (18, 27, 29). Moreover, African green monkeys (Agm), natural hosts of simian immunodeficiency virus, which are known to have characteristically low
frequencies of CD4 T cells, possess a population of CD4$^+$CD8$^{\alpha \text{dim}}$ T cells that elicits functions generally attributed to CD4 T cells (1, 24, 25, 32). Given that progressive CD4 T cell extinction is a hallmark of HIV-1 infection, such a compensatory mechanism could conceivably allow for CD8 T cells to acquire IL-21 competency and subsequent helper function.

To test this hypothesis, we sought to better define the functional significance of IL-21 competent CD8 T cells and their relation to conventional CD4 T cells. We find that IL-21$^+$ CD8 T cells lack CD4 surface expression and that CD4 T cell loss is not a requirement for induction of IL-21-producing CD8 T cells with some, but not all, functional characteristics of classical CD4 T cells. We further demonstrate that these cells arise as a result of the chronic immune activation typically associated with HIV-1 infection. These studies have implications for understanding the factors that contribute to the T cell-driven inflammation that characteristically fuels HIV-1 disease progression.

MATERIALS AND METHODS

Study Participants

A total of sixty-one individuals were included in this study and categorized into three cohorts: 1. A cross-sectional cohort recruited from the University of Alabama at Birmingham (UAB) consisting of twenty chronically HIV-1-infected individuals off antiretroviral therapy (median viral load: 18,282 copies/ml plasma, median CD4 count: 581 cells/mm$^3$), 2. A cohort comprised of twelve HIV-1-infected subjects enrolled from UAB, with longitudinal samples collected prior to ART initiation and 6 to 12 months after
achieving undetectable plasma viremia on ART, 3. A cohort of twenty-nine subjects recruited from the National Institute of Allergy and Infectious Diseases (NIAID) consisting of nine HIV-1 seronegative controls, ten HIV-1-infected individuals off ART, and ten patients with idiopathic CD4 lymphocytopenia (ICL) syndrome. ICL inclusion criteria were at least two documented absolute CD4 T cell counts of less than 300 cells/μl or less than 20% of total T cells in the absence of infection on HIV-1 or HIV-2 testing, other known causes of immunodeficiency, or therapy associated with depressed levels of CD4 T cells. This study was approved by the Institutional Review Boards of the UAB and the NIAID, and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

**Peripheral blood mononuclear cell stimulation**

Cryopreserved peripheral blood mononuclear cells (PBMC), isolated by density gradient centrifugation, were thawed and washed twice in complete R10 medium containing RPMI 1640 (Gibco) supplemented with 10% human AB serum (Gemini Bio-Products), HEPES (25 mM; Gibco), L-glutamine (2 mM; Sigma-Aldrich), penicillin-streptomycin (100 U/ml penicillin and 100 µg/mL, respectively; Sigma-Aldrich), and benzonase nuclease (50 U/ml; Semba Biosciences). PBMC were resuspended at a concentration of 4 million cells/ml in complete R10 medium and activated in the presence of co-stimulatory monoclonal antibodies anti-CD28 (1 µg/ml; BD Biosciences) and anti-CD49d (1 µg/ml; BD Biosciences), GolgiStop (10 µg/ml; BD Biosciences), GolgiPlug (10 µg/ml; BD Biosciences), and anti-CD4-Qdot655 (S3.5; Molecular Probes, Life Technologies). For
assessment of CD40 ligand expression, cells were co-cultured with CD154-APC (TRAP-1; BD Biosciences) in the absence of Golgi-Plug containing brefeldin A. Two million PBMC were stimulated in the presence of Phorbol 12,13-dibutyrate (PDBu) (25 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich) or a CMV-pp65 peptide pool comprised of 69 18-mers overlapping by 10 spanning the entire amino acid sequence of the HCMV protein (2 µg/ml; New England Peptide). An unstimulated (media) control was included for each sample. Cultures were incubated at 37°C and 5% CO₂ for 5 hours and placed at 4°C overnight.

**Intracellular cytokine staining**

Following stimulation, cells were washed twice with phosphate buffered saline (PBS) prior to labeling with fluorescent LIVE/DEAD fixable blue dead cell stain kit for UV excitation (Molecular Probes, Life Technologies) for 30 minutes. Cells were subsequently washed with PBS containing 1% fetal bovine serum (Gemini Bio Products) and surface stained for 30 minutes at 4°C with fluorochrome-conjugated monoclonal antibodies against CD3-Pacific Blue (UCHT1; BD Biosciences), CD3-APCeFluor780 (UCHT1; eBioscience), CD8-PerpCy5.5 (SK1; BD Biosciences), CD45RO-PECy7 (UCHL1; BD Biosciences), CD38-Brilliant Violet 421 (HIT2; BD Biosciences), HLA-DR-PECy7 (G46-6; BD Biosciences), CXCR5-Alexa Fluor488 (RF8B2; BD Biosciences) and ICOS-eFluor450 (ISA-3; eBioscience). Following fixation and permeabilization with BD Cytofix/Cytoperm reagent, cells were washed and intracellularly stained for 30 minutes at 4°C employing monoclonal antibodies against
IL-21-PE (3A3.N21; BD Biosciences), IFN-γ-Alexa Fluor700 (B27; BD Biosciences), perforin-FITC (BD-48; AbCam), and granzyme B-V450 (GB11; BD Biosciences). Following staining, cells were fixed in 2% paraformaldehyde solution (Sigma-Aldrich) prior to flow cytometric analysis within 24 hours.

**Flow cytometric analysis**

Approximately 500,000 to 1,000,000 events were collected per sample on a LSRII multicolor flow cytometer (BD Biosciences). Electronic compensation was performed using antibody capture beads (CompBeads; BD Biosciences) stained separately with individual fluochrome-conjugated monoclonal antibodies used in each experiment. Data were analyzed using FlowJo version 9.2 (Tree Star). Lymphocytes were gated on the basis of light scatter properties [forward scatter area (FSC-A) versus side scatter area (SSC-A)], followed by exclusion of dead cells (viability stain) and doublets by plotting side scatter width (SSC-W) versus side scatter height (SSC-H) and subsequent plotting for forward scatter width (FSC-W) versus forward scatter height (FSC-H). Cells were then gated on CD4 T cells defined as CD3⁺CD4⁺CD8⁻ T cells or CD8 T cells consisting of CD3⁺CD8⁺CD4⁻ T cells. Gates were set based on the unstimulated control for each sample, and all cytokine/effecter functions were background subtracted relative to the unstimulated control values. Contemporaneous assays were performed in HIV-1 seronegative individuals to determine the criteria for a positive response. A positive response was deemed to be any value higher than twice the medium control value for
each individual and greater than the mean plus three standard deviations for all HIV-1 uninfected samples.

**Statistical analysis**

Statistical analysis was performed using Prism software version 5.0 (GraphPad Software). Group medians were compared with the nonparametric Mann-Whitney U test or the Wilcoxon signed rank test for paired samples (pre- and post-ART) derived from the same individual. The Spearman rank test was used to assess correlations between variables. Two-tailed $P$-values of less than 0.05 were considered significant.

**RESULTS**

**IL-21-producing CD8 T cells are selectively induced in the context of HIV-1 infection.**

We recently provided evidence to support that HIV-1-specific IL-21 production is not exclusively restricted to CD4 T cells and described a population of IL-21-producing CD8 T cells which is prominently enriched in HIV-1 controllers, an attribute that may reflect the superior viral control observed in these individuals. Intriguingly, polyclonal CD8 T cells producing IL-21 were significantly expanded in chronically HIV-1-infected persons and not found in seronegative subjects (35). The pronounced induction of IL-21-producing CD8 T cells in the context of HIV-1 infection prompted us to investigate whether persistent infection by other viruses, including cytomegalovirus (CMV), could
give rise to antigen-specific IL-21-producing CD8 T cells. To this end, we stimulated patient and control peripheral blood mononuclear cells (PBMC) \( n = 10 \) HIV+ patients, \( n = 10 \) controls) with overlapping peptides corresponding to the pp65 protein of CMV followed by intracellular cytokine staining for IL-21 and IFN-\( \gamma \) (Fig. 1). Despite the induction of robust CMV-specific CD8 T cell responses as measured by IFN-\( \gamma \) production, neither HIV-1 seronegative nor HIV-1 seropositive subjects elaborated virus-specific IL-21\(^+\) CD8 T cell responses directly \textit{ex vivo} (Fig. 1A). By contrast, CMV-specific and polyclonally-activated CD4 T cells produced IL-21 regardless of HIV-1 infection (Fig. 1B). Therefore, IL-21 competent CD8 T cells comprise a central component of the T cell repertoire that selectively develops during chronic HIV-1 but not CMV infection.
Figure 1. **CMV-specific CD8 T cells do not produce IL-21.** Representative flow cytometric analysis of intracellular cytokine staining for IL-21 and IFN-γ production by CMV-specific CD8 and CD4 T cells. PBMC from HIV-1 seronegative (left panel) and HIV-1 seropositive (right panel) individuals were stimulated with a peptide pool spanning the entirety of the CMV pp65 protein. Numbers represent fraction (%) of total CD3^+CD8^+CD4^- (A) or CD3^+CD4^-CD8^- (B) pp65-specific or PDBu/ionomycin-activated cells producing IL-21 or IFN-γ.
IL-21-producing CD8 T cells lack surface expression of CD4.

Given that IL-21 is traditionally regarded as a CD4 T cell-derived helper factor, we evaluated the extent to which IL-21-producing CD8 T cells expressed the CD4 protein to definitively exclude the possibility that these cells may represent CD4 T cells. Examination of CD3+ T cells after non-specific stimulation with PDBu/ionomycin revealed that the vast majority of IL-21 competent CD8 T cells lacked surface expression of CD4, with only a minor fraction of cells demonstrating co-expression of CD4 and CD8 proteins (consistently <3%) (n = 10 individuals), further validating the existence of mutually exclusive IL-21+ CD4 and CD8 T cell subsets (Supplemental Figure 1). Moreover, analysis of HIV-1-specific IL-21-producing T cell populations yielded similar results (data not shown).

It is well established that profound CD4 T cell depletion is a hallmark of HIV-1 disease progression, and emerging data suggests that in the absence of CD4 T cells, other immune cell subsets can assume helper functions normally performed by CD4 T cells (1, 21, 27, 29, 32). In particular, recent studies in Agm, natural hosts of simian immunodeficiency virus, have shown that the decrease in CD4+ T cells negatively correlates with the increased expansion of a population of CD4−CD8αdim T cells capable of eliciting functional characteristics of CD4 T cells; the emergence of this CD4−CD8αdim population coincided with the downregulation of CD4 and upregulation of CD8α (1). Since CD4 T cells are by far the major producers of IL-21, we reasoned that the deficit of these cells during HIV-1 infection might result in CD8 T cell acquisition of IL-21 competency. Thus, we initiated experiments to address whether IL-21-producing CD8 T cells had a CD4−CD8αdim phenotype similar to the population found in Agm. To address
this, we compared the degree to which polyclonally activated IL-21$^+$ and IFN-γ$^+$ CD4$^+$ T cells expressed the CD8α chain. Strikingly, both IL-21 and IFN-γ-responsive CD4$^+$ T cells resided predominantly within the CD8α$^{hi}$ compartment, suggesting that IL-21-producing CD8 T cells do not develop from CD4 T cells and most likely do not represent CD4 T cells that have lost their CD4 expression and have upregulated CD8α (Fig. 2A).

**IL-21-producing CD8 T cells exhibit CD4-like functions.**

The foregoing result did not preclude the possibility that under conditions of reduced CD4 T cell help, as seen in HIV-1 infection, IL-21-producing CD8 T cells can nonetheless adopt the phenotypic and functional attributes of conventional CD4 T cells. We speculated that this subset of CD8 T cells would be devoid of effector functions typically associated with cytotoxic CD8 T cells while exerting helper activities generally ascribed to CD4 T cells. To test this hypothesis, CD8 T cells producing IL-21 and IFN-γ in response to PDBu/ionomycin were assessed for coexpression of the cytolytic effector molecules perforin or granzyme B as measured by flow cytometry. A comparative analysis of these responses revealed that IL-21$^+$ CD8 T cells exhibited a substantially limited propensity to coexpress perforin than did their IFN-γ$^+$ counterparts ($P=0.04$) (Fig. 2B and C). Additional investigation for granzyme B activity further confirmed the impaired cytotoxic potential displayed by IL-21$^+$ CD8 T cells relative to IFN-γ-producing cells ($P=0.0002$) (Fig. 2B and D). Interestingly, similar analyses performed examining CD4 T cell responses reflected these differences (Fig. 2C and D). To probe for the existence of IL-21-producing CD8 T cells with CD4-like characteristics, we monitored the ability of the latter cells to upregulate CD154 or CD40 ligand (CD40L) upon stimulation. CD40L is highly expressed by activated CD4 T cells and plays a crucial role...
Figure 2. IL-21-producing CD4⁺CD8⁺ T cells exhibit some CD4-like functions. Phenotypic and functional analysis of IL-21-producing T cell populations in HIV-1-infected individuals as determined by flow cytometry. PBMC were polyclonally activated with PDBu/ionomycin and stained for IL-21 and IFN-γ production. Cells were then gated on live CD3⁺CD4⁻ lymphocytes and analyzed for hi or dim expression of the CD8α chain. Data are expressed as percent total IL-21⁺ or IFN-γ⁺ CD4⁺CD8α⁺ T cells (A). Representative flow cytometric plots (B) and compilation graphs showing the percent of total IL-21⁺ or IFN-γ⁺ CD4 and CD8 T cells that were perforin positive (C), granzyme B positive (D) or CD40L positive (E). Percent of total IL-21⁺-producing CD8 T cells that co-expressed IL-2, IL-17, or TNF-α (F). Statistical significance was determined using the two-tailed Mann-Whitney U test; horizontal bars represent median. Representative flow cytometric analysis of CXCR5 or ICOS expression on IL-21- or IFN-γ-producing CD8 T cells (G).
in CD4 T cell help to B cells, thereby facilitating enhanced B cell proliferation, survival, and affinity maturation (6, 33). Indeed, IL-21-producing CD8 T cells expressed CD40L at levels equivalent to that found in CD4 T cells, although the frequency of this population did not exceed the respective proportion of CD40L-expressing IFN-γ+ CD8 T cells (Fig. 2F). Approximately 20% of IL-21-producing CD8 T cells expressed TNF-α and IL-2 upon activation, with a nearly undetectable induction of IL-21/IL-17 co-producers (Fig. 2F). Collectively, IL-21-producing T cells displayed comparable behavior, whether originating from CD4 or CD8 T cells, as no significant differences were observed in the proportion of CD4 or CD8 T cells elaborating CD4-like functions (Fig. 2C-E).

Next, we sought to ascertain whether IL-21-producing CD8 T cells manifested traits that resemble T follicular helper (Tfh) cells, a specialized subset of CD4 T cells that upon triggering can provide help for B cells to support antibody production and isotype class switching (6, 33). Interestingly, a population of human CD8 T cells, marked by expression of the cardinal Tfh-associated molecule, CXCR5, has been described to exist in tonsil B cell follicles and, to a lesser extent in peripheral blood. Importantly, these CXCR5+ CD8 T cells were shown to support B cell survival and IgG production, functions generally attributed to CD4+ Tfh cells (11, 28). Thus, we determined the frequency of IL-21-producing CD8 T cells that demonstrated coordinate expression of Tfh markers including CXCR5 and ICOS. Consistent with published data, we found that less than 2% of total circulating CD8+ T lymphocytes bear the chemokine receptor CXCR5 (Fig. 2G) (11, 28). Furthermore, while only a minor fraction of IFN-γ-producing CD8 T cells were positive for CXCR5 and ICOS, cells producing IL-21 exhibited even
more limited expression of these molecules (Fig. 2G). Taken together, these results demonstrate that IL-21 competent CD8 T cells appear to share some, but not all, characteristics of classical CD4 T cells yet are phenotypically distinct from the Tfh CD4 T cell subset.

**Induction of IL-21 competency by CD8 T cells is independent of CD4 T cell depletion.**

We hypothesized that the emergence of IL-21-producing CD8 T cells was indicative of a compensatory mechanism for the loss of CD4 T cells. However, it remained unclear whether this phenomenon was a direct consequence of CD4 T cell deficiency itself or HIV-1 disease chronicity. To definitively address this issue, we studied the presence of polyclonal IL-21-producing CD8 T cells in a rare cohort of individuals diagnosed with idiopathic CD4 lymphocytopenia (ICL), a syndrome of unknown etiology characterized by low CD4 T cell counts in the absence of HIV-1 infection. Importantly, many of the clinical manifestations associated with ICL are akin to those found in HIV-1 infection, including susceptibility to opportunistic infections, perturbations in T cell homeostasis, and the selective loss of naïve T cells confined predominantly to the CD4 T cell subset (16, 42). Notably, IL-21-producing CD4 T cells were comparably elicited in ICL patients and HIV-1-infected subjects, concomitant with the striking absence of IL-21+ CD8 T cells in ICL patients, suggesting that CD4 T cell depletion cannot fully account for induction of this phenotype in HIV-1-infected individuals (Fig. 3A and B). In line with these observations, there was no association
between the frequency of CD8 T cells producing IL-21 and CD4 T cell count \((r=0.40, P=0.07)\) or CD4:CD8 T cell ratios \((r=0.14, P=0.59; \text{ data not shown})\). Hence, these findings further solidify that the accumulation of IL-21-producing CD8 T cells in chronic, progressive HIV-1 infection is independent of CD4 T cell loss.

**Figure 3. Absence of IL-21-producing CD8 T cells in patients with idiopathic CD4 lymphocytopenia.** PBMC from HIV-1-infected patients, HIV-1 seronegative subjects, and patients with idiopathic CD4 lymphocytopenia (ICL) were stimulated with PDBu/ionomycin. The magnitude of CD4 (A) or CD8 (B) T cells producing IL-21 is shown where horizontal bars indicate median value for each cohort. Statistical significance was determined using the two-tailed nonparametric Mann-Whitney U test.

**CD8 T cell IL-21 production is associated with CD8 T cell activation levels.**

Intriguingly, elevated frequencies of IL-21 competent CD8 T cells have been described in the context of autoimmune conditions including psoriasis, immune thrombocytopenia, and systemic lupus erythematosus \((7, 26, 41)\). Excessive or inadequately controlled T cell responses feature prominently in both autoimmunity and HIV-1 infection, suggesting a common mechanism by which IL-21-producing CD8 T cells arise \((30, 31)\). Therefore, we reasoned that persistent rounds of immune activation
might drive the acquisition of CD8 T cell IL-21 functional competence during chronic HIV-1 infection. To explore this possibility, we studied CD8 T cell activation levels in twelve HIV-1-infected individuals, from whom we collected longitudinal samples prior to antiretroviral therapy (ART) initiation and 6 to 12 months after achieving fully suppressed viral loads. This permitted the assessment of whether viremia and immune activation were causally interdependent. When all samples were grouped together, we found that the frequency of CD8 T cells producing IL-21 modestly correlated with the percentages of activated CD8 T cells as measured by co-expression of CD38 and HLA-DR ($r=0.46$, $P=0.02$) (Fig. 4A). This correlative effect was mainly driven by the association of these parameters found in samples from pre-ART time points ($r=0.57$, $P=0.04$) (data not shown), suggesting an uncoupling of this relationship after ART implementation. We then compared the extent to which IL-21-producing CD8 T cells themselves displayed an activated phenotype relative to cells producing IFN-γ after PDBu/ionomycin stimulation. Overall, IL-21-producing CD8 T cells showed a significantly higher level of activation than IFN-γ-responsive cells irrespective of ART-induced viral suppression (Fig. 4B). In stark contrast, there was no significant difference in activation status of IL-21$^+$ versus IFN-γ$^+$ CD4 T cells before or after ART initiation, indicating that IL-21 competent CD8 T cells were selectively activated (Fig. 4C). While effective ART partially abrogated the intensity of hyperimmune activation, despite ART-induced CD4 T cell rebound, IL-21-producing CD8 T cells continued to persist into chronic infection (Fig. 4D). These results provide further evidence that immune activation, rather than perturbations in CD4 T cell numbers, drives CD8 T cell IL-21 production.
Figure 4. CD8 T cell IL-21 production is associated with CD8 T cell activation levels. Spearman rank correlation between the frequency of IL-21⁺ CD8 T cells and levels of activated CD38⁺HLA-DR⁺ CD8 T cells obtained from HIV-1-infected individuals before and after ART (A). Percent of total CD38⁺HLA-DR⁺ CD8 (B) or CD4 (C) T cells that produced IL-21 or IFN-γ in a cohort of HIV-1-infected individuals (n = 12) with longitudinal samples collected prior to ART initiation and 6 to 12 months after achieving fully suppressed viremia. Statistical comparisons were determined by the Wilcoxon sum rank test for paired samples. Magnitude of CD8 T cell IL-21 production before and after ART (D). Horizontal bars indicate median percentages. Statistical significance was determined by the two-tailed nonparametric Mann-Whitney U test.
DISCUSSION

As reflected by the studies presented herein, we describe a subset of human IL-21-producing CD8 T cells that shares some, but not all, phenotypic and functional attributes with conventional CD4 T cells, enabling new insights into the altered nature and plasticity of T cell responses mounted against HIV-1. Our previous finding that HIV-1 infection leads to the concomitant induction of IL-21-producing CD4 and CD8 T cells, with a biased expansion of the CD8 population, raised important questions regarding the origin and contribution of this subset in immunity to persistent viral infections and HIV-1 disease progression (35). Remarkably, in response to CMV infection, virus-specific CD8 T cells do not readily elicit active IL-21 production, suggesting that induction of IL-21 competent CD8 T cells is not an intrinsic property of all chronic viruses. The foregoing result gave rise to the speculation that since CD4 T cell-derived IL-21 is absolutely critical for viral control, CD8 T cells are poised to secrete IL-21 under circumstances whereby CD4 T cell help is insufficient, namely during HIV-1 infection. Despite lacking surface expression of CD4, IL-21-producing CD8 T cells, could to some extent, perform functions generally attributed to CD4 T cells yet were phenotypically distinct from CD4 Tfh cells. Moreover, this subset was defined by low expression of perforin and granzyme B, indicative of a functional profile divergent from classical cytotoxic CD8 T cells. Nevertheless, acquisition of IL-21 competency by CD8 T cells was not dependent upon CD4 T cell loss but instead contingent upon the extent of peripheral CD8 T cell activation. These results extend the diversity of effects of this pleiotropic cytokine within HIV-1 infection, which coincides with its role in immunopathogenesis during autoimmunity – that is – polyclonal IL-21-producing CD8 T
cells may represent a component of the hyperinflammatory response that results in immune damage (30, 31).

These data highlight the fact that in HIV-1 infection CD4 T cell depletion and immune activation are inextricably linked – that is that immune activation is a consequence of CD4 T cell depletion, which creates a cycle of further infection, depletion, and immune activation. Therefore, addressing whether polyclonal IL-21+ CD8 T cells arise as a consequence of CD4 T cell loss alone or immune activation is difficult to discern. However, the fact that polyclonal IL-21+ CD8 T cells are also observed in autoimmunity, which is generally marked by immune activation but no CD4 T cell depletion, suggests that it is the immune activation rather than CD4 T cell depletion by itself. Given the data showing that IL-21 competent CD8 T cells exhibit some CD4-like functions, it will be important to determine whether HIV-1-specific IL-21-producing CD8 T cells have CD4-like functions and whether they serve as component of intrinsic help to CD8 T cells or play a role in sustaining CD8 T cell quality and maintenance.

The present finding that IL-21 is associated with immune activation initially seems at odds its documented role in controlling chronic viral infections (9, 38). However, these data highlight the contrasting roles of virus-specific versus polyclonal IL-21 competent CD8 T cells. Indeed, we have previously shown that IL-21-producing HIV-1-specific CD8 T cells are almost exclusively observed in elite controllers, which clearly underscores its function in dictating pathogen control (35). By contrast, elevated frequencies of polyclonal IL-21-producing CD8 T cells in chronic HIV-1 infection are consistent with strong evidence that CD8 T cell-derived IL-21, in part, moderates inflammation during psoriasis, immune thrombocytopenia, and systemic lupus.
erythematous (7, 26, 41). It is particularly intriguing that excessive IL-21 production is a hallmark of inflammatory bowel disease, leading to inappropriate T cell responses directed against microbial constituents; this is highly reminiscent of the gastrointestinal pathology observed in patients infected with HIV-1 (2, 23, 36). Thus, it appears that the role of CD8 T cell-derived IL-21 during immune regulation of HIV-1 disease is multifaceted, influencing the balance between the protective virus-specific immune responses needed for pathogen containment and the chronic inflammation driven by constant exposure to peripheral antigens. In support of this idea, we have previously shown that elite controllers have higher HIV-1-specific but lower polyclonal IL-21 production compared to chronic progressors (35).

The latter conclusion is further supported by our finding that CD8 T cells producing IL-21 were completely absent in patients with ICL. While elevated levels of activated CD38\(^{+}\)HLA-DR\(^{+}\) CD8 T cells have been consistently linked with HIV-1 disease progression, previous studies have documented that ICL patients do not exhibit evidence of profound CD8 T cell activation compared to control subjects (16). Hence, this could provide an explanation as to why despite the parallel CD4 T cell depletion seen in HIV-1 and ICL, IL-21-producing CD8 T cells are not observed in persons with ICL.

The data suggesting that CD8 T cells producing IL-21 arise independent of CD4 T cell deficiency raise the question as to which differentiation pathways are involved in driving the development of IL-21-producing CD8 T cells. While no specific molecular mechanisms have been elucidated, a growing number of studies have identified factors that may take part in this process. Interestingly, LCMV-infected mice doubly deficient in the transcription factors T-bet and eomesodermin fail to differentiate into cytotoxic
killers and produce Th17-associated cytokines including IL-21 (15). Moreover, CD8 T cells purified from wild-type mice cultured in the presence of anti-CD3/CD28 and IL-6 or IL-21 upregulate *IL21* mRNA expression (13). Furthermore, a recent report Mittal and colleagues found that IL-27 drives IL-21 production by naïve and total human CD8 T cells; addition of anti-IL-21 neutralizing antibody significantly decreased CD8 T cell proliferation, suggesting that autocrine IL-21 may promote self-expansion of CD8 T cells (22). Clearly, it remains to be seen whether perturbations in the expression of the aforementioned factors impact the expansion of virus-specific or polyclonal IL-21-producing CD8 T cell subsets in HIV-1-infected individuals.

Collectively, these studies support a model in which IL-21 production is remarkably limited to CD4 T cells during the steady state, and CD8 T cells producing IL-21 emerge as a result of an altered CD8 T cell repertoire. Future studies dissecting the inductive pathways for polyclonal CD8 T cell IL-21 competency should be informative in understanding the complex regulation of this pleiotropic cytokine during the course of HIV-1 infection and autoimmunity.
REFERENCES


Supplemental Figure 1. IL-21-producing CD8 T cells do not co-express CD4. Representative flow cytometric analysis of CD4 and CD8 expression on IL-21-producing T cells. PBMC from HIV-1-infected individuals were non-specifically stimulated in the presence of PDBu/ionomycin. Cells were gated on CD3<sup>+</sup>IL-21<sup>+</sup> T lymphocytes and subsequently plotted for CD4 versus CD8 expression. The numbers represent the percentage of gated CD4<sup>+</sup>, CD8<sup>+</sup>, and CD4<sup>+</sup>CD8<sup>+</sup> IL-21-producing T cells.
CONCLUSIONS

It has been suggested that the massive infection and depletion of memory CD4 T cells that occurs during HIV-1 infection disrupts a central process for the successful coordination of the antiviral immune response. In particular, CD4 T cells play a prominent role in providing helper signals to CD8 T cells, especially during infections that are slowly or never controlled. Mouse models of chronic viral infection demonstrate that IL-21, a cytokine produced primarily by CD4 T cells, is required for induction, quality, and longevity of antiviral CD8 T cell responses and viral containment. In light of these observations and to better understand the nature of CD4 T cell help during HIV-1 infection, we investigated the potential role of IL-21 in the provision of help to CD8 T cells and control of HIV-1 replication. Our results demonstrate that both CD4 and CD8 T cells produce IL-21 in response to overlapping HIV-1 peptides, with the latter cell type more closely associated with viral control. Moreover, IL-21-producing CD4 T cells, compared to those producing other cytokines, were the best indicator of functional CD8 T cells. We further show that polyclonal CD8 T cells are permissive to IL-21 production in HIV-1-infected individuals but not in seronegative subjects. The foregoing result led to hypothesis that the selective emergence of IL-21 competent CD8 T cells in HIV-1 infection was indicative of a compensatory mechanism for the loss of CD4 T cells. Notably, our studies reveal that CD8 T cell induction of IL-21 competency is independent of CD4 T cell loss but instead is contingent upon the extent of peripheral CD8 T cell activation. Collectively, these findings suggest that HIV-1-specific IL-21 production,
whether originating from CD4 or CD8 T cells, plays an important role in the control of viremia. In contrast, polyclonal CD8 T cell IL-21 production, uniquely seen in HIV-1 infection, is associated with immune activation.

Prior to initiation of our work, a limited number of studies suggested a role for IL-21 in controlling HIV-1 infection as demonstrated by reduced serum levels in HIV-1-infected AIDS patients on ART compared to uninfected control subjects (94). It was found that the levels of IL-21 directly correlated with CD4 T cell counts, suggesting that CD4 T cells were the principal source of IL-21. However, the impact of immune status, plasma viral load, or the presence or absence of ART on IL-21 secretion was unknown. Furthermore, actual IL-21 production from total or HIV-1-specific CD4 T cells had not been previously reported.

To determine whether IL-21-producing T cells were associated with control of HIV-1 in vivo, we assessed the quantitative and qualitative differences in HIV-1-specific CD4 T cell IL-21 production across an HIV-1 cohort consisting of primary HIV-1 infection (PHI), chronic with undetectable or low viremia (elite controllers [EC] and viremic controllers [VC], respectively), and chronic with uncontrolled viremia (progressors [P]). We sought to methodically evaluate the contribution of CD4 T cells that produce IL-21 from HIV-1 controllers to see if they, unlike chronically infected progressors, were better at maintaining CD8 T cells capable of viral control. As serum IL-21 levels had been previously shown to be diminished in late stage HIV-1, we tested the hypothesis that impaired CD4 T cell function in chronic disease, particularly lack of IL-21 secretion, was associated with viral persistence. Therefore, we compared the
functional profile, frequency, and protein targeting of these populations in HIV-1 controllers versus progressors.

We found similar levels of HIV-1-specific CD4 T cell IL-21 production across the cohorts. Despite the lack of association between the magnitude of CD4 T cell IL-21 production and viral control, elaboration of a polyfunctional IL-21 response reliably predicted viral containment. These data are in agreement with the findings of Yue et al., showing that there were no significant differences in the induction of these responses in long-term nonprogressors versus chronically infected patients with uncontrolled viremia (213). In contrast, Chevalier et al., using a different technical approach, found higher levels of IL-21 secretion by HIV-1-specific CD4 T cells in controllers than in ART-treated individuals, with the lowest levels detected in P (29). Interestingly, this group followed a subset of individuals diagnosed with symptomatic acute HIV-1 infection into chronic infection and found that expansion of HIV-1-specific IL-21+ CD4 T cells in acute infection resulted in lower viral load set points, suggesting a causative link between the IL-21 response and viral control. Although not demonstrated by our group, others have found an inverse correlation between IL-21 levels (plasma) and viral load (92). Yue et al. found that higher frequencies of IL-21+ CD4 T cells in progressors correlated with lower viral loads but did not show this association across the entire cohort (213). In agreement with our findings, Chevalier et al. further noted that some HIV-1 controllers do not mount a detectable IL-21+ CD4 T cell HIV-1-specific response, indicating that there exist alternative, IL-21-independent mechanisms of antiviral function in these subjects (29).
In regards to total CD4 T cell IL-21 production, results are similarly discordant. Both Iannello et al. and Chevalier et al. show that stimulated bulk CD4 T cells from progressors produced less IL-21 than CD4 T cells from controllers. Coinciding with studies by Yue et al., we show the contrary – polyclonal CD4 T cell IL-21 production is elevated in HIV-1-infected individuals compared to control subjects, with higher levels seen in progressors than controllers. However, it is important to note that these studies utilized different methodologies and distinct polyclonal stimuli (ionomycin alone, PMA/ionomycin, SEB, and PDBu/ionomycin), possibly explaining the divergent results.

We hypothesized that the functional impairment of IL-21-producing CD4 T cells compromised CD8 T cell functional quality (i.e. IL-2 production) and memory potential, similar to what was observed in IL-21-deficient mice. We found that HIV-1-specific IL-21, but not IL-2 produced alone by CD4 T cells correlated with CD8 T cell IL-2 production. These results suggest that IL-21 is a better indicator of functional CD8 T cell responses than IL-2, although both cytokines most likely act cooperatively to induce protective, polyfunctional CD8 T cells. Moreover, these findings corroborate studies by Yi et al. showing that IL-21 promotes the differentiation of IL-2-producing CD8 T cells in LCMV-infected mice (212). It would be of great importance to identify the mechanism by which IL-21 regulates IL-2 expression by CD8 T cells. As proposed by Yi et al., the tight clustered genomic organization of IL-21 and IL-2 suggests that IL-21 could modulate the balance between transcription factors, BLIMP-1 or Bcl-6, which have both been shown to reciprocally regulate IL-2 production.

To more directly address the impact of IL-21 on the functional quality of CD8 T cell responses, we performed *in vitro* experiments by stimulating PBMC of HIV-1-
infected individuals with overlapping HIV-1 peptide pools in the presence or absence of recombinant human IL-21 (rhIL-21). We have made the observation that exogenous rhIL-21 addition enhances the capacity of HIV-1-specific CD8 T cells to upregulate effector molecules including IFN-\(\gamma\), TNF-\(\alpha\), and perforin (unpublished results). Notably, these findings have been documented in two recent manuscripts published prior to our paper (29, 206). However, these groups noted differential effects of IL-21 on degranulation of CD8 T cells as measured by CD107a expression, although granzyme A and B effector activities were prominently enhanced after co-incubation with IL-21 (29, 206). In an elegant experiment performed by Chevalier et al., IL-21 production by CD4 T cells from HIV-1 controllers enhanced perforin production by HIV-1-specific CD8 T cells from chronic progressors even in the late stages of HIV-1, further emphasizing the ability of IL-21 to directly modulate CD8 T cell effector functions. Importantly, definitive links to protective immunity came from subsequent studies showing enhanced suppression of HIV-1 replication following IL-21 exposure to CD8 T cells. Taken together, these data suggest that IL-21 may function to increases the killing efficacy of CD8 T cells, which may improve overall viral containment. Although the mechanism by which this occurs remains elusive, recent studies have shown that granulysin, an antimicrobial molecule expressed by CD8 T cells and NK cells, can be induced via IL-21 and IL-15 by STAT3 and STAT5 activation. This suggests that STAT3 and STAT5 possibly regulate induction of other cytotoxic molecules.

Our finding that HIV-1-specific CD8 T cells produce IL-21 is completely novel and somewhat unexpected as IL-21 has been traditionally regarded as a CD4 T cell-derived helper factor. However, we found strong evidence that these cells contribute to
control of viremia, hinging on two major results: (1) EC and VC maintained significantly higher percentages of HIV-1-specific IL-21-producing CD8 T cells compared to P; (2) Gag and Pol-specific IL-21+ CD8 T cells were completely ablated in P, concomitant with their exclusive presence in PHI, EC, and VC. These results are consistent with increasing evidence that preferentially targeting Gag, and possibly Pol, is strongly associated with improved clinical markers of disease progression and suggests that CD8 T cell IL-21 responses directed towards these proteins may contribute to maintenance of viral control. However, in the progressor cohort, the magnitude of CD8 T cells producing IL-21 did not correlate with CD4 count or viral load, indicating that a threshold level of this cytokine may be needed for enhanced viral control. We confirmed our finding that CD8 T cells have the capacity to produce IL-21 by negatively selecting CD8 T cell populations and performing mRNA analysis after stimulation with HIV-1 peptide pools and optimized HLA class I-restricted epitopes for an even more stringent scrutiny of this result. Utilizing transcriptional profiling techniques, we found that controllers uniformly upregulated IL21 mRNA whereas none of the progressors substantially amplified transcripts.

Out of the four independent research laboratories that have reported on the association of IL-21 and control of HIV-1 in vivo, we are the only group that has published on the existence of HIV-1-specific IL-21-producing CD8 T cells. This is likely due to the fact that the other studies were designed to enrich for CD4 populations by CD8 depletion techniques, precluding the examination of CD8 T cell populations for IL-21 production. Moreover, CD8 T cells produce significantly lower levels of IL-21 compared to CD4 T cells, which may make it difficult to detect. Importantly, since our publication,
two independent research groups have now identified virus-specific CD8 T cells producing IL-21 in the setting of HIV-1 and SIV, finding low levels of HIV-1-specific IL-21$^+$ CD8 T cells in the lymph nodes of HIV-1-infected individuals and SIV-specific IL-21$^+$ CD8 T cells in the peripheral blood of SIV-infected non-human primates (personal communications by Hendrik Streeck and Rama Amara, respectively).

An important aspect of our data is that polyclonal CD8 T cell IL-21 production is exclusively found in HIV-1-infected individuals and not seen in seronegative controls. This led us to propose that the selective emergence of IL-21-producing CD8 T cells in HIV-1-infected individuals was indicative of a compensatory mechanism for the quantitative and qualitative decline of CD4 T cells that characteristically marks HIV-1 disease.

To address this hypothesis, we examined IL-21$^+$ CD8 T cells from HIV-1-infected individuals for functions generally attributed to CD4 T cells and compared them to cells producing IFN-γ. We found that IL-21-producing CD8 T cells, despite lacking surface expression of the CD4 molecule, were able to elaborate some CD4-like functions, such as CD40L upregulation and modest production of IL-2 and TNF-α. In fact, IL-21-producing T cells displayed comparable behavior, whether originating from CD4 or CD8 T cells, as no significant differences were observed in the proportion of CD4 or CD8 T cells elaborating CD4-like functions. To examine whether CD4 T cell loss or HIV-1 disease chronicity explained acquisition CD8 T cell IL-21 competency, we studied a rare cohort of ICL patients, who like HIV-1$^+$ patients, have low frequencies of CD4 T cells. Interestingly, IL-21-producing CD8 T cells were completely absent in patients with ICL,
suggesting that CD4 T cell loss is not sufficient for the induction of CD8 T cells endowed with IL-21 production capacity.

Because CD8 T cells producing IL-21 have been identified in the setting of autoimmune diseases, which are marked by excessive T cell responses, we reasoned that CD8 T cells producing IL-21 in HIV-1 infection may arise as a result of immune activation. Indeed, we found an association between the frequency of IL-21-producing CD8 T cells and the level of activated CD8 T cells. Importantly, IL-21⁺ CD8 T cells were preferentially activated compared to their IFN-γ counterparts. Also, the fact ART administration, which induces rebound of CD4 T cells, did not diminish frequencies of IL-21⁺ CD8 T cells, further supports the notion that immune activation, rather than perturbations in CD4 T cell numbers, is driving this phenomenon.

These data highlight the fact that in HIV-1 infection CD4 T cell depletion and immune activation are inextricably linked — that is that immune activation is a consequence of depletion, which creates a cycle of further infection, depletion, and immune activation. Therefore, addressing whether polyclonal IL-21⁺ CD8 T cells, which likely arise as a consequence of immune activation, compensate for the loss of CD4 T cells is difficult to discern. Given the data showing that IL-21 competent CD8 T cells exhibit some CD4-like functions, it will be important to determine whether HIV-1-specific IL-21-producing CD8 T cells have CD4-like functions and whether they serve as component of intrinsic help to CD8 T cells or play a role in sustaining CD8 T cell quality and maintenance. Despite the fact that these cells lack a Tfh-like phenotype, it is still possible that IL-21⁺ CD8 T cells could support B cell function. This could be addressed,
in part, by executing \textit{in vitro} assays by co-culturing IL-21$^+$ CD8 T cells with B cells and monitoring for improved IgG secretion on HIV-1 antigen stimulation (149).

Moreover, further work will be necessary define the inductive pathways of IL-21-producing CD8 T cells. Previous studies have suggested that IL-21 itself or IL-27 could drive IL-21 CD8 T cell production (91, 136). As documented by a mouse model of T-bet and Eomesodermin double deficiency, defective expression of these transcription factors could drive CD8 T cells to produce IL-21 (95). In support of this idea, it has been shown that expression of Eomesodermin and T-bet on HIV-1 tetramer-specific CD8 T cells is intact in acute infection but lost in chronic infection (167). Moreover, the phenotype of these antigen-specific CD8 T cells lacking expression of these factors resembled the impaired cytolytic functional profile of IL-21$^+$ CD8 T cells found in our chronically HIV-1-infected patient cohort.

In light of the data showing both protective and pathogenic consequences of IL-21 production in general, the prospect of modulating IL-21 levels for therapeutic benefit or development of vaccines should be handled with caution. Previous studies in patients with metastatic cancers have demonstrated that after IL-21 administration, tumor regression is mediated by IL-21-dependent increases in NK cell and CD8 T cell cytotoxicity. However, excessive IL-21 levels have been associated with autoimmunity (119). More evidence for the required fine-tuned regulation of IL-21 levels is derived from studies by Yi et al. showing that CD4-deficient mice treated with IL-21 exhibit enhanced CD8 T cell responses and lower viral titers by day 9 post-LCMV infection (211). Despite this, the majority of mice succumbed, most likely due to immunopathology caused by enhanced CD8 T cell responses.
Several studies in recent years have investigated the utilization of γc-chain cytokines as adjunctive therapy for HIV-1 infection. IL-7, IL-2, and IL-15 have been tested in chronically SIV-infected animals. Administration of IL-7 altered T cell homeostasis while IL-15 administration increased the frequency of effector memory CD4 and CD8 T cells. Nevertheless, neither IL-7 nor IL-15 administration reduced viral replication, with IL-15 actually increasing peak viremia in acute infection attributed to increased CD4 T cell proliferation and activation (59, 137, 195). A recent study by Pallikkuth et al evaluated the safety, biological activity, and immunomodulatory effects of IL-21 by means of recombinant mamuIL-21 administration in chronically SIV-infected rhesus macaques (150). IL-21 was well tolerated and enhanced expression of perforin and granzyme B in T cells and NK cells after 48 hours. After each dose, increases were noted in the expression of these molecules in memory and effector CD8 T cells in peripheral and mesenteric lymph nodes, concomitant with increases peripheral blood memory and effector CD4 T cells. However, no changes were noted in T cell proliferation or plasma viral load. No changes were observed in T cell activation markers, a distinguishing feature from the effects seen with IL-7 and IL-15 administration. Future studies will be needed to discern whether IL-21 administration during acute infection affects set point viral load and CD8 T cell exhaustion. Moreover, it is of importance to understand the timing and quantity of IL-21 that is required during chronic viral infection to promote immune control.

In closing, loss of CD4 T cell help in concert with immune activation erodes the immune systems of HIV-1-infected individuals. This deterioration has been associated with impaired CD8 T cell function and presumably results in viral persistence. The
studies presented herein identify IL-21 as an important component of CD4 T cell help to CD8 T cells and preservation of HIV-1 control. It is the first to demonstrate the existence of a novel subset of HIV-1-specific CD8 T cells endowed with IL-21 competency, that when elicited, are associated with superior viral control. Furthermore, we provide evidence that production of IL-21 by polyclonal CD8 T cells is a phenomenon linked to the characteristic immune activation seen in HIV-1-infected individuals, thereby giving IL-21 an even broader role in HIV-1 immunopathogenesis. This work generally contributes to our understanding of the factors that regulate the complexity and quality of anti-HIV-1 immune response. Perhaps more importantly, these studies have raised numerous questions concerning the use of IL-21 as a therapeutic modality to enhance existing immunity, especially in the setting of communicable diseases with latent reservoirs of infected cells. Important next steps will be to further define exactly how IL-21 shapes the CD8 T cell response to chronic viral infections and how to harness this information to instruct the design of future HIV-1 vaccines.
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APPENDIX

IRB APPROVAL FORMS
Protection of Human Subjects
Assurance Identification/IRB Certification/Declaration of Exemption
(Common Rule)

1. Research Type
   [ ] ORIGINAL
   [ ] CONTINUATION
   [ ] EXEMPTION
   [ ] OTHER:

2. Type of Mechanism
   [ ] GRANT
   [ ] CONTRACT
   [ ] FELLOWSHIP
   [ ] COOPERATIVE AGREEMENT

3. Name of Federally Funded Department or Agency and, if known, Application of Proposal Identification No.

4. Title of Application or Activity
   IL-21 and Immune Modulated Viral Control

5. Name of Principal Investigator, Program Director, Fellow, or Other
   GOEPPELT, 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. PWA00005960 the expiration date 08/29/2016
      IRB Registration No. IRB00000196

   [ ] This Assurance, on file with (agency/office), covers this activity.
      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.

   [ ] Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph

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.
      by [ ] Full IRB Review on (date of IRB meeting)
      or [X] Expedited Review on (date) 1-6-12

   [ ] 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.

IRB Approval Issued: 1-6-12

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed until study closure and certification will be provided.

   [ ] Phone No. (with area code) (205) 934-3789
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11. Name of Office
    Marilyn Doss, M.A.

12. Signature

13. Title
    Vice Chair, IRB

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MEMORANDUM

DATE: February 24, 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: IL-21 Immune Mediated Viral Control

Principal Investigator: Paul A. Goepfert, M.D.
IRB Protocol #: X 090223001

Ms. Latonya Williams is a graduate student in the Microbiology department. Ms. Williams joined our group in May 2007. She is currently working on her thesis project, entitled "The role of interferon-21 (IL-21) in promoting immune control of human immunodeficiency virus-1 (HIV-1) infection."

In completing her thesis work, Ms. Williams has been investigating the functional profile of virus-specific CD4+ and CD8+ T cells derived from individuals infected with HIV-1. Her thesis work 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. X 090223001, referenced above.
Signature of Principal Investigator

Date 02-27-09

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