HIV-Specific CD4 T Cells and Viral Control

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HIV-specific CD4 T cells and Viral Control

A dissertation presented

by

Damien Zadour Soghoian

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

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HIV-specific CD4 T cells and Viral Control

Abstract

CD4 T cells play an important and central role in the immune system, coordinating the arms of the adaptive immune system to shape an effective response while simultaneously regulating non-essential or deleterious activities. Their critical necessity is demonstrated most strikingly during acquired immunodeficiency syndrome (AIDS), when depletion of CD4 T cells by human immunodeficiency virus (HIV) type 1 ultimately results in a host of immune dysfunctions and susceptibility to opportunistic pathogens. Although virus-specific CD4 T cell responses are generally vital for the control of viral infections, HIV-specific CD4 T cells have long been recognized to be preferentially targeted and depleted by the virus—raising questions about their utility as immune effectors both during HIV-1 infection and in the context of a prospective HIV-1 vaccine. However, more recent research has challenged the notion that HIV-specific CD4 T cells are only relevant as targets. There is a growing appreciation for the crucial role that these cells may play in mediating anti-HIV immunity through a diverse array of effector functions—including direct anti-viral cytotoxicity. Here we show that HIV-specific CD4 T cell responses are evident throughout the course of HIV disease, including acute infection. In particular, an expansion of HIV-specific CD4 T cells with cytolytic potential early after acute HIV infection is associated with lower viral set point and better clinical progression. This expansion is evident
both as an increase in HIV-specific CD4 T cells able to degranulate upon antigen recognition and as cells with a unique granzyme and perforin expression pattern. Further, HIV-specific cytolytic CD4 T cell responses are functionally enhanced in the setting of durable HIV control during chronic infection, where they exhibit a profile reminiscent of HIV-specific CD8 T cells and are associated with T-bet and Eomesoderm expression. We also show that in HIV controllers, the function of HIV-specific CD4 T cells is very tightly correlated with clinical status. Together these results strongly support the concept that CD4 T cells are critical players in the cellular immune response to HIV, and point to specific CD4 T cell functions—including direct cytolyisis—which may be most important for anti-HIV immunity.
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Dedication: This thesis is dedicated first and foremost to my parents, Stephanie and Richard Soghoian, for their unwavering support over the past three decades. It is also dedicated to the countless friends and family who have encouraged me in my scientific career.
Chapter 1: Introduction
Sections of this chapter are modified from the peer-reviewed publication:

THE GLOBAL HIV/AIDS EPIDEMIC AND THE NEED FOR AN HIV VACCINE

HIV/AIDS represents the most devastating infectious disease epidemic in modern history. Since the first reports of HIV-associated syndromes in the early 1980s, the disease has claimed nearly 35 million lives. There are currently nearly the same number of people infected worldwide, and 1.6 million people die of the disease each year [1]. Due in part to socioeconomic factors, the HIV/AIDS epidemic disproportionately affects Sub-Saharan Africa, particularly South Africa, which is home to the largest number of people living with HIV/AIDS of any country [1].

HIV was first identified as the causative agent of AIDS in 1983, and efforts to identify antiretroviral compounds began shortly afterwards [2]. Zidovudine (AZT) became the first drug approved to treat HIV, in 1987. Since then, an army of drugs have been developed, which block HIV replication by targeting different points of the viral lifecycle [2]. These medications are remarkably effective when used as a multi-drug cocktail and have transformed HIV from a deadly illness to a manageable chronic condition in which life expectancy can approach that of an HIV-uninfected individual [3]. However, anti-HIV medications are expensive and require strict adherence in order to maintain efficacy [4, 5]. Economic factors in particular have hindered the deployment of antiretrovirals in the places that require them most, such as Sub-Saharan Africa. Indeed, only 59 percent of those in this region living with HIV have access to antiretroviral therapy, even as treatment guidelines have shifted to recommend earlier treatment [6]. Moreover, a large proportion of those being treated are not virally suppressed, which encourages the development of resistance to common therapies and additional viral spread [6, 7]. Various prevention strategies have also been initiated or are in development, such as topical
microbicides [8], male circumcision [9], pre- and post- exposure prophylaxis [10] (including long-acting injectable antiretroviral agents [11]), and education to reduce risk behaviors [10]. While these have reduced the growth of the epidemic, especially in certain regions, it is widely believed that only an effective vaccine will truly be able to put an end to it.

Despite decades of research, an HIV vaccine has remained an elusive goal. By virtue of its high rate of mutation, HIV represents an extremely challenging vaccine target: the virus can easily escape from immune pressure, and an inordinately high level of viral diversity exists globally compared to most other disease causing viruses [12]. Although nearly 200 pre-clinical or clinical studies of HIV vaccine candidates in humans have taken place, only five large-scale efficacy trials have been performed to date [13]. Except for one trial, all exhibited either no protection from HIV acquisition or increased viral control upon infection [14]. Notably, the adenovirus type 5 (Ad5) based Merck/HVTN “Step” vaccine trial (and the associated Phambili trial) was halted early after it was determined that vaccinees were at increased risk for HIV acquisition [15]. HVTN 505, a subsequent Ad5 based vaccine trial, was also terminated prematurely after no protection was observed during an interim analysis [16]. Only the RV144 “Thai” vaccine trial—which comprised a canarypox prime and protein boost strategy—exhibited any success, conferring a modest, 31% level of protection [17]. The general lack of success within the HIV vaccine field has illustrated the paucity of understanding of the elements that are required for an efficacious anti-HIV immune response.
HIV-1 PATHOGENESIS AND ACUTE HIV INFECTION

HIV is a *Lentivirus* of the *Retroviridae* family. Two closely related forms of HIV exist—type 1 (HIV-1) and type 2 (HIV-2), although only HIV-1 is primarily responsible for the global HIV/AIDS epidemic [18]. HIV-2 is generally restricted to certain regions of West Africa and countries with historical ties to that region such as Portugal and India; compared to HIV-1, it is less likely to lead to progressive immune deficiency [19]. Both HIV types are the result of zoonoses: HIV-1 originated from apes (primarily chimpanzees), and HIV-2 was transmitted from sooty mangabey monkeys. Although four groups of HIV-1 strains exist—M, N, O, and P—each of which resulted from a separate transmission event, group M comprises the vast majority of all HIV-1 infections [18, 20]. Group M can subsequently be broken down into several subtypes, or clades, which exhibit regional enrichment; clade C affects Sub-Saharan Africa and is the predominant clade worldwide [18].

The envelope proteins on the surface of every HIV virion confer tropism to a relatively narrow range of target cells expressing the glycoprotein CD4 and, depending on viral sequence, the chemokine receptors CCR5 or CXCR4. Activated memory CD4 T cells expressing CCR5 are the dominant targets of HIV, although infection of other cells expressing these proteins like macrophages or dendritic cells may also be infected and have important implications for viral transmission and persistence [10, 21].

Transmission of HIV-1 to target cells occurs primarily through sexual routes, although other modes of transmission such as intravenous drug use or mother to child infection during and after childbirth are also important for fueling the epidemic [10]. Sexual transmission risk varies depending on sex act as well as comorbidities such as infection with other sexually transmitted
diseases, but infection through these routes is generally the result of transmission of virus across the genital or rectal mucosal barriers [22, 23]. The exact mechanism by which the first mucosal cellular infection event is established remains controversial and is likely influenced by many context-specific factors [23]. Evidence from animal models as well as in vitro studies of tissue culture systems suggests that epithelial cells or tissue resident dendritic cells may facilitate the transfer of HIV virions across the mucosal barrier to activated, CCR5+ memory CD4 T cells, which represent the primary targets of the virus [23, 24]. Tissue resident dendritic cells (or macrophages) may also be infected directly, although this likely occurs less often. In cases of tissue damage, HIV may bypass the mucosal membranes entirely and directly access relevant target cells [23, 25]. The stochasticity of viral transmission across an intact mucosal barrier combined with the fact that viruses isolated very early after infection show little sequence diversity suggests that a so-called transmission bottleneck is present; in the majority of cases, therefore, systemic infection results from only a single HIV virion [26, 27]. Regardless of mechanism, however, productive infection of the first target cell is thought to lead initially to uncontrolled viral replication within the local tissue environment. After an “eclipse” phase of several days, the virus engages the target-rich lymphoid system and replicates to extraordinarily high peak levels, disseminating throughout the body and establishing viral reservoirs [28, 29]. Although specific clinical trajectories are unique for each newly HIV infected individual and exact definitions for acute HIV infection vary, the period can be coarsely staged using clinical tests that measure antigenemia and host antibody responses according to the system proposed by Fiebig [26, 30]. In Fiebig stages I and II, only HIV RNA or HIV RNA in combination with Gag p24 protein (respectively) can be detected; Fiebig stages III-VI reflect the onset of the generation
of an HIV-specific antibody response. The early Fiebig I-II stages represent the period during which an individual is most viremic; many but not all of those infected may experience symptoms of acute infection syndrome due to the large amount of viral replication taking place [26]. Critically, patients during these stages are also the most likely to transmit to other individuals due to the high levels of virus and the fact that they may clinically appear HIV negative unless nucleic acid testing is performed. Although next-generation antigen/antibody tests are increasing the ability to diagnose acute HIV infection earlier, up to 50% of all new HIV-1 infections may nevertheless be due to acquisition from individuals undergoing acute HIV infection [27].

THE HIV IMMUNE RESPONSE

The events that take place during acute HIV infection have an overwhelming influence in determining an individual’s clinical progression. Viral dissemination results in massive levels of CD4 T cell depletion, especially within the gut associated lymphoid tissue [31]. These cells are not recovered, even after extended antiretroviral treatment, leaving infected individuals with a permanently reduced arsenal of CD4 T cells to fight disease [32]. Infection fuels extraordinarily high levels of viremia early on, of up to 10^7-10^8 HIV-1 RNA copies/ml blood at peak [33]. Peak viral loads during acute HIV infection are related to severity of disease symptoms and will ultimately resolve to a steady-state “viral set point” within about six months after infection [26]. Viral set point is critically associated with disease outcome: high viral set points are linked to rapid clinical progression, while lower set points are associated with slow progression and a longer disease-free duration before AIDS is reached [34-36]. Set point is related to the ability of the immune system to partially contain viral replication and reflects the interplay between virus
and host. As a result, the factors that contribute to a more effective immune response during acute HIV infection—and ultimately better clinical outcome—have been an important focus of HIV immunology research.

Although most HIV infected individuals reach set points of 10,000-60,000 HIV RNA copies/ml, a small minority of individuals spontaneously controls plasma viremia to extremely low levels and do not follow a standard clinical progression. These individuals are broadly termed “HIV controllers,” although there is heterogeneity within this population [37]. Elite controllers exert such a strong degree of viral control that plasma virus levels are undetectable by standard clinical assays; individuals with less robust control are termed viremic controllers and can control viremia to less than 2,000 RNA copies/ml [38]. Control may last for decades, and considerable effort has been exerted to understand the mechanisms responsible for the phenomenon. Although defective viral replication may be involved in a limited number of cases, most evidence suggests durable, spontaneous viral control is linked to an optimal, highly effective immune response [37].

The earliest immune responses to HIV are from the innate immune system and reflect initial detection of the virus. Infection by HIV results in a massive cytokine storm with markedly enhanced levels of many innate cytokines and signaling factors, including IP-10, IFNα, and IL-15, which peak at seven days post infection [39]. Secretion of these cytokines is likely related to activation of toll-like receptors on plasmacytoid dendritic cells and may contribute to the long-term pathology of HIV-1 infection. Natural killer (NK) cells are also engaged during acute infection; these cells expand early after infection, have the ability to lyse virally infected cells, and therefore may contribute meaningfully to early viral control [40]. Expression of a
particular activating NK receptor, KIR3DS1, and its ligands is associated with slower HIV disease progression [41]. Moreover, sequence analysis of viral strains in HIV-infected individuals reveal NK receptor associated escape mutations, suggesting that NK cells exert immune pressure on the virus [42]. The degree of contribution of these cells to viral control, especially following acute infection, however, remains to be established.

Clearance and control of viral infections is generally related to the activity of the adaptive immune system, and extensive research has been performed on the adaptive immune response to HIV. Within the weeks and months following HIV infection, antibodies against various viral components, including envelope proteins, can be observed in the plasma of infected individuals [26, 43]. However, these are directed against epitopes that are conformationally absent from intact HIV virions and are therefore largely ineffective in neutralizing the virus [43]. While a fraction of antibodies do mediate neutralization activity against the virus, their contribution to long-term viral control is minimal due to rapid evolution of the virus, which results in the constant replacement of circulating viral stains with those resistant to the neutralizing antibodies which may be present at any given time [44]. A small subset of individuals does develop so-called broadly neutralizing antibody responses, but the impact of these antibodies on viremia appears to be minimal as well [45]. B cell depletion studies in monkeys during primary SIV infection reinforce the general lack of contribution of humoral factors to durable viral control [46]. Moreover, HIV infection is also associated with dramatic defects in the B cell compartment, including defects related to proliferation and antigen responsiveness, as well as hypergammaglobulinemia [47].
The vast majority of evidence suggests that a primary source of viral control during natural HIV infection is from the cellular immune response—particularly CD8 T cells—and in many cases differences in clinical trajectory can be linked to enhanced or defective T cell immunity [37]. Several weeks following acute HIV infection, HIV-specific CD8 T cells expand—the early appearance of these cells is temporally associated with the decline of viral load following peak, suggesting that they may play an important role in mediating this drop [48, 49]. HIV-specific CD8 T cells exert many different antiviral functions in response to antigenic recognition, and have the ability to directly kill HIV infected target cells through the release of cytolytic proteins like perforin and granzymes. In HIV controllers, CD8 T cells show enhanced functionality and lytic capacity [50, 51]. Depletion of CD8 T cells in the monkey model results in lack of post-peak viral decline and a more rapid disease progression following SIV infection [52, 53]. The immune pressure exerted by HIV-specific CD8 T cells on the virus can be detected as viral escape mutations, which become evident early after infection [54]. Further, the specific targeting of HIV CD8 T cells has been correlated with an improved disease trajectory and with the ability to spontaneously control viral replication [55-57].

Further evidence for the role that HIV-specific CD8 T cells play in the control of viral replication comes from studies of host genetics as they relate to clinical outcome. Early on it was noted that the expression of certain human leukocyte antigen (HLA; also termed major histocompatibility complex or MHC) class I alleles was linked to better or worse clinical progression. In particular, “protective” HLA alleles such as HLA-B27 and HLA-B57 are enriched in HIV controllers, while additional alleles like HLA-B8 and HLA-B35 are linked with faster progression to AIDS [58]. HLA background dictates the specific HIV peptides any
individual is able to present, and genome wide association studies have confirmed the association between features that affect peptide presentation within certain HLAs with durable HIV control [59]. Although CD8 T cells are not the only cell types that can interact through HLA class I—and indeed, other class I binding partners have been associated with HIV control—these cells nonetheless play an outsized role in contributing to immune control of HIV [60].

Compared to HIV-specific CD8 T cells, HIV-specific CD4 T cells have been relatively less well studied. As detailed in the following sections, CD4 T cells exert a range of functions that could potentially contribute to effective viral control, but in the context of HIV infection, they have been primarily examined as viral targets. However, especially in recent years, there has been a greater appreciation for the role of these cells as critical antiviral effectors. Interestingly, many of the same lines of evidence that have been used to conclude CD8 T cells play an important role in viral control also exist for HIV-specific CD4 T cells. Depletion of CD4 T cells in the monkey model results in a lack of post-peak viral decline following acute SIV infection [61]. As described in Chapter 2 of this thesis, emergence of HIV-specific CD4 T cell responses after acute infection is temporally associated with decline of viral load from peak viremia [62]. Moreover, HIV controllers have been repeatedly shown to have more robust HIV-specific CD4 T cell responses and more effective viral targeting, although it has been difficult to dissect apart whether this represents the cause or consequence of controlled viremia [63-66]. Certain HLA class II alleles have been linked with improved clinical outcome [67], and limited evidence suggests that viral escape from HIV-specific CD4 pressure may take place following acute infection [68-70]. Nonetheless, the exact role that HIV-specific CD4 T cells play in the HIV immune response remains to be fully determined.
CD4 T CELLS

*Antigen Recognition*

The functionality of a CD4 T cell is dependent on the recognition by its T cell receptor (TCR) of specific peptide antigen presented by class II HLA molecules. The CD4 TCR is a heterodimer consisting of structurally similar α and β subunits [71]. Germline TCR rearrangement by V(D)J recombination during early thymocyte development results in the establishment of a highly heterogeneous repertoire of naïve CD4 precursors. The frequency and diversity of the TCR subunits expressed by these is critical in guiding the early immune response to a pathogen. Due to the inclusion of a small diversity segment, the beta TCR subunit (Vβ) is the more polymorphic of the two [72]. Changes in Vβ diversity have been linked to certain disease states. In SIV infection, for example, a loss of Vβ diversity in the CD4 T cell can be observed in rhesus macaques with disease progression, which is related to CD4 T cell depletion [73].

Recognition of a peptide by a CD4 T cell TCR requires antigenic presentation through a compatible MHC class II molecule. MHC class II is expressed at high levels on dendritic cells, macrophages and B cells, and at lower levels on other T cells after activation. The fully assembled class II molecule is a heterotrimer, consisting of an α and a β chain and a bound antigenic peptide. Three different MHC class II molecules on the surface of antigen presenting cells have been identified: HLA-DP, HLA-DQ and HLA-DR [74]. Though there are structural similarities between MHC class I and class II molecules, important distinctions between the two result in vastly different peptide binding characteristics. While the polymorphic residues of MHC
class I molecules are located in the α1 and α2 domain, most of the polymorphisms in MHC class II are located in humans in the β chain [74]. In contrast to MHC class I molecules, the ends of the peptide binding clefts of MHC class II are open, which has important implications for the types of peptides that can be bound. Indeed, peptides of up to 30 amino acids can be presented in the MHC class II binding groove [75].

The pathways by which peptide antigens are processed and presented are also significantly different for MHC class I and II. While MHC class I is mainly specialized in capturing and processing of endogenous antigen, MHC class II is designed for the presentation of peptides derived from exogenous antigens derived from peptide fragments generated in the endosomal compartments of antigen presenting cells [76]. The generation of class II MHC associated peptides from endocytosed antigens involves proteolytic degradation of internalized proteins in endocytic vesicles and the binding of peptide to MHC class II. In addition, cytoplasmic and membrane proteins may also enter the class II pathway through autophagic cross-presentation [74]. It is by this mechanism that viruses like HIV, which replicate in the cytoplasm of infected cells, may enter the MHC class II antigen presentation pathway.

**CD4 T cell helper functions**

The primary function of CD4 helper T cells is to direct and focus immune responses to maximize anti-pathogenic processes, while suppressing non-essential immune responses. This modulatory capacity of CD4 T cells is central to the proper functioning of the immune system. However, recent achievements in CD4 T cell research have changed our thinking about these cells dramatically. While it was commonly believed that Th1 cells provide help to CD8 T cells
and macrophages and Th2 cells generally provide help to B cells, this dichotomy has been revised by the description of additional subsets (Th1, Th2, Th9, Th17, Th21, T follicular helper cells and T regulatory cells), each of which plays a distinct role in the overall immune response.

Upon presentation of viral peptides by antigen presenting cells, CD4 T helper cells become activated, elaborate cytokines, and clonally expand. The differentiation of naïve CD4 T helper cells into distinct subsets occurs during the progression of an infection and depends fundamentally on the effect that the infection has on the antigen presenting cell. The affinity and strength of the TCR-MHC interaction as well as the cytokine/chemokine milieu present during initial CD4 T cell activation greatly influence subsequent T cell differentiation [77]. The CD4 subsets that result from this differentiation are defined primarily on the basis of their ability to secrete different cytokines and the expression of specific transcriptional factors.

Th1 cells are characterized by the expression of the transcription factor Tbet and the production of their cardinal cytokine/chemokine, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), as well as interleukin-2 (IL-2) [78].

Th2 cells, on the other hand, produce IL-4, IL-5, and IL-10 under the control of the transcription factor GATA3 and preferentially act on the humoral arm of the adaptive immune system. These cells have been shown to be important for the induction of antibody class switching to IgE and certain IgG isotypes [79]. A close relative of Th2 cells are IL-9 secreting Th9 cells. The functions and the biology of these cells are not well-understood, although it appears they may have a role in contributing to and regulating inflammation under certain circumstances [80].
T follicular helper (Tfh) cells have been recently described to be central for B cell proliferation, maturation and the induction of somatic hypermutation within the germinal center of B cell follicles. Tfh cells are defined by the transcription factor BCL-6 and surface expression of CXCR5 and inducible costimulator (ICOS) [81]. However, both surface antigens are also present on non-Tfh cells, demonstrating the overlap of effector potential with other CD4 subsets. The functional properties of Tfh cells in humans are currently not well understood; Tfh cells in general produce the cytokine IL-21, which functions to stimulate B cells but may also act in an autocrine fashion to amplify Tfh activity [82]. The production of IL-21 is especially interesting as IL-21 has been linked to important antiviral helper functions for CD8 T cells [63, 83, 84]. It has been suggested that, in addition to Tfh cells, a separate CD4 T cell subset secreting primarily IL-21 may exist to mediate these antiviral activities [85].

Th17 CD4 T cells, which produce IL-17, have also been suggested to secrete IL-21. Th17 cells have been defined on the basis of the transcription factor RORγt and are induced in the presence of IL-6, transforming-growth-factor-β (TGF-β) and IL-1β [86-88]. Interleukin-17 attracts and activates neutrophils and facilitates proinflammatory responses from various other cell types (e.g., endothelial cells).

In contrast to the above subsets, other types of CD4 T cells exert primarily inhibitory functions. T regulatory CD4 T cells (Treg) co-express the zinc-finger transcription factor FoxP3 and IL-2-receptor (CD25). Tregs have been described as a CD4+ subset with suppressive activity on innate and adaptive immunity and have been shown to be enriched in chronic viral infections. Although, it is known that Tregs can be induced through the presence of TGFβ (iTregs), they also can be selected in the thymus in early stages of differentiation (natural Tregs). They are
believed to act by a cell-to-cell contact mediated mechanism that does not depend on soluble factors such as TGF-β or IL-10, although these factors might have an impact in vivo [89, 90].

**Cytolytic CD4 T cells**

Although the majority of CD4 T cell subsets mediate immunoregulatory functions, a growing body of evidence has suggested that certain CD4 T cells may mediate anti-viral effects beyond help and are able to directly lyse virally infected targets [91]. However, relatively little is known about the phenotype, function, and transcriptional profile of cytolytic CD4 T cells. Although cytotoxic activity is traditionally thought to be the role of NK cells or cytotoxic CD8 T lymphocytes, cytolytic CD4 T cells have been implicated in the control of a variety of persistent viral infections, such as Epstein-Barr virus (EBV) and cytomegalovirus (CMV) [92, 93]. Moreover, cyto
tolytic killing of virally infected cells by CD4 T cells has also been observed in vivo in the LCMV mouse model [94]. Although their origin and role in the immune system is controversial, cytolytic CD4 T cells may comprise a novel CD4 subset with a unique lineage and functionality.

Early studies describing the presence of cytolytic CD4 T cells in the context of influenza and poliovirus categorized these cells broadly as belonging to the Th1 subset. This conclusion was based on the cytokine secretion profiles of cytolytic CD4 clones as well as the observation that cells polarized *in vitro* to the Th1 phenotype could exert cytolytic activity in killing assays, in contrast to Th2 cells [95, 96]. These findings have been extended to other viruses, such as West Nile Virus and EBV [97-99] as well as to tuberculosis infection [100] and cancer [101]. However, the functional profile of cytolytic CD4 T cells appears to be distinct from CD4 T cells with a Th1 phenotype. Moreover, while classical Th1 cells show the ability to secrete IL-2, the
The majority of cytolytic CD4 T cells lose this effector function [102]. In contrast, Th1 cells show higher levels of the costimulatory molecules CD28 and CD27 after initial antigenic stimulation, while cytolytic CD4 T cells lack expression of these markers [102, 103]. Similar differences have been observed for the expression of the integrin alpha chains CD11a and CD11b, which are upregulated on CD4 T cells with cytolytic effector potential [102]. This marker profile is suggestive of a terminally-differentiated effector cell phenotype; indeed, the expression patterns of other markers associated with such a phenotype—such as CD57^{high} and CCR7^{low}—are found on perforin or granzyme positive CD4 T cells [102, 104]. Interestingly, perforin or granzyme expressing CD4 T cells with this terminally differentiated phenotype are present even in healthy individuals and are markedly expanded in those with chronic viral infections [102].

Further studies have suggested that cytolytic CD4 T cells may also express molecules that are normally found on NK cells such as NKG2D, KIR2DS2 (CD158j), and KARAP/DAP12 [105-107]. The role of these receptors on cytolytic CD4 T cells, however, is unknown. In CD8+ cells, NKG2D, for example, can act as a costimulatory receptor; signaling through NKG2D on these cells can fine-tune CTL activity in the absence of other costimulatory molecules like CD28 [108-110]. In cytolytic CD4 T cells lacking CD28, it has been hypothesized that NKG2D may serve a similar role [111].

Similarly puzzling is the close phenotypic relationship of cytolytic CD4 T cells with regulatory T cells. Tregs have been defined on the basis of the expression of the transcription factor FoxP3 and the expression of CD25. While the inhibitory action of Tregs is unknown, studies of both mice and human cells suggest that a subset of CD25^{+} CD4 T cells can exert cytolytic activity. CD25^{+} CD4 T cells isolated from human subjects have been shown to express
high levels of perforin and granzyme [112]. Both induced Tregs as well as stimulated CD25+
natural Tregs were confirmed to perform MHC-unrestricted killing of autologous targets [113].
In mice, however, Tregs were observed to lyse target cells in an antigen dependent fashion [114].
Though further studies must be performed to understand the species and context specific
differences behind these activities, work so far has underscored the possibility that Treg
mediated cytotoxicity may play an important role in immune regulation. Most recently, it was
demonstrated that a tumor-antigen specific CD25+ CD4 cell line was able to kill dendritic cells
when infused into tumor bearing mice, suggesting that cytolytic Tregs may be represent a
mechanism to blunt T cell priming by antigen presenting cells [115].

Cytolytic CD4 T cells share common cytolytic pathways with CD8 T cells and NK cells,
including activation of Fas-Fas ligand pathways (CD95L/CD178) [116] and release of cytotoxic
granules containing toxic effector molecules like perforin and granzymes [117]. While the
earliest mechanistic studies of cytolytic CD4 T cells implicated a Fas/FasL based pathway [118],
[119], more recent reports have suggested granule-based mechanisms may be dominant. CD4 T
cells expressing high levels of perforin and granzyme or granulysin have been observed in the
context of several viral infections as well as in healthy subjects. These cells have been shown to
degranulate upon antigenic stimulation directly ex vivo [93, 120-122]. A distinct subset of CD4
T cells expressing both perforin and granzymes has been demonstrated in CMV infected
patients; these cells can release their cytotoxic granules in an antigen-specific manner [93].

Studies using perforin inhibitors or cells from perforin deficient mice have verified the
important role that an exocytotic granule mechanism plays in CD4 cytolysis. Treatment of CD4
T cell clones as well as CD4 T cells directly ex vivo with concanamycin A, a vacuolar type H+-
ATPase inhibitor that inhibits exocytotic granule activity, results in greatly diminished capacity to lyse autologous targets [102, 112]. Though this pathway occurs by a single general mechanism, it is likely it can be differentially regulated within CD4 T cells on the molecular level. For example, mycobacterium- and EBV- specific cytolytic CD4 T cells in particular have been shown to express high levels of granulysin [123, 124]. In studies of cytolytic Tregs, it was found that induced Tregs expressed high levels of Granzyme B, while natural Tregs rather express Granzyme A [113]. The exact conditions that lead to these differences remain unknown but will likely be important for vaccine design.

Additional cytotoxic mechanisms have been described, but their role in mediating cell death is less well studied. In CD8 T cells, it has been demonstrated that the TNF-related apoptosis inducing ligand (TRAIL) plays a significant role in lysing virally-infected target cells, including in HIV-1 and CMV infection [125, 126]. The function of TRAIL in killing by cytolytic CD4 T cells has been evaluated in a limited number of studies, which found that CD4 T cell clones expressed TRAIL and could induce bystander apoptosis in antigen presenting cells [127] as well as in TRAIL-sensitive tumor cell lines [128]. However, the role of the TRAIL pathway in the control of viral infection by CD4 T cells has not been evaluated. Target cells can be sensitized for TRAIL-mediated apoptosis by the presence of inflammatory cytokines such as TNF-α and IFN-γ [126]. In addition to their ability to potentiate apoptosis by other means, both may also exert a direct, contact-independent cytotoxic mechanism [129, 130]. It has been shown that both cytokines can result in cell death by inducing the production of nitric oxide and other free radicals or by activating death pathways within the target cell [131, 132]. Though cytolytic CD4 T cells have been found to secrete these cytokines, their direct impact on CD4-mediated
cytolysis has not been investigated.

Early efforts to quantify the contributions of the different killing pathways to the overall cytolytic activity of CD4 T cells led to the estimate that Fas/FasL mechanisms accounted for 30% of killing and perforin/granzyme pathway only played a negligible role. Cytotoxic cytokines were hypothesized to account for the remainder of the observed activity [133, 134]. However, this work was performed in a mouse model of graft versus host disease and is likely specific for that context. It has also been proposed that a perforin-based pathway is dominant in humans and that Fas-mediated killing is the primary cytolytic pathway for CD4 T cells in mice [135]. Evidence in support of this idea, however, is limited to studies of FasL deficient mice or patients showing a hereditary perforin or Fas deficiency. Indeed, more recent studies in mice have shown that cytolytic CD4 T cells preferentially use a perforin pathway depending on the availability of IL-2 [136, 137]. Though the impact of killing by CD4 T cells through cytotoxic cytokine secretion or a TRAIL-based mechanism has not been evaluated, it is likely that multiple pathways coexist and can be differentially induced depending on immunological context.

Unlike for most of the other CD4 T cell helper subsets, no specific transcription factor or underlying gene pathway has been implicated in mediating cytotoxicity of CD4 T cells. Since cytolytic CD4 T cells have been suggested to resemble Th1 cells, a role for the transcription factor Tbet can be considered. Interestingly, Tbet has recently been found to also regulate the induction of cytolytic programming in CD8 T cells in combination with another T box transcription factor, eomesodermin (Eomes) [138, 139], potentially indicating an overlap in the molecular pathways responsible for cytolytic function in both cell types. Indeed, transcriptional profiling studies have revealed remarkable similarities between the transcriptional profiles of
CD4 and CD8 T cells. Both express high levels of perforin, granzyme, and granulysin, among other proteins [140]. Though the role of Eomes in CD4 T cells is not well characterized, it has been implicated in helping to drive the production of IFN-γ in Th1 cells [77, 141] and has been shown to be able to induce the expression of cytolytic genes when overexpressed in Th2 cells [142]. Nonetheless, CD4 effectors have been found to express Eomes at a considerably lower level than CD8 T cells [143]. A related transcription factor, Runx3, has been shown to be important for the induction of Eomes and the expression of perforin and granzyme B in CD8 T cells [144]. In CD4 T cells, Runx3 is known to play a role in Th1 polarization and IFN-γ production [145, 146]. However, whether it also is involved in the induction of cytolytic activity in CD4 T cells has not yet been established. Also unclear are the polarization conditions that may lead to expression of these transcription factors and cytolytic activity, but high levels of IL-2 likely play a strong role due to specific STAT pathway activation [136]. Indeed, IL-2 has a regulatory effect on perforin and granzyme expression in CD8 T cells and the case may be similar for CD4 T cells [147, 148]. A role in the induction of cytolytic activity in CD4 T cells has also been proposed for IL-15 [102]. Similarly to IL-2, other γ-chain receptor cytokines such as IL-15 and IL-21 can induce expression of perforin and other cytolytic effector molecules [149-151]. However, further transcriptional studies are needed to more rigorously dissect the conditions and effector profiles that lead to the expression of cytolytic function in these cells.

HIV-SPECIFIC CD4 T CELL RESPONSES

Together, the various CD4 T cell subsets synergize to mediate effective anti-pathogen immune responses. Although HIV-infection elicits antigen-specific CD4 T cell responses, the
particular CD4 T cell functions that are most important for anti-viral immunity remain poorly understood. As described above, one hallmark of HIV-1 infection is the progressive depletion of CD4 T cells, with up to 60% of all activated memory CD4 T cells may be deleted in certain tissue compartments [31]. Interestingly, not all of this CD4 T cell loss is due to productive infection by the virus. HIV proteins may directly cause apoptosis, and bystander activation in the context of very inflammatory viral conditions may also lead to cell death [152]. More recently, it has been suggested that abortive infection of CD4 T cells is also an important mechanism for their depletion [153].

**PREFERENTIAL INFECTION**

HIV-specific CD4 T cells are in particular targets for the virus, and early reports suggested that these cells are preferentially infected compared to the general memory CD4 T cell pool. At the time, it was believed this was due purely to antigen-specificity—ongoing HIV replication would result in activation of HIV-specific CD4 T cells, in turn rendering them more susceptible to infection. Indeed, CMV-specific CD4 T cells were shown to be at lower risk for HIV-infection compared to HIV-specific CD4 T cells [154]. More recent evidence has suggested that there may be a functional component to these differences, however. CMV-specific CD4 T cells secrete β-chemokines, which can block viral entry through autocrine binding to the HIV-coreceptor CCR5 [155]. In co-infected individuals, mycobacterium tuberculosis specific CD4 T cells—which generally do not secrete β-chemokines—are at a correspondingly greater risk for HIV infection and are infected at levels similar to HIV-specific CD4 T cells [156]. Regardless of mechanism, however, this preferential infection of HIV-specific CD4 T cells suggested to some that they may have a limited role in HIV control and that elicitation of these cells by a potential
vaccine may be harmful. However, the vast majority of these cells remain uninfected at any given time and available to mediate potentially critical HIV-specific antiviral functions [154].

**HIV-SPECIFIC CD4 T CELL TARGETING**

Although the epitope specificity of HIV-specific CD4 T cells is not as well-characterized as that of HIV-specific CD8 T cells, several studies have pointed to an important role for CD4 T targeting in contributing to an effective antiviral response. During chronic HIV infection, the breadth, magnitude, and specificity of the CD4 T cell response is associated with viral control [64]. Not only do HIV controllers have stronger HIV-specific CD4 T cell responses (as measured by IFNγ ELISPOT), but the breadth of targeting (in terms of number of individual viral epitopes targeted) was inversely associated with viral load. Moreover, HIV controllers preferentially targeted epitopes in the Gag protein compared with other viral proteins, while non-controllers exhibited a dominant targeting of the envelope proteins. Further work as shown that certain HLA-DR molecules are associated with relatively greater levels of viral control and that these alleles have the ability to present a greater breadth of HIV proteins [67]. Interestingly, the immunodominance patterns of HIV-specific CD4 T cell targeting are established early after acute HIV infection and remain relatively static as infection progresses [157]. More effort must be placed on fine mapping individual HIV-specific CD4 T cell epitopes and understanding the functional significance of CD4 targeting of different HIV proteins.
HIV-SPECIFIC CD4 T CELL PHENOTYPE AND FUNCTION

HIV-specific CD4 T cells can be detected in most, if not all, HIV infected subjects, although their frequencies may be extremely low [64, 158]. Examination of CD4 T cell functionality has for the most part been limited to general functions, such as proliferation or Th1 cytokine secretion and targeted, in depth studies of particular CD4 T cell subsets have, for the most part, not been performed. As described earlier, HIV controllers exhibit more robust HIV-specific CD4 T cell responses than individuals with progressive, untreated infection. The HIV-specific CD4 T cells in these subjects are not only stronger, but also appear to be more polyfunctional in terms of the number of different functions they may mediate simultaneously [66]. HIV-specific CD4 T cell responses in controllers show increased proliferative capacity, potentially suggesting that these cells are more readily able to react when expansion of anamnestic responses is required [159-161]. The factors underlying the enhanced functionality of CD4 T cells from HIV controllers are incompletely understood. Virus specific CD4 T cells from these patients are less exhausted, although it is unclear if this is a cause or consequence of controlled viremia [162]. However, several studies have suggested that individuals who control HIV replication have more robust HIV-specific CD4 T cell responses following acute infection, arguing in favor of these cells supporting lower viremia, rather than vice versa [62, 163, 164].

CD4 T cell help for B cells

Due to the importance of CD4 T cells for generating an effective antibody response, and the likely necessity of eliciting a robust antibody response for any protective HIV vaccine, considerable effort has gone into understanding how HIV-specific CD4 T cells may be harnessed to provide optimal help to virus specific B cells [165]. As described above, Tfh cells are the
specialized CD4 T cells that provide critical helper signals to B cells in the germinal centers (GCs) in the lymph node. In the context of HIV infection, perturbations exist within the Tfh compartment [166]. Chronic HIV infection is marked by an expansion of Tfh cells within the lymph nodes; this expansion is associated specific levels of the hypergammaglobulinemia that is characteristic of HIV infection [167]. However, while Tfh cells are expanded during HIV infection, they are also dysfunctional in their capacity to provide help to B cells [168]. Similarly, a longitudinal study of SIV infection has suggested that this accumulating dysfunction may be responsible for lower avidity SIV-specific antibody responses [169]. Further work has shown that B cell helper activity is not only found in the lymph node, but that a particular blood subset of circulating Tfh like cells can also provide help to B cells. Interestingly, the frequencies of these cells correlate with the level of neutralizing antibody breadth in HIV infected individuals and have become an important target for HIV vaccine research [170, 171].

**CD4 T cell help for CD8 T cells**

Another primary mechanism by which CD4 T cells contribute to viral control is through the provision of helper signals to CD8 T cells. Although CD8 T cell responses can be generated in the absence of CD4 T cell help, CD4 T cells are necessary for optimal CD8 T cell function and especially for support of CD8 T cell memory [172]. In HIV infection, the level of IL-2 secreting CD4 T cells correlates inversely with viral load [173]. Although IL-2 likely has several effects, further work described that IL-2 signaling from HIV-specific CD4 T cells plays a role in the proliferation and maintenance of virus-specific CD8 T cell responses. When these signals are lost in the chronic phase of HIV infection, they can be restored in vitro by adding cryopreserved CD4 T cells from acute HIV infection to CD8 T cells from chronic infection. Neutralization of
IL-2 abrogates this effect, suggesting that IL-2 secreting HIV-specific CD4 T cells have a critical role in supporting CD8 T cells [174]. Additional studies have suggested an important helper role for IL-21 secreted by CD4 T cells. IL-21 is important for supporting cytotoxic function in CD8 T cells in the murine model and is secreted by CD4 T cells. In HIV controllers, HIV-specific IL-21 secreting CD4 T cell responses can be found in high levels compared to non-controllers and can enhance HIV-specific CD8 T cell function, including the capacity to inhibit HIV replication [63, 175].

**HIV-specific cytolytic CD4 T cells**

Some of the first HIV-specific CD4 T cell clones isolated from infected individuals were able to kill HIV envelope peptide-pulsed B-cells [176], suggesting a direct cytolytic potential on the part of HIV-1-specific CD4 T cells. Similar reports described cytolytic CD4 T cells obtained from healthy subjects who received a recombinant gp160 subunit vaccine during an early HIV-1 vaccine trial [177-180]. However, these and most other studies of HIV-specific cytolytic CD4 T cells relied on cell lines or clones that were expanded under long-term culture conditions in vitro; it is therefore difficult to draw conclusions about the relevance of cytolytic CD4 T cells to the control of HIV-1 replication in vivo.

Cytolytic CD4 T cells have also been proposed to be a byproduct of chronic viral infection, such as CMV or HIV, where persistent antigenic stimulation could result in the terminally differentiated phenotype that corresponds to cytolytic activity [181]. In the case of HIV infection, a population of perforin positive CD4 T cells can be found in all stages of disease progression; this subset is largest in patients with chronic, untreated disease and can represent up to half of the CD4 T cells in the blood, though this percentage is highly patient dependent [102].
Additional analysis of lymphocytes from HIV-1 infected patients stimulated with gag peptide bolstered these findings, showing that approximately 50% of Gag-specific CD4 T cells are able to degranulate, as measured by the presence of the surrogate degranulation marker CD107a [121]. Several studies have demonstrated cytolytic activity of CD4 T cells from HIV-1 patients directly ex vivo in the killing of peptide-pulsed B cell target cells in chromium release assays [182-185]. In addition, in a recent SIV study, ex vivo peptide-pulsed and fluorescently-labeled target cells disappeared after reinfusion, even when test animals were depleted of CD8 T cells [186]. This suggested a non-CD8 T cell dependent mechanism of target cell lysis and led the authors to speculate that cytolytic CD4 T cells might be involved. However, a direct contribution of these cells to the control of viral replication has not been shown. Thus, it is unknown whether cytolytic CD4 T cells are also able to inhibit HIV-1 replication.

Recent studies have begun to address the question of whether cytolytic CD4 T cells may play a direct role in the control of virus in HIV-1 infected macrophages or CD4 T cells. Sacha et al. demonstrated that macaques able to control SIV infection display strong Gag- and Nef-specific CD4 T cell responses. These CD4 T cell responses were able after long-term culture to inhibit viral replication in SIV-infected macrophages, but were unable to recognize and kill infected CD4 T cells in an in vitro viral replication assay [187]. In contrast, Zheng et al. demonstrated that Nef-specific CD4 T cell clones derived from human elite controllers were able to suppress viral infection in both macrophages and CD4 T cells [188]. Both studies suggested a direct contribution of cytolytic CD4 T cell responses to the control of viral replication. The concept that cytolytic CD4 T cells might recognize virally infected macrophages is interesting and potentially important for HIV-1 vaccine design. Although it has been shown that HIV-1-
specific CD8 T cells are strong contributors to the control of viral replication, escape mutations in the targeted epitopes can substantially impair their efficiency to recognize and kill HIV-1 infected cells [189]. Cytolytic CD4 T cells, however, recognize targets via a class II pathway; infected MHC class II expressing cells—such as other CD4 T cells or macrophages—could therefore be recognized by a second mechanism. It has also been shown that macrophages play a role in the pathogenesis of HIV-1 by acting as long-lived reservoirs for viral persistence [190]. The combined recognition of virally infected antigen presenting cells and CD4 T cells by both cytolytic CD4 and CD8 cells may therefore play an important role in HIV-1 pathogenesis. However, it remains to be seen whether cytolytic HIV-1-specific CD4 T cell responses have any effect on viral inhibition in vivo.
REFERENCES


135. Yanai, F., et al., *Essential roles of perforin in antigen-specific cytotoxicity mediated by human CD4+ T lymphocytes: analysis using the combination of hereditary perforin-


Chapter 2: HIV-specific cytolytic CD4 T cell responses during acute HIV infection predict disease outcome
This chapter is based on the peer-reviewed publication:


ABSTRACT

Early immunological events during acute HIV infection are thought to fundamentally influence long-term disease outcome. Whereas the contribution of HIV-specific CD8 T cell responses to early viral control is well established, the role of HIV-specific CD4 T cell responses in the control of viral replication following acute infection is unknown. A growing body of evidence suggests that CD4 T cells - besides their helper function - have the capacity to directly recognize and kill virally infected cells. In a longitudinal study of a cohort of individuals acutely infected with HIV, we observed that subjects able to spontaneously control HIV replication in the absence of antiretroviral therapy showed a significant expansion of HIV-specific CD4 T cell responses—but not CD8 T cell responses—compared to subjects who progressed to a high viral set point (p=0.038). Strikingly, this expansion occurred prior to differences in viral load or CD4 T cell count and was characterized by robust cytolytic activity and expression of a distinct profile of perforin and granzymes at the earliest time point. Kaplan-Meier analysis revealed that the emergence of Granzyme A+ HIV-specific CD4 T cell responses at baseline was highly predictive of slower disease progression and clinical outcome (average days to CD4 T cell count <350/µl was 575 versus 306, p=0.001). These data demonstrate that HIV-specific CD4 T cell responses can be used during the earliest phase of HIV infection as an immunological predictor of subsequent viral set point and disease outcome. Moreover, these data suggest that expansion of Granzyme A+ HIV-specific cytolytic CD4 T cell responses early during acute HIV infection contributes substantially to the control of viral replication.
INTRODUCTION

Acute HIV infection results in the massive depletion of CD4 T cells throughout all compartments of the body. In particular, HIV-specific CD4 T cells are preferentially targeted, disrupting a central process for the successful coordination of the antiviral immune response [1]. Vaccine design strategies eliciting these responses have consequently been met with skepticism due to the fear that the induction and activation of HIV-specific CD4 T cell responses may fuel, instead of prevent, viral replication. Effector CD4 T cell responses have therefore not traditionally been a primary focus of HIV research, and considerable attention has rather been focused on HIV-specific CD8 T cell responses. Indeed, studies in acute HIV infection have shown that there is a temporal association between the first emergence of HIV-specific CD8 T cell responses and a decrease in viral load to a set point [2, 3]. Further work has shown that this early viral set point is a strong predictor of disease outcome [4, 5].

Nevertheless, an increasing number of reports have suggested that HIV-specific CD4 T cell responses may also play an important role in controlling viral replication in HIV infection [6-9]. In particular, the results of the recent, modestly protective RV144 vaccine trial—which not only induced non-neutralizing antibodies, but also a robust HIV-specific CD4 T cell response [10, 11]—raised important questions regarding the contribution of HIV-specific CD4 T cells to the initial control of HIV viremia.

Besides governing the induction and maintenance of the CD8 T cell response, as well as B cell proliferation and antibody maturation, a growing body of evidence suggests that effector CD4 T cells can themselves display potent antiviral activity by directly killing infected targets (reviewed in [12]). In the context of infection by other viruses, including cytomegalovirus [13],
influenza [14], and Friend virus [15], it has been demonstrated that cytolytic CD4 T cells are readily detectable *ex vivo* and can contribute to viral containment even in the absence of antigen-specific CD8 T cell or B cell responses. Interestingly, CD4 T cells from HIV infected patients have been shown to express large quantities of cytolytic effector molecules like perforin and granzymes, and HIV-specific CD4 T cell clones and cell lines can readily mediate target cell lysis and viral inhibition *in vitro* [16-18]. Moreover, the phenotype of the CD4 T cell response observed in the RV144 trial showed cytolytic activity [11], suggesting that these cells may possibly play a part in the prevention of HIV acquisition.

In the present study, we assessed the dynamics and evolution of the HIV-specific CD4 T cell response in a cohort of highly acutely infected subjects in order to understand the role of HIV-specific cytolytic CD4 T cell responses during acute HIV infection and to determine their impact on subsequent disease outcome, early viral control, and establishment of the early viral set point.

RESULTS

*HIV-specific CD4 T cell responses correlate with control of viral replication after acute HIV infection.*

To assess the role of HIV-specific CD4 T cell responses during acute HIV infection and their subsequent impact on the early viral set point, we selected 11 individuals identified during peak viremia, before seroconversion, with exceedingly high viral loads averaging 3,523,364 HIV RNA copies per milliliter and a negative or indeterminate Western blot test (three or fewer bands; Table 2.1). The study group consisted of a very homogenous population in terms of race,
Table 2.1: Subject characteristics. CD4 T cell responses were longitudinally evaluated in a subset of eleven patients, while baseline CD4 responses were measured in an expanded group, consisting of the longitudinally evaluated individuals and additional subjects meeting the criteria for acute infection.

<table>
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gender, age, and risk factors. All individuals remained off therapy for at least 1 year, and were further divided into two groups on the basis of their viral set points 1 year after acute HIV diagnosis. One group progressed to a low early viral set point (average, 11,234 HIV RNA copies per milliliter), whereas the other group progressed to a significantly higher early viral set point (average, 134,020 HIV RNA copies per milliliter; \( p = 0.0043; \) **Figure 2.1**). However, neither baseline peak viremia nor viral loads at the 2-month time point were significantly different between the two groups (average, 3,768,200 versus 3,319,333 HIV RNA copies per milliliter and 82,232 versus 75,374 copies per milliliter, respectively). Yet, 6 months after initial presentation, viral loads diverged significantly (\( p = 0.004 \)) and remained lower in one group compared to the other. In addition, CD4 T cell counts for both groups did not differ throughout the study period, but began to decline in subjects who reached a high viral set point after 12 months of infection (449 vs. 618 cells/µl, \( p = \text{not significant} \)). Although it has been previously shown that certain human leukocyte antigen (HLA) class I alleles are associated with lower viral loads, none of the subject groups were enriched for protective HLA class I alleles [19] (**Table 2.2**).

To evaluate the impact of HIV-specific CD4 T cell responses on the early viral set point, we longitudinally assessed whether the early emergence of HIV-specific CD4 T cell responses could positively impact the viral load trajectories of these two untreated, acutely infected subject groups or whether—as previously hypothesized—they would have a negative impact because of the generation of new target cells for viral infection. In the high viral set point group, we observed an initial decrease in the Gag-specific interferon-\( \gamma \)-positive (IFN\( \gamma \)+) CD4 T cell response that was maintained at a lower level over the course of the study period. In contrast, in subjects who progressed to a low early viral set point, we observed an early expansion of the
Figure 2.1: Dynamics of viral load during acute HIV infection. 11 acutely infected individuals were split into two groups based on their early viral set points. 5 individuals progressed to a high viral set point of 134,020 HIV RNA copies/ml one year after presentation (red); 6 subjects progressed to a significantly lower viral set point (11,234 copies/ml, $p = 0.004$, Mann-Whitney test) one year after presentation (blue). Average CD4 T cell counts for subjects progressing to high or low viral set points are denoted below the graph in red or blue, respectively, at each analysis timepoint.

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$p = \text{n.s.}$
Table 2.2: Subject HLA class I and class II genotypes.

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HIV-specific CD4 T cell response 2 months after infection \( (p = 0.038; \text{Figure 2.2A}) \). Markedly, this increase was observable before viral loads between the groups diverged, suggesting that the expansion of the Gag-specific CD4 T cell responses plays a positive role in the early control of viral replication. In contrast, no significant difference in the HIV-specific CD8 T cell response between the groups was observed (\text{Figure 2.3A}). Comparable behavior of the HIV-specific CD4 T cell responses to other viral proteins was also detected, but these proteins were observed to be less consistently targeted by CD4 T cells, similar to findings in previous reports [6, 20]. The expression of the CCR5 co-receptor on the HIV-specific CD4 T cells was not significantly different between the two subject groups, suggesting that an elevated activation status of the HIV-specific CD4 T cells did not lead to their preferential depletion in patients progressing to high 1-year viral set points (\text{Figure 2.4}), as previously speculated [21]. Moreover, no difference in proviral HIV DNA levels could be detected within sorted HIV-specific CD4 T cells, with no virus detectable in cells from either subject group (limit of detection: <1 HIV DNA copies/ml). Rather, the simultaneous expansion of virus-specific IFN\( \gamma \)+ CD4 T cell responses and initial decline of viremia preceded the point where differences in viral load became evident between the two groups. This temporal association suggests a possible contribution of HIV-specific CD4 T cell responses to the subsequent control of HIV replication and a lower viral set point.

\textit{Early HIV-specific CD4 T cell responses exhibit cytolytic activity in subjects who control viral replication}

Recent studies of other viral infections like Epstein-Barr virus [22] have indicated that CD4 T cells may also contribute to viral control through potentially independent cytolytic
Figure 2.2: HIV-specific CD4 T cell responses during acute HIV infection. HIV-specific CD4 T cell responses expand in acute HIV infection in subjects progressing to lower viral set points. (A) The Gag-specific IFNγ+ CD4 T response was evaluated longitudinally in individuals who progressed to a lower viral set point (blue, n=6) and in those who progressed to a high viral set point (red, n=5). A significant differences in the IFNγ response levels was noted at 2 months post presentation ($p=0.038$, Mann-Whitney test) (B) Longitudinal monitoring of the Gag-specific CD4 T cell degranulation (CD107a) response was performed following acute HIV infection in subjects progressing to low (blue, n=6) and high (red, n=5) viral set points. Significant differences in CD107a expressing CD4 T cells between patient groups are observable at both two and four months post presentation ($p=0.042$ and $p=0.0097$, respectively, by the Mann-Whitney test).
Figure 2.3: HIV-specific CD8 T cell responses during acute HIV infection are not associated with better viral control. The Gag-specific CD8 T cell response was evaluated in subjects who progressed to a high (red, n = 5) or low (blue, n = 6) viral set point after one year of infection. The expansion of the Gag-specific CD8 IFNγ response (A) and CD107a degranulation response (B) were assessed longitudinally following acute infection in both groups. Differences between subject groups were non-significant at any timepoint (p>0.05, Mann-Whitney test).
Figure 2.4: **CCR5 expression on Gag-specific CD4 T cells does not differ between subjects progressing to high or low viral set points.** In order to assess the influence of HIV-specific CD4 T cell activation and preferential depletion of these cells, the CCR5 expression pattern on HIV-specific CD4 T cells was determined in patients progressing to high (red, n=5) or low (blue, n=6) viral set points. This is expressed both as (A) the percentage Gag-specific IFNγ+ CD4 T cells expressing CCR5 as well as (B) the median-fluorescence intensity (MFI) of CCR5 on Gag-specific IFNγ+ CD4 T cells. Differences between subject groups were non-significant at any timepoint (p>0.05, Mann-Whitney test).
effector functionality, raising the possibility that a similar mechanism may be taking place during acute HIV infection. Indeed, cytolytic CD4 T cells have not only been described in HIV infection [17] but have also been shown to have the ability to kill virally infected cells [16, 18, 23, 24]. Therefore, to assess the direct cytolytic activity of HIV-specific CD4 T cell responses during acute infection, we first longitudinally evaluated CD107a expression after HIV peptide stimulation in each patient group as a surrogate measure of degranulatory activity [25]. As with HIV-specific IFNγ-secretion, in individuals who progressed to a high viral set point, we observed an early decline in the HIV-specific CD107a response (Figure 2.2B). This was in stark contrast to individuals who spontaneously controlled viral replication and progressed to a low viral set point. In these individuals, there was a significant early expansion of CD107a responses to a level significantly higher both at 2 months and 6 months after infection (p=0.042 and p=0.0097, respectively; Figure 2.2). This difference in CD107a expression became evident before the divergence of viral load or CD4 T cell count, again suggesting a causal relationship between the specific enrichment of HIV-specific CD107a+CD4 T cells and viral load in individuals who progress to a low viral set point. As before, no significant difference was observed for the HIV-specific CD8 T cell response at any time point (Figure 2.3B). Differences in CD4 T cell CD107a expression were also not due to a general enrichment of degranulatory CD4 T cell responses in one subject group over the other, as no significant differences were observed between patient groups at any time point in the ability of bulk CD4 T cells to degranulate after polyclonal stimulation with phorbol 12-myristate 13-acetate (PMA)/ionomycin (Figure 2.5).
Figure 2.5: Cytolytic CD4 T cell responses following PMA/ionomycin stimulation do not differ between set point groups. (A) The longitudinal CD4 T cell degranulation (CD107a) response was assessed following non-specific stimulation with PMA/ionomycin in both subject groups (high set point: n=5; low set point: n=6). Differences between subject groups were non-significant at any timepoint ($p>0.05$, Mann-Whitney test). (B) The cytolytic phenotype of IFN$\gamma^+$ CD4 T cells from patients in these groups was examined following PMA/ionomycin stimulation to verify that the granzyme A enriched phenotype observed in the HIV-specific IFN$\gamma^+$ CD4 T cell response at baseline in individuals who progressed to a lower viral set point was not due to a general higher level of granzyme A expression (orange arcs) in the bulk CD4 cells of these individuals.
**CD4 T cells from HIV infected individuals mediate direct cytolytic activity**

To establish that HIV-specific CD4 T cells from acutely HIV-infected patients can exert a cytolytic effect that directly contributes to the control of HIV replication, we developed a single-cycle, flow based viral inhibition assay optimized for the detection of antiviral CD4 T cell function. This assay makes use of autologous monocyte-derived macrophages as optimal target cells [18] and expanded CD4 T cells as effectors. Macrophages were infected with a single-cycle, ZsGreen expressing, pseudotyped HIV to facilitate a high level of synchronized target cell infection and to prevent any reverse infection of the effector cell population. Expanded CD4 T cells from the chronic phase of the infection from nine subjects within our cohort were purified and then co-incubated with autologous, infected macrophages at different effector to target (E:T) ratios. Interestingly, we observed a significant, dose-dependent suppressive effect of up to 40% reduction in HIV-infected macrophages mediated by CD4 T cells (E:T 5:1 range: 5-40%; p=0.004; **Figure 2.6A** and **2.6B**). To determine whether short term HIV-specific expansion could augment the CD4 T cell antiviral capacity, we expanded purified CD4 T cells from one individual for 7 days by culture with Gag peptide pools. Indeed, this short term antigen specific expansion resulted in a significant increase in the suppressive capacity of the CD4 T cells from 14% to 35% compared to direct ex vivo viral suppression (E:T 10:1; p = 0.014; **Figure 2.6C**). To further confirm our findings, we generated HIV-specific CD4 T cell clones from the same individual specific for an epitope within p24/Gag (TAPPEESFRFGEETTPSQK). In a 7 day viral inhibition assay using HLA-DR matched H9 CD4 T cell line targets, these HIV-specific CD4 T cell clones were able to inhibit viral replication up to 1000-fold. Moreover, this effect was almost completely abrogated when HLA-DR was blocked using a neutralizing antibody
Figure 2.6: CD4 T cells from HIV-infected subjects mediate viral suppression.
Functional assays were performed to assess the ability of CD4 T cells from HIV-infected subjects to directly exhibit an antiviral effect. (A) Representative example of the results of one single-cycle macrophage inhibition assay; CD4 T cell effector cells were tested for their ability to reduce the number of macrophages infected with a ZsGreen reporter HIV at various effector to target (E:T) ratios. Percentages represent the number of HIV+ macrophages remaining at the end of the assay period. (B) CD4 T cells from a subset of the cohort of subjects analyzed longitudinally (n=9) were assessed for suppressive capacity in the single-cycle inhibition assay. Results are expressed as percentage of HIV+ autologous macrophages remaining and have been normalized to the respective maximum for each set of conditions. (C) CD4 T cells from a chronically HIV-infected patient were used as effectors in the single-cycle macrophage inhibition assay ex vivo or following non-specific (CD3.8) or Gag-specific expansion. (D) CD4 T cell clones were generated from the same chronically infected patient and assessed for their ability to inhibit viral replication in a standard 7 day viral inhibition assay using infected HLA-DR matched H9 cells as targets.
(Figure 2.6D). Our data therefore suggest that the HIV-specific CD4 T cells in our cohort may indeed have direct cytolysis activity against HIV infected cells and directly contribute to the control of viral replication.

**Individuals who progress to low viral set point exhibit a unique HIV-specific cytolytic CD4 phenotype at baseline**

To further investigate the differences in the HIV-specific cytolytic CD4 T cells in both groups, we examined the cytolytic phenotype of the HIV-specific CD4 T cell response at each time point. Using intracellular cytokine staining, we assessed the presence of cytolytic effector molecules granzyme A, granzyme B, granzyme K, and perforin in HIV-specific IFNγ-secreting CD4 T cell responses (Figure 2.7). Using a boolean gating strategy, we observed that the HIV-specific IFNγ+ CD4 T cell response at baseline was unexpectedly cytolytic—nearly 75% of the IFNγ responding cells contained at least one type of granzyme or perforin. Interestingly, however, the baseline HIV-specific IFNγ response in subjects who progressed to a low viral set point was substantially different than in those who progressed to a high set point (Figure 2.7B), at a time when viral loads and CD4 T cell counts were not significantly different. The baseline response in the low set point group not only contained an increased number of responses expressing all four cytolytic molecules but was also strikingly dominated by granzyme A (GrzA). Expression of GrzA—but not any other measured effector molecule—was significantly associated with lower viral set point (p<0.0001, Figure 2.7C). These differences were only observed at baseline and the cytolytic profile of the responses at later time points did not differ substantially between the two subject groups. Additional analysis revealed that these differences were HIV-specific and were not due to a general elevation of GrzA in IFNγ-responsive CD4 T
Figure 2.7: Baseline HIV-specific CD4 T cell responses and viral control. Baseline HIV-specific CD4 T cell responses enriched in Granzyme A are associated with viral control. (A) Representative example of flow cytometric analysis of the expression of the cytolytic effector molecules granzymes A, B, K, and perforin in HIV-specific IFNγ+ CD4 T cells. (B) Co-expression analysis of the expression of granzymes and perforin in Gag-responding CD4 T cells was performed to determine if differences in the cytolytic profile of these cells could be detected in the cohort of subjects analyzed longitudinally (n=11). Pie slices are colored according to the number of cytolytic molecules expressed in the Gag-specific CD4 T cell response (0: pattern; 1: yellow; 2: green; 3: blue, 4: red); the orange colored arc represents the fraction of the total HIV-specific IFNγ+ CD4 T cells expressing granzyme A. (C) Phenotypic analysis was performed to determine the ratio of Granzyme A to other cytolytic effector molecules within HIV-specific IFNγ-secreting CD4 T cells expressing at least one cytolytic enzyme in both patient groups (low set point, 43.2%, versus high set point, 13.4%; p = 0.019, Mann-Whitney test).
cells in individuals who progressed to a low viral set point. Indeed, no difference was observed in the baseline cytolytic phenotype of IFNγ positive cells after PMA/ionomycin stimulation in both groups (Figure 2.5B). Further examination of HIV-specific GrzA+ CD4 T cell responses revealed an almost exclusive co-secretion of IFNγ; GrzA+ CD4 T cells expressed other markers like IL-2, TNFα or CD40L to a lesser extent (Figure 2.8). Nonetheless, a second analysis performed using antigen-specific CD40L upregulation to define HIV-specific CD4 T cells provided further verification that the presence of HIV-specific GrzA+ CD4 T cells at baseline is significantly enriched in those individuals subsequently controlling viremia (p=0.038; Figure 2.8B). To confirm a role for GrzA expressing HIV-specific CD4 T cells in the initial control of HIV viremia, we next examined the cytolytic phenotype of the HIV-specific IFNγ+CD4 T cell responses at baseline in an expanded cohort of 26 acutely infected individuals without prior knowledge of their clinical course. This cohort consisted of the original subjects studied longitudinally as well as an additional group of individuals who met the same criteria for acute infection (Table 2.1). We observed a striking association between the proportion of the Gag-specific IFNγ+ CD4 T cells expressing GrzA (relative to the other cytolytic effector molecules) and ultimate clinical outcome (Figure 2.9). Kaplan-Meier analysis revealed that patients who exhibited GrzA<sup>high</sup> Gag-specific CD4 T cell responses at the time of initial presentation with acute HIV infection maintained CD4 T cell counts above the treatment initiation threshold of 350 cells/μl (US Department of Health and Human Services [26]) significantly longer than subjects with GrzA<sup>low</sup> responses (avg. 575 days vs. 306 days; Log-rank p=0.0012, Wilcoxon p=0.0019; Fig. 2.9A). Similarly, analysis of the time after initial presentation until initiation of antiretroviral therapy demonstrated that subjects with GrzA<sup>high</sup> Gag-specific CD4 T cell
Figure 2.8: Alternate markers of HIV-specificity may be used to define Granzyme A+ CD4 T cells. (A) ICS was performed to determine which markers of HIV-specificity co-expressed best with Granzyme A within HIV-specific CD4 T cells. Staining was carried out for IFNγ, TNFα, IL-2, and CD40L. Percentages displayed represent the fraction of the displayed response that can be detected using only IFNγ. Representative staining is displayed from one subject. (B) Baseline samples from patients progressing to high (n=4) or low (n = 6) viral set points were stimulated with Gag and stained for CD40L as well as granzymes A, B, K, and perforin. The cytolytic phenotype was then analyzed to determine the proportion of granzyme A expressing HIV specific responses, where HIV-specific CD4 T cells were defined as CD69+CD40L+, rather than IFNγ+ (p=0.038, Mann-Whitney test).
Figure 2.9: Cytolytic phenotype of HIV-specific CD4 T cells predicts clinical outcome. The cytolytic phenotype of the HIV-specific CD4 IFNγ response was measured at baseline in an expanded cohort of 26 patients comprised of the original patients evaluated longitudinally and additional patients meeting the same criteria for acute HIV infection. Subjects were stratified into two groups based on the presence of GrzA\textsuperscript{high} CD4 T cell responses (blue lines, n = 13) or GrzA\textsuperscript{low} CD4 T cell responses (red lines, n = 13). Kaplan-Meier analysis was then performed to determine if differences between the two groups were present in (A) the time until CD4 counts declined to 350 cells/µl, (B) the time subjects remained off antiretroviral treatment, or (C) the length of time individuals were able to control viremia to levels below 100,000 HIV RNA copies/ml. \( p \) values denoted on the graphs represent Log-rank test results.
responses at baseline remained off therapy significantly longer than individuals with GrzA\textsuperscript{low} responses (avg. 716 days vs. 423 days; Log-rank p = 0.0039, Wilcoxon p=0.0026; \textbf{Figure 2.9B}). Moreover, subjects with elevated HIV-specific GrzA+ CD4 T cell responses at baseline demonstrated significantly longer control of viral load to levels below 100,000 HIV RNA copies/ml (BII recommendation for treatment initiation [26]) compared to subjects with lowered responses (649 vs. 258 days before reaching this threshold; Log-rank p=0.0138, Wilcoxon p=0.007; \textbf{Figure 2.9C}). Our data therefore demonstrate that the expansion of specific GrzA-expressing, HIV-specific IFN\textgamma+ CD4 T cell responses during the earliest phase of HIV infection is highly predictive of subsequent clinical outcome and may potentially represent a prognostic tool to provide an early estimate for long-term disease progression risk prior to the establishment of viral set point.
DISCUSSION

The contribution of CD4 T cells to the HIV-specific immune response has remained unclear. Although CD4 T cells and the helper and effector functions they employ are recognized to be necessary for optimal antiviral responses, the fact that HIV-specific CD4 T cells are preferentially depleted has cast doubt on their ability to effectively contribute to HIV control. Whereas HIV-specific CD4 T cell activity has previously been associated with viral control in studies of chronically infected patients, the kinetics and character of these responses have not been fully assessed, especially during primary HIV infection when initial immune control results in establishment of the viral set point. Here, we therefore investigated the HIV-specific CD4 T cell response longitudinally following acute HIV infection in two patient groups with very similar baseline characteristics but progressing to divergent viral set points, with a specific focus on the cytolytic CD4 T cell response. We observed that the HIV-specific CD4 T cell response, and not the HIV-specific CD8 T cell response, was enhanced in individuals progressing to a lower viral set point following acute HIV infection. In these subjects, greater CD4 T cell cytolytic activity was observed, reflected both as an expansion of CD107a+ CD4 T cell responses as well as an enrichment of granzyme A expressing cytolytic CD4 T cells at baseline. These findings raise the possibility that the involvement of HIV-specific CD4 T cell responses in controlling viral replication may be particularly important in the context of a less effective HIV-specific CD8 T cell response. Our cohort was not enriched for protective HLA class I alleles that have been previously associated with HIV control, suggesting that inefficient CD8 T cell activity can potentially be compensated for by a strong HIV-specific CD4 T cell response. Conversely, in elite HIV controllers who have protective HLA class I alleles such as HLA-B57 and -B27, it is
possible that the role of HIV-specific cytolytic CD4 T cell responses is comparatively attenuated and that viral control may be mediated dominantly through CD8 T cell responses.

Our results are in agreement with previous, albeit smaller, studies suggesting that individuals who control HIV replication have more robust HIV-specific CD4 T cell responses following acute infection [27, 28]. Enhanced levels of IFNγ production by HIV-specific CD4 T cells and particularly high avidity CD4 T cells may provide important helper signals to aid in the antiviral response mediated by CD8 T cells or B cells [29]. However, the ability of HIV-specific CD4 T cells to degranulate and release cytolytic effector molecules may also have special significance for HIV pathogenesis, especially during the first phases of infection. During acute infection, the virus irreversibly establishes viral reservoirs in long-lived cells such as memory CD4 T cells or macrophages. Due to their residence in the tissues, macrophages in particular may represent critical early targets for viral infection and dissemination [30, 31]. By virtue of their expression of high levels of MHC class II molecules, they may also be important targets for lysis by cytolytic CD4 T cells. Our inhibition assays using short-term expanded CD4 T cells suggest that macrophages can consistently be targeted by suppressive CD4 T cell responses. Moreover, our data demonstrate dose-dependent cytolytic CD4 T cell activity against HIV infected macrophages that was significantly enhanced after HIV-specific expansion, in line with previous reports [18, 24]. HIV-specific cytolytic CD4 T cells may therefore be uniquely suited to lysing macrophages (as well as other MHC class II expressing cells) and, with CD8 T cells, provide a second route of cytolytic pressure on the virus. Further evidence for the importance of these cells is provided by the SIV model. Not only does the presence of strong virus-specific CD4 T cell responses correlate with protection after vaccination, but cytolytic SIV-specific CD4
T cells that are able to degranulate and express perforin are also associated with enhanced viral control in the context of attenuated SIV infection [32-34]. Additionally, a recent study by Ortiz et al has demonstrated a lack of post peak viral decline in SIV infected macaques when CD4 T cells were depleted prior to SIV infection. Interestingly, no association with humoral or CD8 T cell mediated factors was observed, suggesting that CD4 T cells may indeed play an important direct antiviral role during acute SIV infection [35]. Although further studies are required to evaluate the mechanisms used by HIV-specific cytolytic CD4 T cells, our results nonetheless suggest that cytotoxicity may be an important CD4 T cell function in the context of the early control of HIV infection. However, it is important to note that the contribution of HIV-specific CD4 T cell responses in the control of HIV infection is most likely multifactorial and includes not only direct cytolytic activity, but also help for B cells and CD8 T cells. Our data suggest a critical role for HIV-specific cytolytic CD4 T cells early during acute infection that is maintained into chronic HIV infection but ultimately lost, suggesting that alternate factors may play a role in the continued control of viral replication in later stages. In particular, broadly neutralizing antibody responses that have been shown to emerge late in chronic HIV infection might additionally contribute to the containment of viral replication [36]. Thus, the enhanced early HIV-specific CD4 T cell response we observe may also play an important role in priming later immune function for chronic control of viremia. Further studies will be necessary to examine the interplay of the various short and long-term antiviral effects mediated by virus specific CD4 T cells during acute HIV infection.

The relevance of enriched GrzA expression in cytolytic CD4 T cells to the control of HIV replication is unclear. Although the expression of granzyme B and perforin in CD8 T cells has
been linked to efficient cytotoxic functionality and to HIV control [37, 38], granzyme A is nonetheless the protease most prevalent in the granules of cytotoxic cells [39]. Although its specific functions remain incompletely characterized, GrzA has been shown to be important for recovery from poxvirus infections in the murine model, for example [40]. The enzyme is known to act at least in part by proteolytically destroying elements of the SET complex, a group of proteins which plays an important role in stress-response and DNA-damage repair pathways [39]. Interestingly, it has recently been shown that the SET complex facilitates chromosomal integration of the HIV-1 genome by preventing abortive autointegration events [41]. It is therefore possible that GrzA produced by HIV-specific cytolytic CD4 T cells may be uniquely able to inhibit this complex, preventing HIV-integration in newly infected cells and reducing the early establishment of viral reservoirs. Indeed, the strongest cytolytic activity observed in the subjects in our cohort was directed towards Gag peptides, which can be presented as early as two hours after infection in infected cells, prior to the occurrence of HIV integration [42]. GrzA additionally has been shown to mediate extracellular and pro-inflammatory activity in certain contexts. Additional research will be required to investigate the functional effects of granzyme A produced by cytolytic CD4 T cells and determine how this protease may facilitate viral control.

Our findings showing robust IFNγ+ and cytolytic CD4 T cell responses in individuals who spontaneously control viral replication to a lower viral set point after acute infection have important implications regarding the role of CD4 T cells in the early control of HIV. The fact that the expansion of the HIV-specific CD4 T cell response—but not CD8 T cell response—was evident before viral load differences and correlated with lower subsequent viral set point
suggests the possibility that HIV-specific CD4 T cell activity, and especially cytolytic CD4 T cell activity, may indeed have a contributing role in ultimately determining viral set point. Here, in particular, the granzyme A status of HIV-specific CD4 T cell response at baseline appears to predict subsequent clinical outcome, suggesting that GrzA may have a unique role in shaping the early antiviral response against HIV. Thus, our findings not only provide an additional example of the importance of cytolytic CD4 T cells in the human antiviral response, but will also help guide future HIV vaccine design strategies as the field builds on the results of the RV144 trial.
MATERIALS AND METHODS

Subjects. All primary subjects were identified during acute HIV infection (Table S1) and were recruited as subjects following written informed consent. Subjects were enrolled at either the Jessen-Jessen-Stein clinic in Berlin, Germany, the Fenway Community Health Center in Boston, MA, or the Massachusetts General Hospital in Boston, MA. For all subjects, acute infection was defined clinically as a negative or indeterminate (less than 3 bands) HIV western blot test and positive HIV RNA.

Stimulation. Cryopreserved PBMCs were thawed and allowed to rest overnight at 37° C, 5% CO₂ at a concentration of 2x10⁶/ml in R10 media (RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES). The following day, PBMCs were washed and resuspended at a concentration of 5-10 million cells/ml in R10 medium containing anti-CD28/49d costimulatory antibodies (1 µg/ml, clones L293 and L25, BD Biosciences). For detection of CD107a, a pretitered amount of PE-Cy5 conjugated antibody to CD107a (clone H4A3, BD Biosciences) was added to the medium. 1-2 million cells were stimulated for six hours with 18-mer overlapping peptide pools comprising HIV clade B Gag, Pol, Nef, Gp120, or Gp41 at a concentration of 2 µg/ml. As a positive control, cells were stimulated with a combination of PMA and ionomycin. An unstimulated (medium only) sample served as a negative control. 30 minutes into the stimulation, the transport inhibitors brefeldin A (Sigma) and monensin (BD Biosciences) were added as previously described [43] to facilitate detection of T cell responses. For detection of CD40L, cells were stimulated in H10 medium (RPMI-1640 supplemented with 10% heat-inactivated human AB serum [Gemcell], 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml
streptomycin, and 10 mM HEPES) without CD107a-PECy5 and without costimulatory antibodies (to minimize CD40L background).

**Flow cytometric staining.** *Longitudinal analysis.* Following stimulation, cells were washed with phosphate buffered saline (PBS) and stained with a UV-excitable, amine-reactive viability dye (LIVE/DEAD Blue, Invitrogen). Cells were subsequently washed with staining buffer (PBS containing 2% fetal calf serum and 0.09% sodium azide) and stained with CD4-BD Horizon v450 (clone RPA-T4, BD Biosciences), CD8-eFluor 650NC (clone RPA-T8, eBioscience), and CCR5-APC-Cy7 (clone 2D7/CCR5, BD Biosciences). After surface staining, cells were fixed and permeabilized (FIX/PERM, Invitrogen) and stained intracellularly using the following antibodies: CD3-Qdot 605 (clone UCHT1, Invitrogen), IFNγ-PE-Cy7 (clone B27, Biolegend), Granzyme A-Alexa 647 (clone CB9, Biolegend), Granzyme B-Alexa 700 (clone GB11, BD Biosciences), Granzyme K-FITC (clone GM6C3, Santa Cruz Biotechnology), and Perforin-PE (clone B-D48, Santa Cruz Biotechnology). *CD40L analysis.* Cells were stained as described above, with the following modifications: CD107a-PE-Cy5 and CCR5-APC-Cy7 were excluded; CD4 was stained with CD4-Qdot 705 (clone S3.5); CD40L and CD69 were stained for intracellularly using CD154-Brilliant Violet 421 (clone 24-31, Biolegend) and CD69-APC-Cy7 (clone FN50, Biolegend), respectively. *Polyfunctionality analysis.* Cells were stained as described above, with a modified antibody panel. Surface staining was performed with CD4-BD Horizon v450 (clone RPA-T4, BD Biosciences) and CD8-eFluor 650NC (clone RPA-T8, eBioscience). Cells were stained intracellularly using CD3-Qdot 605 (clone UCHT1, Invitrogen), IFNγ-PE-Cy7 (clone B27, Biolegend), Granzyme A-Alexa 647 (clone CB9,
Biolegend), IL-2 FITC (clone 5344.111, BD Biosciences), TNFα Alexa Fluor 700 (clone MAb11, BD Biosciences), and CD40L PE (clone TRAP1, BD Biosciences).

**Flow cytometric analysis.** Flow cytometric data was collected using a special order 5-laser LSR Fortessa and FACSDiva software (BD Biosciences). Compensation was performed using single-stained antibody capture beads (CompBeads, BD Biosciences) and amine-dye reactive beads (ArC, Invitrogen). Cytometer settings were standardized and tracked between runs using multi-fluorescent calibration beads (Rainbow Fluorescent Particles, Spherotech). Data were analyzed using FlowJo version 9.2 (TreeStar). Initial gating was performed using a forward scatter (area) vs side scatter (area) lymphocyte gate, followed by a forward scatter (area) vs. forward scatter (height) doublet exclusion gate. CD4 cells consisted of live (viability stain negative), CD3+ T cells, CD4+, CD8- T cells (Figure 2.10). For analysis of CD40L expression, CD40L positive CD4 T cells were defined as CD69+CD40L+ CD4 T cells. All response data shown have been background subtracted based on the unstimulated control for each sample set. Coexpression analysis of Granzyme A, B, K, and Perforin was performed using a Boolean gating strategy and the PESTLE and SPICE software suite (NIH/Mario Roederer [44]).

**Detection of cellular HIV-1 DNA.** Cryopreserved PBMC were thawed and stimulated as described above. Following stimulation, cells were labeled with LIVE/DEAD Violet (Invitrogen) and surface stained with CD3-Alexa 700 (clone UCHT1, BD Biosciences), CD4-FITC (clone RPA-T4, Biolegend), CD8-APC-Cy7 (clone SK1, Biolegend), and CD45RO-APC (clone UCHL1, Biolegend). Following fixation and permeabilization (FIX/PERM, Invitrogen), cells were stained intracellularly with IFNγ-PECy7 (clone B27, Biolegend). HIV-specific IFNγ+ CD4 T cells and CD45RO+ memory CD4 T cell subsets were sorted using a FACS Aria Ilu
Figure 2.10: Flow cytometry gating strategy. Initial gating was performed using a FSC-A vs SSC-A lymphocyte gate, followed by a FSC-A vs. FSC-H doublet exclusion gate. CD4 T cells consisted of live (viability stain negative) CD3+, CD4+ CD8- cells. Subsequent gating on CD4 T cells was used to evaluate the expression of IFNγ, CD107a, CCR5, granzyme A, granzyme B, granzyme K, and perforin. IFNγ and CD107a responses were background subtracted using the unstimulated control as a reference.
using FACSDiva software (BD Biosciences). Sorted cells were then treated with 25 µL of a 1:100 dilution of proteinase K (Roche, Indianapolis, IN) in 10mM Tris buffer. Quantitative PCR was carried out using 5 µL of each of cell lysate per reaction as template, as previously described [45]. Thermal cycling was carried out as follows: 95°C holding stage for 5 minutes, and 50 cycles of 95°C for 15 seconds followed by 60°C for 1 minute using the Taq DNA polymerase kit (Invitrogen, Carlsbad, CA). The sequence of the forward primer for HIV is GGTGCGAGAGCGTACGTTATAAG. The reverse primer sequence is AGCTCCCTGCTTGCCCATA. The probe sequence is AAAATTCGGTTAAGGCCAGGGGGAAAGA. For cell number quantification, albumin was measured as previously described [45]. The qPCR instrument used was the StepOne Plus (Applied Biosystems); analysis was performed using StepOne software (Applied Biosystems).

**Single-cycle viral inhibition assay.** *Generation of macrophages and CD4 effector cells.* CD14+ monocytes were purified from cryopreserved PBMC samples by positive magnetic selection (EasySep, Stemcell technologies). Monocytes were differentiated into macrophages by culture for seven days in H10 supplemented with 50 ng/ml recombinant human M-CSF (R&D systems) in ultra-low adhesion flasks (Corning). CD4 T cells were expanded from the CD14-depleted PBMC fraction by seven day culture in H10 supplemented with 100 U/ml IL-2 (NIH AIDS Reagent Program), 5 µM nevirapine (NIH AIDS Reagent Program), and 0.5 µg/ml bispecific CD3.8 antibody (Dr. Johnson Wong, Massachusetts General Hospital), which activates CD4 T cells and depletes CD8 T cells [46]. For the inhibition assay, macrophages were transferred into R10 and plated at 15,000 cells per well in an ultra-low adhesion 96-well plate (Corning).
Effector cells were enriched for high purity CD4 T cells by negative magnetic enrichment prior to use (EasySep, StemCell technologies). *Viruses and infection.* Production of HIV-1 vectors and SIVmac virus-like particles (VLPs) has been described previously [47]. Briefly, VSV-G-pseudotyped HIV-1 vectors were produced by co-transfecting 293T cells (ATCC) with pAGM, psPAX2, and pMD2.G using Lipofectamine 2000 (Invitrogen). VSV-G-pseudotyped SIVmac251 VLPs were produced by co-transfecting 293T cells with pSIV3+ and pMD2.G using Lipofectamine 2000. SIVmac VLPs were harvested 48 h post transfection, clarified by centrifugation at 200 x g, filtered through a 0.45 micron syringe filter (Whatman), and added to macrophages 3 h prior to overnight challenge with HIV-1 vectors. After extensive washing of the targets, expanded CD4 effectors were added at 1:1, 5:1 and 10:1 effector:target ratios and incubated for 36 hours at 37ºC. At least three replicates were performed for each condition. To assess infectivity, cell mixtures were stained with APC conjugated anti-CD11b antibody (clone ICRF44, Biolegend) in the presence of 2 mM EDTA for 15 minutes and fixed. Samples were acquired on an LSR Fortessa (BD Biosciences) and data were analyzed using FlowJo (Treestar). Macrophages were identified by scatter properties and high CD11b expression; percent infection was determined by analyzing the percentage of ZsGreen positive events in the macrophage gate. ZsGreen gates were established based on uninfected controls for each individual. Inhibition data for each subject were normalized to the maximum respective positive control (HIV-infected, no effector) condition.

**Traditional viral inhibition assay.** *CD4 T cell cloning.* CD4 T cell clones were generated from cryopreserved PBMC samples by stimulation with the HIV Gag peptide TAPPEESFRFGEETTPSQK in the presence of IL-2 followed by limiting dilution cloning.
Inhibition assay. Viral inhibition assays using the CD4 T cell clones were performed as previously described [8] using the CD4 T cell line H9 as the target population, which express the matching restricting HLA-DR molecule DRB1*0401 [48]. Briefly, H9 target cells were infected with NL4-3 at an MOI of 0.01. Effector cells were added at a 1:1 effector:target ratio and incubated for 7 days. Supernatants were sampled on days 3 and 7 and assayed for p24 level using the Alliance p24 ELISA kit (Perkin-Elmer). To block the MHC class II HLA-DR presentation pathway, purified HLA-DR blocking antibody (low-endotoxin, azide-free, clone L243, Biolegend) was added to the culture medium at a concentration of 100 µg/ml.

Statistical analysis. Statistical analysis was performed using Prism (GraphPad). Parameters and responses were compared between subject groups using a Mann-Whitney analysis. Kaplan-Meier survival outcomes were compared by the Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests.
REFERENCES


Chapter 3: HIV-specific cytolytic CD4 T cell function is enriched in HIV controllers and associated with coordinated transcription factor expression
Acknowledgements: Primary experimental design and execution were performed by D.Z.S. Oliver Davis provided technical assistance. Experimental and analytical discussion and direction were provided by B.D.W. Funding for this project was provided by the National Institutes of Health grant UM1AI100663 (CHAVI-ID).
ABSTRACT

HIV specific CD4 T cells mediate many different effector functions, including direct cytotoxicity, which has been linked to improved clinical outcome after acute HIV infection [1]. The features of HIV specific cytolytic CD4 cells and their contribution to viral control during chronic infection, however, are incompletely understood. In this study, we seek to explore the nature of cytotoxicity in HIV-specific CD4 T cells as they relate to viral control and to determine which phenotypes and transcriptional features may contribute this type of CD4 function in the context of HIV infection. Ex vivo CD4 T cell responses were examined in HIV controllers and non-controllers after HIV Gag peptide stimulation by 15- and 18- color polychromatic flow cytometry and intracellular cytokine staining. HIV-specific CD4 T cell clones derived from an HIV infected elite controller were used to perform in vitro assessments of CD4 cytotoxicity. We find that HIV-specific cytolytic CD4 T cells are enriched in HIV controllers. These cells exhibit a functional profile that is distinct from non-cytolytic CD4 T cells and more similar to that of HIV-specific CD8 T cells; this profile was most pronounced in elite controllers. Moreover, CD4 T cell cytolytic potential is tightly associated with the coordinated expression of the transcription factors T-bet and Eomesodermin both ex vivo and in vitro in CD4 T cell clones. HIV-specific cytotoxic CD4 T cells may represent an important complement to the HIV-specific CD8 T cell response and may be an important component of HIV control.
INTRODUCTION

HIV-specific T cell responses play a critical role in controlling HIV infection. Although untreated HIV ultimately leads to T cell depletion and death from AIDS related disorders in most individuals, the immune system is nonetheless able to exert a relative degree of viral control for some time after infection, and this control is mediated in part by HIV-specific T cell responses [2]. During acute HIV-infection, viral loads peak to exceedingly high levels, but then decrease to the viral set point. This early decline corresponds temporally to the emergence of HIV-specific CD8 T cell responses [3, 4], as well as HIV-specific CD4 T cell responses, as we showed in Chapter 2 [1]. The importance of T cell responses has been further illustrated through depletion experiments in animal models, where depletion of CD8 T cells during acute or chronic SIV infection results in uncontrolled viremia [5]. Similarly, depletion of CD4 T cells prior to acute SIV infection leads to the inability of the immune system to mediate post-peak viral decline [6]. Further, escape mutations in CD8 T cell epitopes can be detected in HIV infected individuals, reinforcing the immune pressure these cells exert on the virus [7]. CD4 T cell escape mutations have also been described, although the evidence for this phenomenon is less clear [8-10].

While CD8 T cells function primarily by killing virally infected cells, CD4 T cells are able to exert a multitude of different effector functions [11]. Secretion of γ-chain cytokines like IL-2 and IL-21 by HIV-specific CD4 T cells, for example, has been linked to better CD8 T cell function and viral control [12, 13]. However, CD4 T cells are not only helper cells—in certain cases, they may mediate direct cytolytic effects on target cells [14]. We have previously shown that the presence of HIV-specific cytolytic CD4 T cell activity during acute HIV-infection is
associated with lower viral set point, and the CD4 T cell responses elicited by the marginally successful RV144/Thai vaccine also exhibited cytolytic activity [15, 16]. This and other evidence [17, 18] therefore suggests that cytolytic CD4 responses may actively contribute to viral control during HIV infection.

Nonetheless, CD4 T cell responses of this type are extremely poorly understood during chronic HIV infection—especially in comparison to their CD8 T cell counterparts, for which the factors related to cytotoxic function have been extensively dissected [19]. We therefore sought to understand whether HIV-specific cytolytic CD4 T cells may play a role in viral control during chronic HIV infection and to dissect the phenotype, function, and transcriptional program of these cells. Here, we perform an in depth analysis of HIV-specific cytolytic CD4 T cells in HIV elite controllers (individuals able to spontaneously control viral replication to undetectable levels in the absence of therapy) and in non-controllers (“chronic progressors,” individuals with chronic, progressive HIV disease). Comparison of these subject groups revealed that elite controllers exhibited a greater frequency of HIV-specific cytolytic CD4 T cell responses, and that responses in these individuals were functionally enhanced and more similar to CD8 T cells. Further, HIV-specific cytolytic CD4 T cell activity was associated with the expression of specific T-box transcription factors, T-bet and Eomesodermin.
RESULTS

HIV-specific cytolytic CD4 T cells are present in most chronically HIV infected subjects but enriched in HIV controllers

We previously demonstrated that HIV-specific cytolytic CD4 T cell responses expand during acute HIV-infection in individuals who progress to lower viral set points compared to those who progress to significantly higher set points (Chapter 2). However, it is important to note that none of the subjects in that study were HIV controllers—indeed, even the individuals in the low set point group (average viral load, 11,234 RNA copies per milliliter) had dramatically higher levels of viremia than the undetectable viral loads observed in elite controllers [1]. This led us to ask the question of whether HIV-specific cytolytic CD4 T cell responses would be observed during chronic infection in HIV infected individuals and in the setting of durable, spontaneous HIV elite control.

We therefore examined a group of 14 HIV elite controllers (EC) and 15 chronic progressors (CP, Table 3.1) and assessed the frequency and function the HIV Gag-specific CD4 T cell responses by intracellular cytokine staining flow cytometry directly ex vivo. We initially examined the expression levels of the effector molecules perforin, granzyme A, and granzyme B in bulk CD4 T cells. Interestingly, in each individual studied we found a population of CD4 T cells expressing one or more of these effector proteins (Figure 3.1A). Similarly to what has been shown for cytolytic protein expression in bulk CD8 T cells [20], we did not find any differences between the levels of effector molecules in bulk CD4 T cells in HIV controllers or chronic progressors (p = n.s., Figure 3.1A).
Table 3.1. Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Elite controllers (EC)</th>
<th>Chronic progressors (CP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
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</tr>
<tr>
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<td>4</td>
</tr>
<tr>
<td>Viral load, RNA copies/ml, average (range)</td>
<td>64 (1-192)</td>
<td>49306 (4350-155000)</td>
</tr>
<tr>
<td>CD4 T cell count, cells/µl, average±SD</td>
<td>1001 ± 236</td>
<td>440 ± 240</td>
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</tbody>
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Figure 3.1. Bulk and HIV-specific cytolytic CD4 T cells during chronic infection. (A) Top: representative staining showing expression of granzyme A, granzyme B, and perforin in CD4 T cells from an HIV-infected individual; bottom: percentages of CD4 T cells expressing each of these proteins is shown for 14 elite controllers (EC) and 15 chronic progressors (CP). Overall levels of bulk expression of each of these proteins were not significantly different between EC and CP (p = n.s., Mann-Whitney test, all comparisons). (B) The magnitude of the general IFNγ+ HIV-specific CD4 T cell response is greater than that of IFNγ+perforin+ CD4 T cells (p < 0.0001, Wilcoxon matched pairs test). (C) Significant enrichment of HIV-specific responses in perforin+ CD4 T cells versus the bulk CD4 T cell population (p < 0.0001, Wilcoxon matched pairs test). (D) Compared with non-controllers, HIV controllers exhibit a higher level of HIV-specific CD4 T cell responses by IFNγ secretion (p = 0.0181, Mann-Whitney test) and (E) CD107a+ degranulation (p = 0.0361, Mann-Whitney test).
We first studied the frequency of the HIV-specific CD4 T cell response by assessing the IFNγ+ response after Gag peptide pool stimulation of PBMC ex vivo followed by intracellular cytokine staining. In line with our previous study of acute HIV infection, we found that the overall magnitude of the HIV specific IFNγ+ CD4 T cell response was approximately ten-fold greater than that of HIV-specific CD4 T cells with cytolytic potential (either as CD107a+ CD4 T cells or IFNγ+perforin+ CD4 T cells, p < 0.0001, Figure 3.1B). Interestingly, however, the perforin expressing fraction of CD4 T cells exhibited a relative enrichment for HIV Gag-specific responses compared to the overall CD4 T cell population (p < 0.0001, Figure 3.1C), potentially reflective of the idea that antigen-specific CD4 T cells with cytolytic character are induced by chronic viral infections like HIV [14].

We next compared the magnitude of the HIV-specific CD4 T cell responses between elite controllers and individuals with progressive infection. HIV controllers exhibited a higher level of HIV-specific CD4 T cell responses by IFNγ secretion (p = 0.0181, Figure 3.1D), in line with previous reports of enhanced Gag-specific CD4 T cell function in controllers compared with chronic progressors [21, 22]. Most notably, however, we found that the frequency of degranulating CD107a+ CD4 T cells specific for Gag was also higher in controllers than in progressors (p = 0.0361, Figure 3.1E), further suggesting an enrichment of cytolytic CD4 T cells in these patients, echoing our previous study [1] and reinforcing the idea that these cells may have an important role in HIV control.
Expression of cytolytic proteins in HIV-specific CD4 T cells is associated with multiple memory states

The acquisition of cytolytic functionality in CD4 T cells has been repeatedly linked to memory differentiation, although the specific phenotypes described for these of these cells are often inconsistent [14, 23, 24]. Indeed, some reports suggest that cytolysis is a feature only of terminally differentiated, replicatively senescent CD4 T cells, while others demonstrate that cytolytic functionality can occur in less mature subsets as well [14, 23, 25]. The situation is further complicated by the fact that a plethora of T cell memory markers exist and there are different strategies for determining memory phenotypes. The most commonly employed approaches for human memory profiling rely on either the RA or RO isoforms of the protein tyrosine phosphatase CD45 in combination with either the chemokine receptor CCR7 or the CD62L (L-selectin) [26, 27]. However, alternate approaches are also used, and the addition of markers such as the costimulatory molecules CD27 and CD28 can provide a greater level of granularity [28]. We therefore utilized four separate differentiation markers—CD45RA, CCR7, CD27, and CD28—to survey the differentiation states exhibited by HIV-specific cytolytic CD4 T cells in controllers and progressors. In general, the vast majority CD4 T cells responding to HIV Gag stimulation were either central memory (CD45RA-CCR7+) or effector memory (CD45RA-CCR7-). Perforin expressing CD4 T cells also express granzyme A and B, therefore perforin can be used as a marker for fully cytolytic CD4 T cells [23]. Gag-specific CD4 T cells expressing perforin were more skewed towards an effector memory phenotype, but some central memory cells with a cytolytic phenotype were also detectable (Figures 3.2A and 3.2B).

Moreover, the majority of HIV-specific CD4 T cells were therefore not terminally differentiated
**Figure 3.2.** Expression of cytolical proteins in HIV-specific CD4 T cells is associated with increasing memory differentiation (A) Representative flow plots showing the breakdown of bulk CD4 T cells, total Gag-specific CD4 T cells, and variously defined cytolytic Gag-specific CD4 T cells into memory subsets based on CD45RA and CCR7 (Naive, CM, EM, and TEMRA). (B) The stratification of total Gag-specific CD4 T cells and Perforin+ Gag-specific CD4 T cells into CM and EM does not reveal striking differences associated with cytotoxicity, although the perforin expressing cells are enriched for EM cells (p < 0.0001, Mann-Whitney test). (C) The breakdown of Gag-specific CD4 T cells according to CD27 and CD28. HIV-specific CD4 T cells expressing all three measured cytolical proteins are made up of significantly fewer CD27+ and CD28+ cells compared to those expressing only granzyme A (p < 0.0001 for both CD27 and CD28, Mann-Whitney test) or granzymes A and B (CD27: p = 0.0012, CD28: p = 0.0002, Mann-Whitney test).
TEMRA (CD45RA+CCR7-) cells. These findings suggest that end stage differentiation is not completely required for cytolytic functionality in CD4 T cells, and that cytolytic CD4 T cells can be found in less differentiated memory states. We also sought to determine if the costimulatory molecules CD27 and CD28 [28, 29] could be used to better stratify cytolytic functionality (Figure 3.2C). While our data do indicate that bulk and HIV-specific CD4 T cells expressing perforin are largely (> 50 percent) negative for CD27 and CD28, subdivision of cytolytic CD4 T cells according to these markers does not appear to be absolute. Indeed, although the majority of HIV-specific CD4 T cells expressing both granzymes and perforin are negative for CD27 and CD28, not all are. Late differentiation—either measured by CD45RA/CCR7 staining or CD27/CD28 staining—is therefore not a requirement for cytolytic functionality within HIV-specific CD4 T cells. Rather, cytolytic protein expression occurs across a spectrum of memory phenotypes, potentially suggesting that it may not only be a byproduct of maturation as has been hypothesized [23], but rather may represent a unique CD4 T cell functional program.

**HIV-specific cytolytic CD4 T cells in controllers exhibit a functional profile similar to that of HIV-specific CD8 T cells**

Because of the natural role of CD8 cells as the major cytotoxic T cell population, we sought to compare cytotoxic CD4 T cells to CD8 T cells to determine if they shared common features. Using perforin expression as a surrogate marker for cytolytic potential, we assessed the ability of perforin+ CD4 T cells to mediate multiple effector functions (CD107a, IFNγ, TNFα, MIP1β, and IL-2) in response to HIV Gag peptide pool stimulation and performed comparisons to CD8 T cells as well as non-cytolytic (perforin-granzyme-) CD4 T cells (Figure 3.3A) in both
Figure 3.3. HIV-specific cytolytic CD4 T cells exhibit a functional profile similar to that of HIV-specific CD8 T cells. (A) The Gag-specific functional profile was measured by ICS for five T cell responses in EC and CP. Non-cytolytic gag-specific (perforin-, granzyme-) CD4 T cells are compared to cytolytic (perforin+) and CD8 T cells. Pie slices represent the number of functions expressed simultaneously (blue = 1, red = 5); pie arcs show the particular function expressed, as specified. (B) The dominant Gag-specific CD8 functional profile (MIP1β+IFNg+CD107a+) is significantly enriched in perforin+ CD4 compared to non-cytolytic CD4 in both EC and CP (p = 0.0007 and p = 0.002, respectively, Wilcoxon). EC additionally have a significantly higher proportion of this phenotype than CP (p = 0.0369, Mann-Whitney). (C) Representative stain showing Gag-specific cytolytic CD4 T cells and CD8 T cells share a similar CD27/CD28 memory profile. (D) MFI of CD27 and CD28. Perforin+ CD4 T cells showed equivalent CD27 median fluorescence intensity (MFI) and an intermediate CD28 MFI to CD8 T cells (indicated p values, Wilcoxon matched pairs test). Comparisons between EC and CP were not significant (p > 0.05, Mann-Whitney) and were excluded for clarity.
Figure 3.3 (Continued).
EC and CP. In general, the cytolytic CD4 T cells displayed an intermediate profile and were more similar to CD8 T cells compared to non-cytolytic CD4 T cells in terms of functions expressed, including degranulation. Interestingly, cytolytic HIV-specific CD4 T cells secreted reduced levels of TNFα as well as minimal IL-2 compared to non-cytolytic CD4 T cells, perhaps suggesting reduced helper capacity. However, they expressed far greater levels of MIP1β—in fact, a majority of the Gag-specific perforin+ CD4 T cells in both controllers and progressors were positive for MIP1β (Figure 3.3A). The dominant CD8 T cell functional phenotype (MIP1β+, IFNγ+, CD107a+) was also represented at significantly greater level in perforin+ versus non-cytolytic HIV-specific CD4 T cells (Figure 3.3B). The secretion of MIP1β in particular may have important anti-viral implications, as strong expression of this chemokine may block infection through occupation of the CCR5 coreceptor. Expression of MIP1β by CD4 T cells may also prevent self-infection, potentially indicating that cytolytic CD4 T cells are less susceptible to HIV-infection [30]. Although the functional profile of perforin expressing CD4 T cells was more similar to CD8 T cells in both patient groups, the perforin+ Gag specific cells were notably more functional in EC than CP. The level of the dominant, trifunctional CD8 T cell functional profile was significantly greater in EC than in CP, reinforcing the idea there is a functional enhancement of CD4 T cells with cytolytic potential in these subjects.

We also examined the differentiation phenotype of the non-cytolytic and cytolyic HIV-specific CD4 T cells in comparison to CD8 T cells and assessed the expression levels of CD27 and CD28 (Figure 3.3C and 3.3D). In accordance with the similar functional profiles, the Gag-specific cytolytic CD4 T cells were more similar to the Gag-specific CD8 T cells than were the non-cytolytic CD4 T cells. CD27 expression levels were equivalent between the two cell types.
CD28 expression on perforin expressing CD4 T cells was higher than that of CD8 T cells, but lower than the non-cytolytic CD4 T cells. However, within these specific CD4 T cell populations, there were not notable differences between patient groups.

**Cytolytic protein expression within HIV Gag-specific CD4 T cells correlates with viral control**

In our previous study of acutely infected individuals, we found that the particular combination of perforin and granzymes expressed in Gag-specific CD4 T cells was linked with clinical outcome. Specifically, we showed that the relative expression level of granzyme A was associated with lower viral set point. Individuals who progressed to lower viral set points exhibited proportionally higher levels of granzyme A (versus the other granzymes or perforin) within their Gag-specific CD4 T cells early after infection compared to individuals who progressed to higher set points [1]. This led us to ask the question of whether a similar effect could be observed for granzyme A within HIV-specific cells during chronic HIV infection. Using our intracellular staining data along with a Boolean gating strategy, we determined the combinational breakdown of perforin, granzyme A, and granzyme B expression within the Gag-specific CD4 T cells (defined as positive for at least one cytokine or CD107a following Gag stimulation) in controllers and progressors. In line with our previous data, HIV elite controllers expressed a significantly greater proportion of granzyme A within their Gag-specific CD4 responses (p = 0.0011, Figure 3.4A). Further, we found that this proportional level of granzyme A correlated inversely with viral load within these subjects (p = 0.0002, Figure 3.4B), with a high degree of granzyme A being representative of better viral control. These results therefore
Figure 3.4. **Granzyme A expression in HIV-specific CD4 T cells is linked to HIV control** (A) Boolean gating was used to characterize the combinations of perforin and granzyme expressed within the Gag-specific CD4 T cells. Pie slices represent the number of cytolytic proteins expressed simultaneously (blue = 1, red = 3) in Gag-specific CD4 T cells bearing at least one cytolytic protein. The arc (orange) represents granzyme A expression. The relative proportion of granzyme A is higher in EC than CP (p = 0.0011, Mann-Whitney test). (B) The relative granzyme A level is inversely correlated with viral load (p = 0.0002).
again suggest that granzyme A expressing cytolytic CD4 T cell responses may play an important role in viral control, not only early during acute HIV infection but potentially during chronic HIV infection as well.

**HIV-specific CD4 T cell cytolytic potential in controllers is associated with the combined expression of T-bet and Eomesodermin.**

The functional characteristics of differentiated CD4 T cells are determined by the activation of transcriptional programs dictated in large part by specific key transcription factors. The commitment of CD4 T cells to the Th1 lineage, for example, is controlled by expression of the T-box transcription factor T-bet (tbx21) [31]. Further, expression of T-bet in concert with another T-box transcription factor, Eomesodermin (Eomes), has been linked to cytotoxicity in CD4 T cells, especially in the mouse model [11]. We therefore sought to determine whether expression of T-bet and Eomes was linked to cytotoxic potential in HIV-specific CD4 T cells. Intracellular transcription factor staining was performed in combination with cytokine staining after peptide stimulation in a set of six elite controllers. Based on T-bet and Eomes expression, CD4 T-cells can be stratified into at least four populations: negative, Eomes single positive, T-bet single positive, and T-bet/Eomes double positive (Figure 3.5A). Staining for three functions—TNFα, IFNγ, and CD40L (as a universal functional marker for cytolytic CD4 T cells [32])—allowed for the identification of Gag-specific CD4 T cells, which could then be divided according to T-bet and Eomes expression. Strikingly, Gag-specific CD4 T cells expressing CD107a or perforin consisted primarily of Eomes and T-bet double positive cells (Figure 3.5B). Significantly more Gag-specific CD4 T cells with cytolytic potential were therefore positive for
Figure 3.5. Markers of HIV-specific cytolytic CD4 T cell function are linked to the combined expression of T-bet and Eomesodermin. (A) CD4 T cells can be divided into four subsets based on T-bet and Eomesodermin expression. (B) Representative staining of T-bet and Eomesodermin in Gag-specific CD4 T cells. (C) The vast majority of Gag-specific CD4 T cells with the ability to degranulate (left) or express perforin (right) co-express T-bet and Eomesodermin (p < 0.05, as indicated, Wilcoxon matched pairs test). (D) The percent of Gag-specific CD4 T cells expressing both T-bet and Eomesodermin is inversely correlated with the expression of the memory marker CD27 (p = 0.0168).
both transcription factors, reinforcing their strong linkage to cytotoxic functionality (Figure 3.5C). The proportion of Gag-specific CD4 T cells double positive for both factors was also inversely correlated with expression of CD27 on the Gag-specific CD4 T cells, in line with the general loss of CD27 with cytotoxic potential seen earlier (Figure 3.5D).

**HIV-specific cytotoxic activity in vitro is associated with the expression of perforin, T-bet, and Eomes.**

We next sought to determine the features associated with cytolytic activity by HIV-specific CD4 T cells. Because of the inherent difficulty in performing cytotoxicity assays ex vivo due to the generally low frequency of virus specific CD4 T cells, we generated a series of HIV-specific CD4 T cell clones to use for the assessment of CD4 T cell mediated cytotoxicity in vitro. These clones were generated by tetramer sorting single-cells from a CD4 T cell line derived from an HIV elite controller and specific for the immunodominant Gag epitope DV16 (DRFYKTLRAEQASQE) [22]. These clones were then subjected to a cytometric killing assay using autologous, peptide loaded B cell line targets to determine the level of specific cytolytic activity, as previously described [33]. Autologous B cells were stained with either CFSE and peptide loaded or with CellTrace Violet and left unloaded. These B cell populations were then mixed equally and incubated with individual effector clones at a 1:1 ratio. Specific lysis could be evaluated by determining the percentage of CFSE labeled targets lost after incubating with the effector clones (Figure 3.6A). Surprisingly, the clones were extremely variable in killing ability. While many HIV specific CD4 T cell clones exhibited high cytolytic capacity, several clones showed reduced killing ability, and others were unable to lyse targets at a detectable level.
Figure 3.6. HIV-specific cytolytic CD4 T cell activity in vitro is associated with increased CD4 T cell cytolytic potential in controllers is associated with the expression of perforin, T-bet, and Eomesodermin. (A) Representative example of the cytomeric killing assay. Targets are labeled with either CFSE and peptide pulsed or CellTrace Violet and left unpulsed (left). After incubation of the mixed target populations with effector cells, the ratio of the pulsed to unpulsed targets will change to reflect killing, allowing for the calculation of specific lysis (right). (B) HIV-specific CD4 T cell clones mediate variable levels of cytotoxic activity in vitro, as determined by the cytomeric killing assay. (C) Specific lysis by HIV-specific CD4 T cell clones is correlated with perforin expression. (D) Intranuclear transcription factor staining of three representative clones (low cytotoxicity, [blue], intermediate cytotoxicity [green], and high cytotoxicity [red]) show that perforin expression is linked to the expression of Eomes in combination with T-bet, but not T-bet alone.
Moreover, the expression of perforin within these clones correlated well with the level of specific lysis each clone was able to mediate (Figure 3.6C). Interestingly, clones could be divided into essentially two groups based on perforin expression: those with high levels of this critical cytotoxic protein were robust killers; those with minimal perforin expression exhibited correspondingly minimal cytolytic activity. To further understand the basis for this, we examined the expression of T-bet and Eomes within a subset of three clones with differing levels of specific lysis. While each of the clones expressed similar levels of T-bet as determined by intranuclear transcription factor staining, the clones could be stratified by Eomes expression according to their killing capacity (Figure 3.6D). The low-cytotoxicity clone expressed the lowest level of Eomes, and the best killer expressed the highest level of this transcription factor. These data reinforce the potential importance of Eomes as a key transcription factor for cytotoxicity in CD4 T cells, and potentially suggest that its expression may be necessary for full cytolytic activity in this cell type.
DISCUSSION

The role of HIV-specific CD4 T cells in contributing to the control of viral replication during HIV infection is incompletely understood [34]. Although these cells are preferentially infected and depleted, they still likely mediate important effector functions that are critical components of the overall HIV-specific immune response. We previously showed that after acute HIV infection, HIV-specific CD4 T cell responses—and in particular cytolytic CD4 T cell responses—are enhanced in individuals who reached lower viral set points in chronic infection. We therefore sought to better understand the participation of cytolytic CD4 T cell responses to HIV in viral control in settings where a high level of control is continuously maintained. Here, we performed a detailed characterization of the HIV-specific cytolytic CD4 T cell response in HIV elite controllers and in individuals with uncontrolled, progressive infection. We observed that HIV-specific cytolytic CD4 T cells were commonly detectable, but were enriched in elite controllers. Moreover, the HIV-specific cytolytic CD4 T cells from elite controllers exhibited a functional profile closer to HIV-specific CD8 T cells than did HIV-specific cytolytic CD4 T cells from non-controllers. Cytolytic activity within controller HIV-specific CD4 T cells was strongly associated with the presence of the transcription factors T-bet and Eomes.

Our results bolster the notion that HIV-specific CD4 T cells, including those with cytolytic potential, may indeed be an important element of the immune response to HIV. In HIV elite controllers, the dominant contributing factor to viral control is likely the expression of protective MHC class I alleles [2]. However, associations of viral control with MHC class II molecules have also been shown [35], and it is possible that HIV-specific CD4 T cell responses may play an auxiliary, albeit still necessary, role in supporting HIV-specific CD8 T cell
responses. The maintenance of higher levels of HIV-specific CD4 T cell responses we observed in the elite controllers may be important for continued, durable viral control. These results are in line with previous studies showing enhanced HIV-specific CD4 T cell responses in the context of viral control: High avidity HIV-specific CD4 T cells, for example, have been suggested to be important for long-term help to HIV-specific CD8 T cells in HIV controllers [36]. Ferrando-Martinez, et al also found higher HIV-specific CD4 T cell responses in HIV elite controllers, although their findings also suggested that the HIV-specific CD4 T cell type most important for elite control was a CD27+CD57- memory phenotype [37], which is generally non-cytolytic [23]. In this study, we determined that HIV-specific cytolytic CD4 T cells are also enriched in HIV elite controllers, a finding which is reinforced by studies comparing elite controllers with subjects virally suppressed on antiretroviral therapy [38] as well as our own study of acute HIV infection [1].

The HIV-specific cytolytic CD4 T cell responses we observe in HIV controllers may have an additional supportive role for HIV-specific CD8 T cells beyond classical helper signals. By virtue of their recognition of peptides through MHC class II, HIV-specific cytolytic CD4 T cells may complement CD8 T cell activity through MHC class I. Class I associated viral escape mutations in CD8 targeted epitopes, for example, can significantly hamper the ability of CD8 T cells to recognize and destroy HIV-infected cells [39]. Cytolytic CD4 T cells may therefore help to better target MHC class II expressing cells like macrophages, dendritic cells, or activated CD4 T cells. As long-lived target cells, macrophages in particular are of critical importance in the immunopathogenesis of HIV, acting as reservoirs for viral persistence [40] and may be more resistant than other targets to lysis by CD8 T cells [41]. Depletion of CD4 T cells in rhesus
macaques prior to SIV-infection results not only in the abrogation of a post-peak viral decline, but also leads to significantly increased levels of infected macrophages and other antigen-presenting cells [42]. The synergistic dual-recognition of both HIV infected CD4 T cells and these antigen presenting cells by cytolytic CD4 T cells and CD8 T cells together may therefore represent an important factor in the control of viral replication.

In addition to being of higher magnitude in elite controllers, HIV-specific CD4 T cell responses with cytolytic potential also show enhanced functionality in these subjects, with both greater secretion of MIP1β as well as increased degranulatory ability, similar to CD8 T cells. Degranulation is the necessary step for a cytolytic T cell to release its cytotoxic granule content to destroy a target cell, and a greater ability to do this has natural implications for increased killing capacity. However, the increased expression of MIP1β is also particularly notable. MIP1β is a natural ligand for CCR5, the primary HIV-1 coreceptor [43], and MIP1β can block HIV entry to target cells, suppressing viral replication [44]. Autocrine expression of MIP1β by CMV-specific CD4 T cells has been shown to be an important factor that contributes to their relatively lower level of infectability [30]. Therefore, high levels of secretion of this chemokine by HIV-specific cytolytic CD4 T cell may be dually beneficial: MIP1β may not only contribute to viral control by these cells via a second, non-cytolytic mechanism, but may also put these cells at a lower risk of HIV infection compared to other types of HIV specific CD4 T cells. Although the infectability of HIV-specific cytolytic CD4 T cells has not been directly studied, these cells are largely (but not completely) CD57 positive [45], and CD57+ HIV-specific CD4 T cells have been shown to harbor significantly less HIV than CD57- HIV specific CD4 T cells [46]. However, it remains to be determined what the specific factors are that render these cells less
infectable, as well as the exact degree of infectability of specifically cytolytic CD4 T cells (rather than only CD57+ cells).

Interestingly, we also noted that a relatively greater expression level of the cytolytic protease granzyme A was associated with lower viremia, as we had shown previously during acute HIV infection [1]. The repeated appearance of granzyme A as a signal of better viral control raises important questions about the role of this protein as it relates to the HIV immune response. Unlike granzyme B or perforin, granzyme A is comparatively less well characterized, although it is known to function by acting to induce DNA damage [47, 48]. While it is known to be important for antiviral immunity in certain contexts [49, 50], granzyme A also appears to have functions beyond cytotoxicity that may also be relevant [51], including those related to inflammatory signaling. It is possible that the cytotoxic and non-cytotoxic aspects of this protein may make it an important contributing factor in viral control by HIV-specific CD4 T cell responses.

Phenotypically, cytolytic CD4 T cells have been previously described to be terminally differentiated effector memory cells [14, 23, 24]. Although this varies based on marker strategy used for memory determination, many HIV-specific cytolytic CD4 T cells do show features of end stage differentiated cells (like lack of CD27 and CD28). However, our data also indicate that less mature CD4 T cells may exhibit cytolytic potential, suggesting that while cytolysis is often a feature associated with end stage differentiation, the latter may not be a requirement for the former. Non-terminally differentiated T cells have greater replicative capacity [52, 53], so
cytolytic CD4 T cells within these categories may potentially be an important source of class II restricted, self-renewing killer cells.

Very late-stage T cell maturation is usually reached after persistent antigenic stimulation [52, 54]. For this reason, cytotoxic CD4 T cells are often seen in the context of chronic viral infections, where antigenic stimulation resulting from ongoing viremia leads to high levels of T cell differentiation [14]. However, as described above, most HIV-specific cytolytic CD4 T cells are not terminally differentiated, suggesting that other molecular mechanisms may underlie the acquisition of cytolytic function by CD4 T cells. We determined that HIV-specific CD4 T cell cytolytic potential was linked to the expression of T-bet and Eomes, as has been described in the mouse model and for human CD4 T cells in other settings [55, 56]. The manner in which this transcriptional program is induced is not entirely dissected, however. In mice, costimulation through OX40 and 4-1BB pathways results in the induction of T-bet and Eomes and the corresponding development of cytolytic CD4 T cell activity [55]. In humans, costimulation through at least OX40-OX40L appears to be important as well [56]. Interestingly, activation of OX40 on HIV-specific CD4 T cells does improve their functionality in vitro [57], and higher expression of 4-1BB is associated with increased HIV-specific CD4 T cell function ex vivo as well as reduced viral loads in vivo [58]. However, it remains to be determined if this pathway is active in HIV-specific cytolytic CD4 T cells as well.

The data presented in this study add to the growing body of evidence that HIV-specific cytolytic CD4 T cells may contribute meaningfully to the antiviral immune response. Although their exact role in terms of target cells remains to be fully determined, induction of cytolytic CD4
T cell responses may be beneficial in terms of viral control during HIV infection. As the HIV vaccine field shifts increasingly to build vaccine modalities that preferentially elicit virus-specific CD4 T cell responses, it will be important to understand how to induce HIV-specific cytolytic CD4 T cells, as well as to determine the degree to which they may contribute to the viral reservoir.
MATERIALS AND METHODS

Subjects. Study subjects were recruited according to guidelines approved by the Massachusetts General Hospital Institutional Review Board following informed written consent. Subjects defined as elite controllers had viral loads consistently below detection limits (blips permitted if sufficient longitudinal data available); subjects defined as progressors had viral loads above 2000 RNA copies/ml.

Stimulation. Cryopreserved PBMCs were thawed and allowed to rest for three hours at 37º C, 5% CO2 at a concentration of 2x10^6/ml in R10 media (RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES). PBMCs were washed and resuspended at a concentration of 5-10 million cells/ml in R10 medium. For detection of CD107a, a pre-titered amount of PE-Cy5 conjugated antibody to CD107a (clone H4A3, BD Biosciences) was added to the medium. 1-2 million cells per condition were stimulated overnight with pooled 15-20 mer HIV Gag peptides (a combination of overlapping peptide pools comprising HIV clade B and sequence optimized cross-clade peptides to maximize clade coverage (Gag Ultra, JPT Peptide)). As a positive control, cells were stimulated with staphylococcal enterotoxin B (1 mg/ml, Sigma). An unstimulated (medium only) sample served as a negative control. 30 minutes into the stimulation, the transport inhibitors brefeldin A (0.5 mg/ml, Sigma) and monensin (GolgiStop, 0.3 µl/ml, BD Biosciences) were added to facilitate detection of T cell responses.

Intracellular cytokine staining. Following stimulation, cells were washed with phosphate buffered saline (PBS) and stained with a UV-excitable, amine-reactive viability dye
(LIVE/DEAD Blue, Invitrogen). Cells were subsequently washed with staining buffer (PBS containing 2% fetal calf serum and 0.09% sodium azide) and stained with CD3-Brilliant Violet 510 (clone OKT3, Biolegend), CD4-Brilliant Violet 650 (clone OKT4, Biolegend), CD8-APC-eFluor780 (clone RPA-T8, eBioscience), CD14-biotin (clone HCD14, Biolegend), CD19-biotin (clone HIB19, Biolegend), CD45RA-Brilliant Violet 570 (clone HI100, Biolegend), CCR7-Brilliant Violet 785 (clone G043H7, Biolegend), CD27-Brilliant Violet 605 (clone O323, Biolegend), and CD28 PE-CF594 (clone 28.2, BD Biosciences). For detection of CD14 and CD19, cells were washed and incubated with Streptavidin-Alexa 350 (Life Technologies). After surface staining, cells were fixed and permeabilized (Cytofix/Cytoperm, BD Biosciences) and stained intracellularly using the following antibodies: IL-2 FITC (clone 5344.111, BD Biosciences), TNFa PerCP-Cy5.5 (clone MAb11, Biolegend), MIP1β Brilliant Violet 421 (clone D21-1351, BD Biosciences), IFNγ-PE-Cy7 (clone B27, Biolegend), Granzyme A-Alexa 647 (clone CB9, Biolegend), Granzyme B-Alexa 700 (clone GB11, BD Biosciences), and Perforin-PE (clone B-D48, Biolegend).

**Intracellular transcription factor staining.** Following stimulation (to identify HIV Gag-specific cells), cells were washed with phosphate buffered saline (PBS) and stained with a UV-excitable, amine-reactive viability dye (LIVE/DEAD Blue, Invitrogen). Cells were subsequently washed with staining buffer (PBS containing 2% fetal calf serum and 0.09% sodium azide) and stained with CD3-Brilliant Ultra Violet 395 (clone UCHT1, BD Biosciences), CD4-Brilliant Violet 650 (clone OKT4, Biolegend), CD8-PerCP-eFluor710 (clone SK1, eBioscience), CD19-PE-Cy5.5 (clone SJ25-C1, Life Technologies), CD45RA-Brilliant Violet 510 (clone HI100, Biolegend), CCR7-Brilliant Violet 605 (clone G043H7, Biolegend), CD27-APC-H7 (clone M-
T271, BD Biosciences), CD57-biotin (clone HCD57, Biolegend), and GPR56-PE-Cy7 (clone CG4, Biolegend). For detection of CD57, cells were washed and incubated with Streptavidin-Brilliant Violet 786 (BD Biosciences). After surface staining, cells were fixed and permeabilized for transcription factor staining (Transcription Factor Buffer Set, BD Biosciences) and stained with the following antibodies: IFNγ-Brilliant Violet 711 (clone B27, BD Biosciences), Granzyme A-Alexa 488 (clone CB9, Biolegend), Granzyme B-Alexa 700 (clone GB11, BD Biosciences), Perforin-Brilliant Violet 421 (clone B-D48, Biolegend), Eomesoderm-eFluor 660 (clone WD1928, eBioscience), and T-bet PE (clone O4-46, BD Biosciences).

Flow cytometric analysis. Flow cytometric data were collected using a special order 5-laser LSR Fortessa and FACSDiva software (BD Biosciences). Compensation was performed using single-stained antibody capture beads (UltraComp eBeads, eBioscience) and amine-dye reactive beads (ArC, Invitrogen). Cytometer settings were standardized and tracked between runs using multi-fluorescent calibration beads (Rainbow Fluorescent Particles, Spherotech). Data were analyzed using FlowJo version 9.6 (TreeStar). Polyfunctionality analysis was performed using Pestle and Spice [59].

Derivation of CD4 T cell clones. Thawed PBMC from an HIV controller were stimulated in vitro using the immunodominant Gag peptide YVDRFYKTLRAEQASQEV in R10 medium supplement with 50 U/ml IL-2. After 14 days, the cells were stained with a PE-conjugated HLA class II tetramer presenting the optimal epitope DRFYKTLRAEQASQEV. Single tetramer positive CD4 T cells were subsequently sorted using a FACSria IIu sorter (BD Biosciences) into single wells of a 96 well plate containing allogeneic irradiated feeder PBMC and stimulatory
anti-CD3 antibody (clone OKT3, Biolegend). Clone wells showing positive growth were expanded by restimulation with anti-CD3 and allogeneic feeders every 14 days.

**In vitro cytotoxicity assessment.** Flow based assessment of cytotoxic activity was performed using a modified version of the “VITAL” assay, as previously described [33]. Briefly, to generate targets, EBV-transformed B cell line (BCL) cells (autologous to the effector cells) were stained with either CFSE (as CFDA-SE, Life Technologies) or CellTrace Violet (CTV, Life Technologies) according to the manufacturer’s recommendations. CFSE stained BCL were then pulsed with the peptide YVDRFYKTLRAEQASQEV for one hour at 37°C; CTV labeled cells were left unpulsed (DMSO control). After six washes with 1X PBS (to ensure removal of all unbound peptide), identical numbers of the CFSE and CTV labeled BCL targets were combined in a 1:1 ratio with the YVDRFYKTLRAEQASQEV-specific effector CD4 T cell clones and incubated for six hours at 37°C. A no-effector control condition was also included, and each condition was performed in at least three replicates. Specific lysis was determined by calculating the ratiometric change in the peptide-pulsed (CFSE) to unpulsed BCL (CTV), normalized to the effector-free condition, as previously described [33].
REFERENCES


Chapter 4: Identification of virus-specific CD4 T cell signatures associated with spontaneous HIV-1 control by automated dimensionality reduction
Acknowledgements: The work described here represents a collaborative project between Damien Z. Soghoian and Dr. Karthik Shekhar, a former graduate student in the lab of Dr. Arup Chakraborty at MIT. D.Z.S. designed the experimental protocols and collected all biological data. K.S. developed and programmed the ACCENSE algorithm for multidimensional analysis [1]. D.Z.S and K.S. analyzed and interpreted the data. Experimental and analytical discussion and direction were provided by B.D.W. and A.C.
ABSTRACT

CD4 T cells are essential for proper immune function, including the clearance and control of viral pathogens. While CD4 T cells have been studied extensively in the context of HIV-1 infection in their role as viral targets, comparatively less is known about these cells as anti-viral effectors. Here we perform a detailed study of the function and memory phenotype of HIV-specific CD4 T cells in the context of spontaneous HIV control. We apply a dimensionality reduction and cellular classification tool—ACCENSE—to flow cytometry immunophenotyping data and isolate key phenotypic subsets of antigen-specific CD4 T cells enriched in HIV elite controllers, viremic controllers, and non-controllers. Our analysis reveals specific, multi-cytokine functional signatures of Gag-specific CD4 T cells that correlate directly with the specific level of viremia and clinical status within these subjects and bolsters the idea that CD4 T cell polyfunctionalilty is a critical feature for effective viral control.
INTRODUCTION

HIV-1 infection normally results in persistent viral replication that, if left untreated, leads to progression to AIDS. However, a very small subset of HIV-1 infected individuals follow a different clinical trajectory and are able to control HIV viremia without therapy. Elite controllers exhibit undetectable plasma HIV-1 RNA by commercial assays; viremic controllers, on the other hand, maintain detectable, but low, levels of HIV-1 RNA (< 2000 copies/ml) without progression [2]. The factors that contribute to durable control of HIV-1 are varied and complex [3]. Although there are some reports which suggest that the viruses infecting HIV controllers may be replicatively deficient, the vast majority of evidence indicates that HIV control is the result of an effective immune response often related to host genetic factors [4, 5]. A more complete understanding of the multitude of immune features that result in spontaneous HIV control in viremic and elite controllers will facilitate the rational design of therapeutic interventions or HIV-1 vaccines [3].

HIV control is associated with a vigorous and robust cellular immune response [3]. CD8 T cells in particular have been linked to viral control, and there is a wealth of evidence supporting the importance of HIV-specific CD8 cells in mediating HIV control and establishing HIV controller status [6]. However, virus specific CD4 T cells also likely play an important role in controlling HIV infection [7]. CD4 T cells provide a variety of different helper signals to other immune subsets, including CD8 T cells. Moreover, like CD8 T cells, CD4 T cells also have the ability to directly kill target cells in certain circumstances [8]. During HIV infection, HIV-specific CD4 T cells undergo preferential depletion compared to the general memory pool, but
the majority of these cells are nonetheless still available to potentially exert effector functionality [9].

The particular effector functions and cellular properties that contribute to the HIV-specific CD4 T cell response are not well understood. Robust HIV-specific proliferative responses were linked to HIV control early on [10]. HIV-specific CD4 T cells have been shown to be inversely correlated with HIV viral load and are directly correlated with the level of HIV-specific CD8 T cell responses, suggesting that these cells may be providing supportive signals to CD8 T cells [10, 11]. Further work has identified the secretion of helper cytokines, including IL-2 and IL-21 by HIV-specific CD4 T cells as potentially important functions [12-14]. HIV-specific CD4 T cells with cytolytic potential have also been suggested to play a role in HIV immunity through direct lysis of infected target cells [15]. It remains unclear, however, what the most important functional effector phenotypes of HIV-specific CD4 T cells are as they relate to viral control. Such an understanding will be critical as HIV vaccine efforts increasingly target CD4 T cells [16].

To evaluate the features of virus-specific CD4 T cells that may contribute most to viral control, we performed in-depth, 15-color flow cytometry phenotyping and intracellular cytokine staining followed by unbiased, automated computational profiling based on the expression of multiple markers. The CD4 T cell signatures we discover using this approach correlate strongly with clinical status and offer insight into the biology of the HIV-specific CD4 T cell response. Moreover, they represent an important proof of concept of a novel computational approach (ACCENSE) to visualize high-dimensional cellular expression data and automatically identify
expression signatures that correlate with viral control. Our data reinforce the notion that particular CD4 T cell functionality may play an important role in contributing to the control of HIV infection.
RESULTS

HIV-1 control is associated with higher frequencies of Gag-specific CD4 T cell responses

To understand the role of HIV-specific CD4 T cell responses as they relate to viral control during chronic HIV-1 infection, we selected a group of 14 elite controllers (EC), 4 viremic controllers (VC), and 15 individuals with uncontrolled viremia (chronic progressors, CP) (Table 4.1), which included the same subjects analyzed in Chapter 3. In order to probe the HIV-specific CD4 T cell response, we stimulated cryopreserved PBMC from these subjects with HIV Gag peptide pool and performed intracellular cytokine staining flow cytometry to measure five effector functions: MIP1β, TNFα, IL-2, IFNγ, and CD107a. In line with previous reports that have suggested an enrichment of various HIV-specific CD4 T cell functions in the context of HIV control [13, 14, 17-20], we found that HIV controllers exhibited significantly higher frequencies of certain functions than non-controllers. This effect was most striking for IFNγ secretion, but was also significant for CD107a, as discussed earlier in Chapter 3 of this thesis (Figure 4.1A-E).

Dimensionality reduction using ACCENSE enables the analysis of Gag-specific CD4 T cell responses

To further analyze the differences between Gag-specific CD4 T cells in HIV controllers and non-controllers, we extracted phenotyping data for the Gag-specific CD4 T cells (defined as positive for at least one function) from a subset of 20 total ECs (n=7), VCs (n=4), and CPs (n=9) for whom complete polyfunctionality and memory marker staining was available to generate a Gag-specific CD4 dataset. Only parameters relevant to CD4 T cells were retained, leaving 12-
Table 4.1: Subject characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Elite controllers (EC)</th>
<th>Viremic controllers (VC)</th>
<th>Chronic progressors (CP)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. of patients</strong></td>
<td>14</td>
<td>4</td>
<td>15</td>
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</tr>
<tr>
<td>African American, n</td>
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<td>4</td>
</tr>
<tr>
<td><strong>Viral load, RNA copies/ml, average (range)</strong></td>
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<td>800 (400-1205)</td>
<td>49306 (4350-155000)</td>
</tr>
<tr>
<td><strong>CD4 T cell count, cells/µl, average±SD</strong></td>
<td>1001 ± 236</td>
<td>800 ± 269</td>
<td>440 ± 240</td>
</tr>
</tbody>
</table>
Figure 4.1: CD4 T cell effector functions after stimulation with Gag peptides. Intracellular cytokine staining (ICS) was performed on PBMC from elite controllers (EC), viremic controllers (VC), or chronic progressors (CP) stimulated with overlapping HIV Gag peptide pools for six hours. Expression of each function was assessed in the live, CD14-CD19-CD3+CD4+ population. (A) IFNγ, (B) CD107a, (C) TNFα, (D) MIP1β, and (E) IL-2 levels are expressed as frequencies of the CD4 T cell parent population. Only significant differences (p < 0.05, Mann-Whitney test) are displayed.
parameters of data for any given single-cell: the five effector functions mentioned above, four memory markers (CD45RA, CCR7, CD27, and CD28), and three cytolytic proteins (Granzyme A, Granzyme B, and Perforin). The cells for all subjects were pooled, resulting in a dataset consisting of 13062 Gag-specific CD4 T cells with 12 marker measurements per cell. Expression data were mean-centered and normalized to facilitate comparison, and the data were then embedded on a two-dimensional map using ACCENSE, as previously described [1]. In brief, ACCENSE uses t-distributed stochastic neighbor embedding (t-SNE) to describe the 12 measurements per cell in just two parameters that can then be represented on a biaxial plot. The distances between datapoints on this plot represent the degree of similarity across all 12 markers. An iterative k-means approach [21] was then used to cluster the Gag-specific CD4 T cells on this map into 25 subsets based on phenotypic similarity (Figure 4.2A and Table 4.2).

The general distribution of marker expression across the ACCENSE map begins to enable broad characterization of the virus-specific CD4 T cells (Figure 4.2B). Although the positioning of the cells on the map is defined by all twelve parameters together, in general, cells which very highly express any particular marker tend to cluster together. Moreover, cells expressing related markers also tend to cluster together. For example, perforin, granzyme A, and granzyme B expression marks similar groups of cells due to the nature of related co-expression of these parameters, as reflected in the ACCENSE map or the derived heatmap illustrating the expression of parameters within each clustered subpopulation (Figure 4.2C). At a high-level, the map also allows for the identification of phenotypic signatures, such as the large group of cells expressing high levels of IFNγ, TNFα, and IL-2, which may potentially have biological significance (Figures 4.2B and 4.2C).
Figure 4.2: Analysis of HIV Gag-specific CD4 T cell responses by ACCENSE. (A) Gag-specific CD4 T cells (defined as positive for at least one function) were subjected to dimensionality reduction by ACCENSE to generate a two-dimensional map that was subsequently clustered into 25 clusters based on phenotypic similarity. (B) Distribution of marker expression across the ACCENSE map. Relative expression level of each marker after normalization is shown based on the color key. (C) A derived heatmap representation of the expression levels of each marker within each cell (y-axis, stratified by ACCENSE map subset).
**Table 4.2: Phenotypic signatures of the 25 computed subpopulations.** The superscripts for each marker represent the expression level of that marker relative to expression within other populations in the dataset. The percentile ranges for the different expression level categories are: ‘- - -’ (0-5), ‘- -’ (6-25), ‘-’ (26-45), ‘0’ (46-55), ‘+’ (56-75), ‘++’, (76-95), ‘+++’ (96-100).

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<th>Phenotype</th>
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<tr>
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</tr>
<tr>
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<tr>
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<tr>
<td>S20</td>
<td>IL2°TNFα°GrA°GrB°MIP1β°CD45RA°CD27°CCR7°Pfn+CD28°CD107a°IFNγ°</td>
</tr>
<tr>
<td>S21</td>
<td>IL2°TNFα°GrA°GrB°MIP1β°CD45RA°CD27°CCR7°Pfn+CD28°CD107a°IFNγ°</td>
</tr>
<tr>
<td>S22</td>
<td>IL2°TNFα°GrA°GrB°MIP1β°CD45RA°CD27°CCR7°Pfn+CD28°CD107a°IFNγ°</td>
</tr>
<tr>
<td>S23</td>
<td>IL2°TNFα°GrA°GrB°MIP1β°CD45RA°CD27°CCR7°Pfn+CD28°CD107a°IFNγ°</td>
</tr>
<tr>
<td>S24</td>
<td>IL2°TNFα°GrA°GrB°MIP1β°CD45RA°CD27°CCR7°Pfn+CD28°CD107a°IFNγ°</td>
</tr>
<tr>
<td>S25</td>
<td>IL2°TNFα°GrA°GrB°MIP1β°CD45RA°CD27°CCR7°Pfn+CD28°CD107a°IFNγ°</td>
</tr>
</tbody>
</table>
ACCENSE reveals phenotypic Gag-specific CD4 T cell subpopulations enriched in HIV controllers

Given the ability to use ACCENSE to derive the primary phenotypic subsets of Gag-specific CD4 T cells, we next sought to determine whether certain subtypes of cells were preferentially represented in specific patient groups. We first calculated the log odds ratio for representation of controller and progressor HIV-specific CD4 T cells on the ACCENSE map (Figure 4.3A), which revealed a several fold-enrichment of controller cells within specific phenotypic areas of the map. This could be determined more rigorously by calculating the proportion of cells within each of the 25 identified subsets for each individual (Figure 4.3B). This analysis revealed a relative enrichment of phenotypic subsets 7-13 in HIV controllers compared to chronic progressors. We assessed the statistical significance for phenotypic enrichment in EC versus VC versus CP for any given population using a nonparametric Mann-Whitney U test. Multiple hypothesis testing was accounted for through a positive false discovery rate (pFDR) approach[22], which allowed for the ranking of subpopulation associations with clinical category by q value with a cutoff of q=0.035 (Table 4.3). The q-value cutoff was chosen such that the expected number of false discoveries among the subpopulation associations classified as significant was less than 1.

The most striking association of phenotypic enrichment with controller status was for subpopulation S8, which was significantly over-represented in elite controllers (median proportion 6.2%) versus chronic progressors (median proportion 1.1%). This subset is characterized by a tri-functional phenotype with dominant expression of the cytokines IFNγ,
Figure 4.3: Enrichment of phenotypic subpopulations according to controller status. (A) The log odds ratio for representation of controller and progressor HIV-specific CD4 T cells on the ACCENSE map (red, enriched in controllers; blue, enriched in progressors). (B) Heatmap representation of the relative frequency of each subject’s Gag-specific CD4 T cells into the 25 ACCENSE subpopulations (red, high enrichment; black, low enrichment). (C) Frequencies and relative marker expression levels for S8 and S9, the two subpopulations most significantly enriched in elite controllers. Plots on the left indicate frequencies of HIV-specific cells for each patient group within these populations (red lines indicate median values, whiskers indicate interquartile ranges). Charts on the right indicate the relative expression levels of each of the markers within those populations (green) compared to their global medians (blue lines).
Table 4.3: Significant subpopulation frequency differences between clinical groups. Key phenotypic features are indicated for each subpopulation. Only significant differences between the indicated tested subject groups are shown in the table. $p$ values are determined with the Mann-Whitney test; $q$ values are determined with the pFDR approach, as described in the text.

<table>
<thead>
<tr>
<th>No.</th>
<th>Subpopulation</th>
<th>Group 1</th>
<th>Group 2</th>
<th>$p$-value</th>
<th>$q$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$S_8$ (IL2^{++}+TNFα^{++}+IFNγ^{+})</td>
<td>CP (1.1%)</td>
<td>EC (6.2%)</td>
<td>1.7 x 10^{-4}</td>
<td>1.6 x 10^{-3}</td>
</tr>
<tr>
<td>2</td>
<td>$S_9$ (IL2^{++}+TNFα^{++}+IFNγ^{+}+CD107a^{+})</td>
<td>CP (0.3%)</td>
<td>EC (5.3%)</td>
<td>1.7 x 10^{-4}</td>
<td>1.6 x 10^{-3}</td>
</tr>
<tr>
<td>3</td>
<td>$S_{12}$ (IL2^{+}+TNFα^{+}+IFNγ^{+}+Pfn^{+}CD107a^{+})</td>
<td>CP (0.5%)</td>
<td>EC (3.7%)</td>
<td>1.7 x 10^{-4}</td>
<td>1.6 x 10^{-3}</td>
</tr>
<tr>
<td>4</td>
<td>$S_{22}$ (TNFα^{−}+CD27^{+}+CD107a^{+}+IFNγ^{−})</td>
<td>CP (10.2%)</td>
<td>EC (1.8%)</td>
<td>2.1 x 10^{-3}</td>
<td>9.4 x 10^{-3}</td>
</tr>
<tr>
<td>5</td>
<td>$S_9$ (IL2^{++}+TNFα^{++}+IFNγ^{−}+CD107a^{+})</td>
<td>CP (0.3%)</td>
<td>VC (1.9%)</td>
<td>2.8 x 10^{-3}</td>
<td>9.4 x 10^{-3}</td>
</tr>
<tr>
<td>6</td>
<td>$S_{12}$ (IL2^{+}+TNFα^{−}+IFNγ^{+}+Pfn^{−}CD107a^{+})</td>
<td>CP (0.5%)</td>
<td>VC (4.4%)</td>
<td>2.8 x 10^{-3}</td>
<td>9.4 x 10^{-3}</td>
</tr>
<tr>
<td>7</td>
<td>$S_{14}$ (TNFα^{+}+CD27^{−}+CD45RA^{−}CCR7^{−})</td>
<td>CP (3%)</td>
<td>VC (8.7%)</td>
<td>2.8 x 10^{-3}</td>
<td>9.4 x 10^{-3}</td>
</tr>
<tr>
<td>8</td>
<td>$S_{23}$ (TNFα^{−}+CD28^{+}+CD107a^{−}+IFNγ^{−})</td>
<td>CP (5.1%)</td>
<td>VC (1%)</td>
<td>2.8 x 10^{-3}</td>
<td>9.4 x 10^{-3}</td>
</tr>
<tr>
<td>9</td>
<td>$S_{10}$ (IL2^{++}+TNFα^{+}+IFNγ^{+}+CD27^{+})</td>
<td>CP (0.9%)</td>
<td>EC (4.9%)</td>
<td>5.2 x 10^{-3}</td>
<td>1.37 x 10^{-2}</td>
</tr>
<tr>
<td>10</td>
<td>$S_{11}$ (TNFα^{−}+CD27^{−}+CD28^{−}+IFNγ^{−})</td>
<td>CP (0.9%)</td>
<td>EC (3.2%)</td>
<td>5.2 x 10^{-3}</td>
<td>1.37 x 10^{-2}</td>
</tr>
<tr>
<td>11</td>
<td>$S_{19}$ (CD27^{++}+CD28^{−})</td>
<td>CP (3.5%)</td>
<td>VC (6.6%)</td>
<td>5.6 x 10^{-3}</td>
<td>1.37 x 10^{-2}</td>
</tr>
<tr>
<td>12</td>
<td>$S_{11}$ (TNFα^{−}+CD27^{−}+CD28^{−}+IFNγ^{+})</td>
<td>CP (0.9%)</td>
<td>VC (4.5%)</td>
<td>1.1 x 10^{-2}</td>
<td>2.3 x 10^{-2}</td>
</tr>
<tr>
<td>13</td>
<td>$S_{22}$ (TNFα^{+}+CD27^{+}+CD107a^{+}+IFNγ^{−})</td>
<td>CP (10.2%)</td>
<td>VC (3.5%)</td>
<td>1.1 x 10^{-2}</td>
<td>2.3 x 10^{-2}</td>
</tr>
<tr>
<td>14</td>
<td>$S_{14}$ (TNFα^{+}+CD28^{−}+CD45RA^{−}CCR7^{−})</td>
<td>VC (8.7%)</td>
<td>EC (1.9%)</td>
<td>1.1 x 10^{-2}</td>
<td>2.3 x 10^{-2}</td>
</tr>
<tr>
<td>15</td>
<td>$S_7$ (IL2^{++}+TNFα^{++}+CD28^{−})</td>
<td>CP (1.5%)</td>
<td>VC (6.2%)</td>
<td>2 x 10^{-2}</td>
<td>3.5 x 10^{-2}</td>
</tr>
</tbody>
</table>
TNFα, and IL-2 (Figure 4.3C) at extremely high levels—74th, 80th, and 98th percentiles, respectively, among all the cells in the dataset. Subpopulations S9 and S10 were also enriched in elite controllers and displayed a similar phenotype with respect to these three cytokines. Interestingly, subset S9 exhibited additional characteristics of cytotoxicity, including the expression of CD107a and Perforin above median levels (within the 56th-75th percentiles, Figure 4.3C and Table 4.3). These data support the idea of multiple cytokine secretion, particularly IL-2, as being important for HIV control, as well as potentially indicate some degree of contribution of responses with cytolytic character.

The subsets described above were not at significantly enriched levels in viremic controllers, although this likely is due to the low number of viremic controllers within the dataset. However, certain additional subsets were preferentially enriched in viremic controllers at significant levels (Table 4.3), including S7, S12, and S14. These phenotypes were all characterized by high (85th-97th percentile) TNFα expression, but were either monofunctional or bifunctional with only one other cytokine co-expressed at high levels. This suggests that while three functions are optimal—even if not they are not expressed at the absolute highest levels—TNFα may still mediate a beneficial effect alone or in combination with either IL-2 or IFNγ.

**Modeling relative viral control based on subpopulation frequencies**

Although certain subsets were enriched in controllers, we sought to determine the phenotypes that likely are most relevant for viral control by examining the subpopulations that correlate best with plasma viral load and CD4 T cell count (Table 4.4). Of the 25 subsets identified by the ACCENSE analysis, six significantly correlated both with viral load and with
Table 4.4: Correlations of subpopulation frequencies with clinical status. The pearson correlation coefficients for log viral load ($R_{VL}$) and CD4 T cell count ($R_{CD4}$) were calculated for the within patient frequencies for each subpopulation ($p$ values computed using the Student’s $t$-test). Significant associations (red text) were determined using a pFDR approach.

<table>
<thead>
<tr>
<th>Subpop</th>
<th>$R_{VL}$</th>
<th>$p_{VL}$</th>
<th>$R_{CD4}$</th>
<th>$p_{CD4}$</th>
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</thead>
<tbody>
<tr>
<td>$S_1$</td>
<td>0.45</td>
<td>4.5 $\times$ 10$^{-2}$</td>
<td>−0.41</td>
<td>7.1 $\times$ 10$^{-2}$</td>
</tr>
<tr>
<td>$S_2$</td>
<td>0.32</td>
<td>0.17</td>
<td>−0.29</td>
<td>0.22</td>
</tr>
<tr>
<td>$S_3$</td>
<td>0.29</td>
<td>0.22</td>
<td>−0.245</td>
<td>0.29</td>
</tr>
<tr>
<td>$S_4$</td>
<td>−0.23</td>
<td>0.34</td>
<td>0.19</td>
<td>0.42</td>
</tr>
<tr>
<td>$S_5$</td>
<td>−0.57</td>
<td>8.6 $\times$ 10$^{-3}$</td>
<td>0.37</td>
<td>0.11</td>
</tr>
<tr>
<td>$S_6$</td>
<td>0.54</td>
<td>1.5 $\times$ 10$^{-2}$</td>
<td>−0.54</td>
<td>1.4 $\times$ 10$^{-2}$</td>
</tr>
<tr>
<td>$S_7$</td>
<td>−0.19</td>
<td>0.43</td>
<td>0.13</td>
<td>0.59</td>
</tr>
<tr>
<td>$S_8$</td>
<td>−0.71</td>
<td>4.4 $\times$ 10$^{-4}$</td>
<td>0.57</td>
<td>9.2 $\times$ 10$^{-3}$</td>
</tr>
<tr>
<td>$S_9$</td>
<td>−0.62</td>
<td>3.6 $\times$ 10$^{-3}$</td>
<td>0.37</td>
<td>0.11</td>
</tr>
<tr>
<td>$S_{10}$</td>
<td>−0.65</td>
<td>1.9 $\times$ 10$^{-3}$</td>
<td>0.58</td>
<td>7.8 $\times$ 10$^{-3}$</td>
</tr>
<tr>
<td>$S_{11}$</td>
<td>−0.49</td>
<td>2.7 $\times$ 10$^{-2}$</td>
<td>0.59</td>
<td>6.2 $\times$ 10$^{-3}$</td>
</tr>
<tr>
<td>$S_{12}$</td>
<td>−0.58</td>
<td>7.3 $\times$ 10$^{-3}$</td>
<td>0.54</td>
<td>1.3 $\times$ 10$^{-2}$</td>
</tr>
<tr>
<td>$S_{13}$</td>
<td>−0.50</td>
<td>2.2 $\times$ 10$^{-2}$</td>
<td>0.62</td>
<td>3.7 $\times$ 10$^{-3}$</td>
</tr>
<tr>
<td>$S_{14}$</td>
<td>−0.08</td>
<td>0.73</td>
<td>0.11</td>
<td>0.64</td>
</tr>
<tr>
<td>$S_{15}$</td>
<td>0.09</td>
<td>0.71</td>
<td>0.10</td>
<td>0.68</td>
</tr>
<tr>
<td>$S_{16}$</td>
<td>0.006</td>
<td>0.98</td>
<td>0.25</td>
<td>0.27</td>
</tr>
<tr>
<td>$S_{17}$</td>
<td>0.25</td>
<td>0.28</td>
<td>−0.29</td>
<td>0.21</td>
</tr>
<tr>
<td>$S_{18}$</td>
<td>−0.14</td>
<td>0.56</td>
<td>0.10</td>
<td>0.66</td>
</tr>
<tr>
<td>$S_{19}$</td>
<td>−0.39</td>
<td>0.09</td>
<td>0.36</td>
<td>0.12</td>
</tr>
<tr>
<td>$S_{20}$</td>
<td>0.32</td>
<td>0.17</td>
<td>−0.30</td>
<td>0.20</td>
</tr>
<tr>
<td>$S_{21}$</td>
<td>0.22</td>
<td>0.35</td>
<td>−0.14</td>
<td>0.57</td>
</tr>
<tr>
<td>$S_{22}$</td>
<td>0.70</td>
<td>4 $\times$ 10$^{-4}$</td>
<td>−0.59</td>
<td>6.6 $\times$ 10$^{-3}$</td>
</tr>
<tr>
<td>$S_{23}$</td>
<td>0.40</td>
<td>7.7 $\times$ 10$^{-2}$</td>
<td>−0.36</td>
<td>0.12</td>
</tr>
<tr>
<td>$S_{24}$</td>
<td>0.15</td>
<td>0.54</td>
<td>−0.37</td>
<td>0.11</td>
</tr>
<tr>
<td>$S_{25}$</td>
<td>0.49</td>
<td>2.9 $\times$ 10$^{-2}$</td>
<td>−0.39</td>
<td>8.4 $\times$ 10$^{-2}$</td>
</tr>
</tbody>
</table>
CD4 T cell count. Interestingly, only four correlated inversely with viral load and directly with CD4 T cell count (S8, S10, S11, and S12), which were generally enriched in elite controllers and characterized by multifunctionality. The remaining two subsets (S6 and S22) were directly correlated with viral load and inversely with CD4 T cell count, potentially indicating an ineffective or detrimental role for cells with these phenotypes, which were largely monofunctional in nature (primarily MIP1β or CD107a, respectively). The negative correlation of these monofunctional subsets possibly indicates that multiple functions must be combined for a beneficial effect, or that monofunctionality may represent a negative biomarker of HIV-specific CD4 T cell dysfunction.

As each of the above populations correlated significantly with clinical status by themselves, we next investigated whether combinations of these functional subsets could be used to build a model that more strongly describes a subject’s clinical status. We therefore attempted to fit viral load and CD4 T cell count measurements to a linear model that employs frequencies of different subpopulations as predictors. To avoid over-fitting the data, we utilized a model-shrinkage technique, LASSO regularization [23]. This method was used to ensure that the inferred model was sparse, including only predictors with significant explanatory power and allowing for the selection of the “minimal set” of subpopulation frequencies that explain a substantial proportion of the variation within CD4 T cell count or viral load. With this approach, we determined a linear model based on the intra-patient frequencies of only five phenotypic subpopulations as predictor values (S5, S6, S8, S10, and S22 for viral load; S6, S10, S11, S13, and S22 for CD4 T cell count). These models explain upwards of 80% of the variance in the viral loads (Figure 4.4A) or CD4 T cell counts (Figure 4.4B). Moreover, the regression coefficient of
Figure 4.4: Multivariate linear regression modeling to describe clinical parameters. A LASSO regularization approach was utilized to build models incorporating the most significant predictor variables (subpopulation frequencies) for both (A) viral load (as log viral load) and (B) CD4 T cell count (cells/µl). Each model incorporated five subsets as predictor values (S5, S6, S8, S10, and S22 for viral load; S6, S10, S11, S13, and S22 for CD4 T cell count).
each multivariate model is substantially better than any single subpopulation alone (Figure 4.4 versus Table 4.4), indicating the value in combining phenotypes. These results demonstrate that a large degree of variance within these critical clinical parameters can be accounted for by the frequencies of key phenotypes within the HIV-specific CD4 T cell response. Although this analysis cannot infer cause and effect relationships, it nonetheless further supports the idea that particular CD4 T cell functions may contribute meaningfully to relative and durable viral control during chronic HIV infection. Future studies should explore these associations more deeply by combining high-dimensional function and immunophenotyping data with robust measurements of clinical parameters.
DISCUSSION

The power to distill multi-dimensional data to reveal key features of biological relevance is increasingly critical within immunology, as experimental modalities which can assess many parameters simultaneously on single cells become more commonplace [24, 25]. While traditional approaches to flow cytometry datasets (primarily gating on biaxial plots) are tractable for low-parameter datasets, these methods become exponentially more cumbersome with each additional dimension of data. Standard approaches therefore involve manual searching for known cell phenotypes or combinations of functions, resulting in biased analyses that may fail to identify unexpected cellular signatures. Moreover, biaxial projections may conceal potentially important multivariate relationships between different parameters, such as those reported here.

In this study, we describe the application of a nonlinear dimensionality reduction and classification algorithm, ACCENSE, to understand the most important cellular features of antigen-specific CD4 T cells related to the spontaneous control of HIV infection while avoiding the bias of manually examining pre-selected CD4 phenotypes. Our results demonstrate that, separate from magnitude, the functional phenotype of the HIV-specific CD4 T cell response is tightly linked to spontaneous viral control. Moreover, they suggest that particular CD4 T cell responses may be important determinants of better or worse clinical status, and provide an example of how non-traditional analytical approaches may be used to understand immune correlates.

Controversy persists regarding whether HIV-specific CD4 T cell responses actually contribute to viral control [26]. Although their levels are enhanced in settings of both relative and
durable HIV control, their presence may merely reflect a lower level of CD4 T cell depletion in the setting of reduced viremia attained through other mechanisms. It is therefore unclear whether HIV-specific CD4 T cell responses represent a cause or consequence of controlled viral infection. Several lines of evidence, however, suggest that these cells may be important for the direct participation in an effective HIV immune response. Studies of acute HIV infection have shown that more robust HIV-specific CD4 T cell responses early after infection are associated with improved clinical outcome later on [27]. Moreover, the emergence of the HIV-specific CD4 T cell responses occurs coincident with the initial decline of viremia following peak viral load [15]. Although there is disagreement related to the prognostic value of early HIV-specific CD4 T cell responses with relative long-term control, this may be related to the specific CD4 T cell functions measured [15, 28]. The particular functional phenotype of the CD4 T cell response is therefore likely of great importance, a concept reinforced by studies of infections other than HIV. In influenza infection, for example, the presence of a particular type of virus specific CD4 T cell response is associated with protection from greater disease severity in a human challenge model [29]. In mice, the elicitation of strong, multifunctional pathogen-specific CD4 T cell responses to *Mycobacterium tuberculosis* through vaccination is the defining feature of long-term protection [30].

In our multidimensional analysis, HIV-specific CD4 T cells can be divided into 25 subsets based on the fine expression levels of the functional and phenotypic markers we measured. HIV controllers showed a marked enrichment of CD4 T cell responses exhibiting a dominant trifunctional IFNγ+TNFα+IL-2+ phenotype. Based on the expression of memory
markers, these cells were primarily CD27 low effector memory cells with a mixed CD28 phenotype.

Our results confirm and build on prior studies suggesting multifunctional HIV-specific CD4 T cells—and particularly those expressing IFNγ, TNFα, and IL-2 simultaneously—may be important for HIV control during chronic infection [13, 18, 31]. The repeated association of CD4 T cells expressing specifically these cytokines with improved clinical status indicates the potential importance of these three functions in the setting of HIV control. IFNγ is the classical cytokine used to measure antiviral responses, as it is generally the last cytokine to be lost as cells become exhausted [32]. Beyond experimental practicality, however, as a Th1 effector cytokine, IFNγ is also critical for the clearance many pathogens through activation of various immune signaling pathways [33]. Its effect on macrophages in particular may explain the importance of this cytokine in controlling *M. tuberculosis* infection, although its most critical functional mechanism in the context of HIV is less clear [34]. Like IFNγ, TNFα is another inflammatory cytokine that can mediate a variety of immune functions; TNFα signaling appears to play an important contribution to both HIV pathogenesis and HIV control, reflective of its pleiotropic nature [35]. Alone and in combination with IFNγ, TNFα can sensitize target cells to killing via cell death pathways [36].

IL-2 secretion by CD4 T cells likely also plays a central role in HIV control. Although IL-2 cannot directly exert an antiviral effect like IFNγ or TNFα, it can function indirectly through the support of other CD4 and CD8 T cells. In HIV infection, IL-2 secreting CD4 T cells have been suggested to be most important for the maintenance of an effective CD8 T cell
response [13]. Loss of IL-2 secreting HIV-specific CD4 T cells following acute HIV infection has been associated with reduced HIV-specific CD8 T cell functionality. This loss of CD8 T cell function is recoverable—at least \textit{in vitro}—through the provision of IL-2 producing CD4 help [12].

The relevance of these CD4 T cell functions appears to be most striking when they are expressed together, as highlighted by our current study, where viral load is closely associated with the presence of particular multifunctional phenotypes. However, it is not fully clear why polyfunctional CD4 T cell responses are most important, and why distinct, monofunctional subsets could not collaborate to potentially produce the same antiviral effect. However, a prior study of CD4 T cells specific for viruses other than HIV suggests that the benefit of polyfunctional capacity may be related in part to the quantity of cytokines produced by a cell—polyfunctional cells secreted more cytokine on a per-cell basis than monofunctional cells did [19]. Indeed, our data support this concept, and the Gag-specific cell type associated most significantly with HIV control exhibited some of the highest expression levels of those three cytokines (Table 4.2). The multiple functions expressed by polyfunctional HIV-specific CD4 T cells may also act synergistically to create a microenvironment most conducive to an antiviral effect [37].

One key limitation of this study is that although the analysis of the dataset is unbiased, the cytokines in the flow cytometry panel used are primarily Th1 effector functions. Future work should examine other CD4 functions, such as IL-4 or IL-21 secretion, in combination with these to determine which mediate the most prominent effects. Nonetheless, as Th1 responses have
been repeatedly shown to correlate with better virological outcome after infection with HIV and other viruses [37], there is still relevance in examining Th1 responses by themselves—as evidenced by the highly significant subset correlations we observe here.

It is also important to mention that we did note an association of cytotoxic markers within HIV-specific CD4 T cells with viral control (in population S9). Although this population was not the one most associated with controller status, it was significantly enriched in elite controllers, reinforcing concepts presented in Chapter 3, where we applied a more traditional approach focused only on cytolytic CD4 T cell responses. In that analysis, we similarly observed greater multifunctionality of HIV-specific cytolytic CD4 T cell responses in controllers, especially those expressing CD107a in combination with at least MIP1β and IFNγ. Although it is difficult to equate the two analytical approaches, the subsets that primarily contain these specific three functions together in the ACCENSE analysis are S5, S11, and S12. The abundance of cells in these populations all correlated significantly with better clinical status (either directly with CD4 count, inversely with viral load, or both; Table 4.4), in line with the results in the previous chapter.

In summary, the findings described here support the idea that the qualitative features of the HIV-specific CD4 T cell response may be of particular importance to HIV control. As vaccine candidates move forward that seek to engage cellular immune responses to control or abort HIV infection, it will be critical to take into account the functional phenotypes elicited not only of HIV-specific cytotoxic CD8 T cells, but virus specific CD4 T cells as well.
MATERIALS AND METHODS

Subjects. Study subjects were recruited according to guidelines approved by the Massachusetts General Hospital Institutional Review Board following informed written consent. Subjects defined as elite controllers had viral loads consistently below detection limits (blips permitted if sufficient longitudinal data available); viremic controllers had detectable viral loads under 2000 RNA copies/ml; subjects defined as progressors had viral loads above 2000 RNA copies/ml.

Ex vivo stimulation: Cryopreserved PBMCs were thawed and allowed to rest for three hours at 37º C, 5% CO2 at a concentration of 2x10^6/ml in R10 media (RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES). PBMCs were washed and resuspended at a concentration of 5-10 million cells/ml in R10 medium. For detection of CD107a, a pre-titered amount of PE-Cy5 conjugated antibody to CD107a (clone H4A3, BD Biosciences) was added to the medium. 1-2 million cells per condition were stimulated overnight with pooled 15-20 mer HIV Gag peptides (a combination of overlapping peptide pools comprising HIV clade B and sequence optimized cross-clade peptides to maximize clade coverage (Gag Ultra, JPT Peptide)). As a positive control, cells were stimulated with staphylococcal enterotoxin B (1 mg/ml, Sigma). An unstimulated (medium only) sample served as a negative control. 30 minutes into the stimulation, the transport inhibitors brefeldin A (0.5 mg/ml, Sigma) and monensin (GolgiStop, 0.3 µl/ml, BD Biosciences) were added to facilitate detection of T cell responses.

Intracellular cytokine staining. Following stimulation, cells were washed with phosphate buffered saline (PBS) and stained with a UV-excitable, amine-reactive viability dye
Cells were subsequently washed with staining buffer (PBS containing 2% fetal calf serum and 0.09% sodium azide) and stained with CD3-Brilliant Violet 510 (clone OKT3, Biolegend), CD4-Brilliant Violet 650 (clone OKT4, Biolegend), CD8-APC-eFluor780 (clone RPA-T8, eBioscience), CD14-biotin (clone HCD14, Biolegend), CD19-biotin (clone HIB19, Biolegend), CD45RA-Brilliant Violet 570 (clone HI100, Biolegend), CCR7-Brilliant Violet 785 (clone G043H7, Biolegend), CD27-Brilliant Violet 605 (clone O323, Biolegend), and CD28-PE-CF594 (clone 28.2, BD Biosciences). For detection of CD14 and CD19, cells were washed and incubated with Streptavidin-Alexa 350 (Life Technologies). After surface staining, cells were fixed and permeabilized (Cytofix/Cytoperm, BD Biosciences) and stained intracellularly using the following antibodies: IL-2 FITC (clone 5344.111, BD Biosciences), TNFa PerCP-Cy5.5 (clone MAb11, Biolegend), MIP1b Brilliant Violet 421 (clone D21-1351, BD Biosciences), IFNγ-PE-Cy7 (clone B27, Biolegend), Granzyme A-Alexa 647 (clone CB9, Biolegend), Granzyme B-Alexa 700 (clone GB11, BD Biosciences), and Perforin-PE (clone B-D48, Biolegend).

**Flow cytometric analysis.** Flow cytometric data were collected using a special order 5-laser LSR Fortessa and FACSDiva software (BD Biosciences). Compensation was performed using single-stained antibody capture beads (UltraComp eBeads, eBioscience) and amine-dye reactive beads (ArC, Invitrogen). Cytometer settings were standardized and tracked between runs using multi-fluorescent calibration beads (Rainbow Fluorescent Particles, Spherotech). Standard data analysis was performed using FlowJo version 9.6 (TreeStar). For ACCENSE analysis, data were pre-processed using FlowJo to isolate antigen-responsive cells (defined as positive for at least one function following peptide pool stimulation); embedding and multivariate analysis were then
performed as previously described [1]. Matlab was used for all statistical calculations and building of the linear regression model using LASSO regularization, as described in the text.
REFERENCES


Chapter 5: Conclusions
Sections of this chapter are modified from the peer-reviewed publication:

OVERVIEW

The identification of the immunologic determinants of HIV control in vivo has proven to be one of the most challenging endeavors in HIV research, but will be absolutely critical for the development of an effective HIV vaccine. Decades of research have so far yielded mixed results, primarily with regards to MHC class I genotype, as well as the induction and function of HIV-specific CD8 T cell responses [1]. While CD8 T cell activity is undoubtedly of paramount importance during the natural control of HIV, a growing number of studies—including those described in this thesis—have revealed an increasing recognition of the critical role CD4 T cells play in modulating and directly participating in multiple features of the antiviral immune response.

An effective adaptive immune response generally requires some level of participation of CD4 T cell responses, especially in the case of chronic viral infections [2]. Indeed, the importance of virus-specific CD4 T cells has been repeatedly established in multiple animal model systems [3]. In humans, CD4 T cell effector responses have been examined extensively in chronic viral infections [2]. Evidence for the involvement of CD4 T cell responses partially comes from the study of HIV infected individuals, where reactivation of chronic viral infections, like cytomegalovirus, is associated with low CD4 T cell levels [4]. However, assessing the importance of HIV-specific CD4 T cells to HIV control has not been as straightforward.

As natural targets of the virus, the mere presence of activated HIV-specific memory CD4 T cells may have a deleterious effect—nonetheless, they persist and can be found in most HIV infected individuals [5, 6]. Compared to CD8 T cells, HIV-specific CD4 T cells are of relatively
low frequency—making them more intractable to study and leading some to speculate that they may be present at an insufficient level to truly affect the virus [7]. However, these frequencies are reflective of normal CD4 T cell biology and similar to that of other chronic viral infections [8]. The HIV-specific CD4 T cells that are present in infected individuals may mediate a variety of functions, including direct cytolytic activity [9]. We therefore investigated the role of several effector functions in mediating viral control during acute and chronic infection. The studies described in this thesis add to the increasing body of evidence suggesting that HIV-specific CD4 T cells may be a fundamentally important component of HIV immunity—and, by extension, potentially critical to induce for optimal efficacy of future HIV vaccines.

Much of the evidence for the importance of cellular immunity in HIV infection—particularly HIV-specific CD8 T cell responses—comes from studies of acute infection, a period that has tremendous impact on the clinical trajectory of an HIV infected person long-term. As described in Chapter 2, we examined the HIV-specific CD4 T cell response early after acute infection in subjects who progressed to either high or low one-year viral set points [10]. All subjects remained treatment-naïve, enabling the tracking of their immunological responses over the course of one year post infection. Interestingly, we found no significant difference between HIV-specific CD8 T cell response levels between the two groups, but the individuals who progressed to lower viral set points exhibited significantly higher HIV-specific CD4 T cell responses. Previous studies showed HIV-specific CD4 T cells may exhibit direct cytolytic capacity [11, 12], and additional reports suggested an enrichment of these responses during acute HIV infection that wane into chronic infection [13]. We therefore examined differences in the cytotoxic capacity of HIV-specific CD4 T cells between the high and low viral set point groups.
Our data demonstrate that in individuals who progress to a low viral set point, there is a significant early expansion—before changes in the viral set point occur—of HIV-specific CD4 T cell responses with cytolytic activity that is absent from individuals who progress to a high viral set point. We further isolated a particular cytolytic phenotype at the time of initial presentation as being associated with better or worse long-term clinical progression, implying that CD4 T cells may possibly be mediating some level of viral control early on and suggesting that cytolytic capacity may be an important functional trait.

The responses we observed after acute infection ultimately decreased with the transition to chronic infection to levels that were not significantly different between the two set point groups. However, the cohorts examined in Chapter 2 did not consist of traditional HIV controllers—they were merely individuals who had relatively lower or higher normal viral set points. This led us to examine, in Chapter 3, the characteristics of HIV-specific cytolytic CD4 T cells during chronic infection and to investigate whether these cells are functionally enhanced in the setting of spontaneous, durable viral control compared to progressive infection. Elite controllers exhibited higher frequencies of HIV-specific CD4 T cells with cytolytic capacity. More interestingly, however, the functional profile of these cytolytic CD4 T cells in elite controllers more closely resembled that of HIV-specific CD8 T cells than those from untreated individuals with progressive infection. These data suggest that in elite controllers, HIV-specific cytolytic CD4 T cells are better able to collaborate with CD8 T cells to directly kill HIV infected target cells. In further examining the phenotype of these cells, we found that cytolytic potential in HIV-specific CD4 T cells was closely associated with the expression of the transcription factors T-bet and Eomesodermin. These transcription factors are associated with changes in
functional capacity in CD8 T cells, and our data provide more information on the influence of T-bet and Eomesodermin on CD4 T cell effector function during HIV control [14].

Even though HIV controllers exhibit higher levels of virus-specific cytolytic CD4 T cells, cytolytic cells represent an overall minority of the HIV-specific CD4 T cell response. In Chapter 4, we therefore performed an unbiased, multidimensional computational analysis of five different effector functions (as well as surface markers) to determine which HIV-specific CD4 T cell functional phenotypes correlate most closely with viral control. Most broadly, our results confirm prior suggestions that HIV-specific CD4 T cells that exert multiple effector functions simultaneously may be more effective in helping to mediate HIV control than less multifunctional cells. Although the strongest signal was observed for cells expressing IL-2 in combination with the inflammatory cytokines TNFα and IFNγ, we did see a weaker signal for cells also co-expressing perforin and the degranulatory marker CD107a.

**HIV SPECIFIC CD4 T CELLS AND CONTROL – A CAUSE OR CONSEQUENCE?**

Despite the wealth of recent evidence suggesting that HIV-specific CD4 T cells may contribute to viral control, including the several studies described above and presented in this thesis, a definitive answer to the question of whether these cells are actually beneficial overall remains elusive. In the context of an HIV vaccine, elicitation of an aggressive HIV-specific CD4 T cell response may enhance viral infection through the creation of a target cell population that becomes more infectable upon recognition of the virus.

This question is extremely difficult to address in human cohorts. At the earliest, most cohorts only enroll subjects when they report for medical treatment for symptoms of acute HIV
infection—likely several weeks after actual infection took place and far after virus specific CD4 responses have initially emerged. Newer, prospective cohorts—in which high-risk subjects are enrolled and monitored prior to infection—may be useful for determining the relationship between the very early trajectory of CD4 T cell responses and virus. These cohorts also may be extremely useful in light of recent evidence suggesting that many HIV-uninfected individuals harbor low level HIV-specific CD4 T cell responses that are present due to priming by other, cross-recognized pathogens [15]. Examining in prospective cohorts whether the presence or absence of these pre-existing HIV-specific CD4 T cell responses enhances (or diminishes) the risk of HIV infection may help answer some of these remaining questions about their overall impact on HIV infection. By extension, a study of the phenotypes of these cells may also provide insight into the CD4 T cell subsets that may promote or inhibit acquisition as well as modulate extremely early viral dynamics. Interestingly, there have been several reports of an enhancement of detectable CD4 T cell responses in highly exposed seronegative individuals [16, 17]. These results hint at a potential involvement of CD4 T cells in protection, or at the very least suggest that the presence of HIV-specific CD4 T cells may not increase infection risk, but nonetheless remain extremely controversial.

Vaccine studies in humans and animals—where virus specific CD4 T cells can be elicited by prior to infection—have also been used to approach these questions. Animal studies additionally afford the ability to artificially manipulate the levels of CD4 T cells through antibody depletion. Attenuated strains of SIV have shown particular efficacy against subsequent superinfection upon challenge with pathogenic SIV strains [18]. These attenuated SIV strains induce robust virus-specific CD4 T cell responses—including those with a cytolytic effector
phenotype—and some degree of this protection has been attributed to these CD4 T cells [19]. However, attenuated SIV is a relatively unique model that is not directly translatable to human HIV vaccinology due to safety considerations [20]. Studies of more traditional vaccine concepts in animals have been less conclusive. Vaccination with a DNA prime, adenovirus boost regimen has been shown to elicit strong virus-specific CD4 T cell responses; animals which exhibit higher peak levels of these cells after vaccination survive longer after challenge with a pathogenic SIV strain, potentially suggesting a contribution of these cells to viral control [21]. However, studies of other SIV vaccine concepts have suggested, quite to the contrary, that virus specific CD4 T cells may actually enhance viral replication [22]. Several groups have attempted to deplete CD4 T cells to understand the contribution of virus-specific CD4 T cell responses to viral control in SIV models. In one case, this resulted in the lack of viral control following acute SIV infection [23]. However, another study of brief CD4 depletion during chronic infection in an SIV model of elite control showed no impact on viremia [24]. Further work is clearly necessary to reconcile these differences, which may be context dependent and suggest that the participation of CD4 T cells changes as infection progresses.

Although far fewer HIV vaccine efficacy studies have been performed in humans, they still can offer insight into the HIV-specific CD4 T cell response. The first vaccine aimed at eliciting T cell immune responses and also tested in a large-scale efficacy trial was the Merck recombinant adenovirus type 5 (rAd5) vaccine. This vaccine consisted of recombinant adenoviral vectors expressing HIV Gag, Pol, or Nef and was designed to induce HIV-specific CD8 T cell responses [25]. It was tested in the HVTN 502 “STEP” trial and HVTN 503 “Phambili” trial and represented a shift in vaccine philosophy from earlier efficacy trials of vaccines designed to
protect through the induction of antibody responses against the virus [26]. The vaccine offered no protective or controlling effects and rather appeared to increase the risk of infection in both HVTN 502 and 503, which were halted early [25, 27]. In HVTN 502, this risk appeared to be associated at least partially with pre-existing adenoviral serostatus as well as circumcision status [25]. A later trial of a similar, Ad5-based concept, HVTN 505, also showed no protective effect and was terminated early [28].

The Merck rAd5 vaccine could elicit CD8 T cell responses in a majority of vaccinees, as well as CD4 T cell responses in close to half of tested subjects [29]. In those subjects who did generate a CD4 T cell response, it was equivalent in magnitude to what is seen during natural HIV infection. Moreover, the CD4 T cell responses were characterized by IL-2 secretion alone or in combination with other cytokines [29], a similar phenotype to that we have linked to better HIV control in Chapter 4. No differences in protection were seen in those individuals who did or did not generate T cell immunity in the STEP trial. The lack of protection despite presence of HIV-specific T cells with hypothetically appropriate functional phenotypes raises important questions about the role of cellular immunity in an HIV vaccine. It is possible that the responses exhibited sub-optimal targeting of the virus, especially for CD8 T cells [29]. Although post-hoc sieve analysis suggests that CD8 T cells did exert immune pressure on the virus, this was clearly not enough to affect protection or control [30]. It is also possible that the observed phenotypes—such as the IL-2 secreting CD4 phenotype—which appear to be relevant for control during chronic HIV infection, may not be most important in the context of a vaccine.
The only HIV vaccine to show any degree of protective efficacy is the ALVAC/AIDSVAX prime boost concept tested in the RV144 “Thai” trial [29]. This trial, which many in the field believed should not have taken place at the time it was initiated, showed a modest 31 percent reduction in infection [31, 32]. The vaccine was designed to engage both cellular and humoral HIV immunity, although controversy persists regarding whether protection was actually observed in this trial. The RV144 trial enrolled primarily individuals at low risk of HIV infection to begin with, resulting in a relatively low number of infections to base statistical inferences on [32]. Vaccine efficacy was lower in a post-hoc analysis of high-risk individuals, and efficacy overall seemed to wane with increasing time from vaccination [33]. An immune correlates analysis showed that the protection observed correlated with non-neutralizing IgG antibody responses directed against the V1V2 loop of HIV Env. An inverse effect was observed for IgA responses, which appeared to mediate a detrimental effect [34]. Although CD4 T cell responses did not survive the first correlates analysis, the combination of ALVAC and AIDSVAX used in the trial does induce modest CD4 T cell responses (but not CD8 T cell responses) against Env [35]. Interestingly, the majority (80%) of these responses were CD107a+ degranulating cells, potentially suggesting a contributing role for CD4 T cell cytotoxicity in vaccine induced protection and indicating the importance of examining this relatively underappreciated CD4 T cell function as future candidates are evaluated [35].
HIV-SPECIFIC CYTOLYTIC CD4 T CELLS: A MODEL FOR DUAL PATHWAY TARGETING

Compelling evidence suggests an unprecedented and critical role for cytolytic CD4 effector responses in the control and clearance of pathogens [36]. Although early studies have already reported on the presence of cytolytic CD4 T cells, their existence has only recently been widely acknowledged and investigated in greater detail. Especially in viral infections—where pathogens can replicate unhindered within the confines of a cell—recognition through the antigen presentation machinery is pivotal for viral containment [37]. Historically, the effector CD8 T cell response has been recognized to be a major contributor to the control of chronic viral infection—including and especially in HIV. Virus-specific CD8 T cells recognize target cells after viral antigen presentation through the MHC class I complex, and significant associations exist between the expression of certain HLA class I alleles and the ability to control the replication of viruses like HCV and HIV-1 [38]. Thus, there is strong evidence that CD8 T cells play a major role in the lysis of infected target cells and therefore contribute to the control of viral replication. However, CD8 T cells are also often unable to control viral replication alone, emphasizing the need for potential alternative pathways that can be induced by vaccines.

Although the overall role of cytolytic CD4 T cells in the containment of infections in vivo remains to be fully determined, the presence of these responses raises important considerations for vaccine design. While MHC class I is ubiquitously expressed, MHC class II expression is limited to professional APCs such as B cells, monocytes/macrophages, dendritic cells and some epithelial cells [39]. The induction of cytolytic CD4 T cell responses by a vaccine might be
especially important in the case of HIV-1 infection, where error-prone reverse transcription during viral replication results in a vast diversity of circulating strains and the rapid generation of escape mutations in CD8 T cell targeted epitopes. These escape mutations often impair CD8 T cell recognition and contribute to an accelerated disease progression [40, 41]. Viral immune escape has also been observed at CD4 T cell targeted epitopes in several infections, including HCV and the LCMV mouse model [42, 43]. While this phenomenon has not been widely studied to date, CD4 T cell driven escape mutations have also been suggested to occur in the case of HIV infection, indicating the potential importance of CD4 T cell mediated immune selection pressure in the control of viral replication [44]. Thus, it is possible that the simultaneous induction of cytolytic CD4 T cell responses as well as CD8 T cell responses bears several advantages in the context of viral infection: First, dual recognition through MHC class I and II increases the chance of killing virally infected target cells (Figure 5.1). Second, the nature of the MHC class II structure allows for a greater diversity in the type and number of viral epitopes that can be presented in comparison to MHC class I. Thus, the potential ability of a vaccine to induce virus-specific cytolytic CD4 T cells to recognize and kill virally infected targets in concert with cytolytic CD8 T cells represents a unique opportunity to contain viral replication early after HIV-1 acquisition. Infected antigen presenting cells like macrophages may be especially important targets of such a strategy, as these cells are believed to act as long-lived reservoirs [45].

Nonetheless, the induction of cytolytic CD4 T cell responses can be a double-edged sword. Research in LCMV infected mice, for example, has shown that an induction of CD4 effector responses can result in an increase in inflammation and a corresponding enhancement of
Figure 5.1: Hypothetical model for dual-pathway killing by cytolytic CD4+ and CD8+ T cells. Certain viruses like HIV-1 are able to establish infection within cells that express both MHC class I and class II molecules, such as antigen-presenting cells. Antigen presentation by these molecules is critical for recognition by circulating cytolytic CD4+ and CD8+ T cells. Even if the virus were to inhibit recognition through one pathway – for instance by the acquisition of escape mutations – effective recognition and lysis of the infected cell could still be achieved through the other pathway. From [12].
viral pathogenesis [46]. Most broadly, the function and role of cytolytic CD4 T cells in vivo must be evaluated to determine their impact on the modulation of other antigen specific B and CD8 T cell responses.

**A PATH AHEAD: EVALUATING HIV-SPECIFIC CD4 T CELLS FOR HIV VACCINES**

Eliciting particular types of HIV specific CD4 T cell responses—including cytolytic CD4 responses—*in vivo* will require the development of innovative vaccines consisting of intelligently designed antigens and immunogens. In particular, vaccines designed to specifically induce cytolytic CD4 T cell responses have not been studied, primarily due to the lack of understanding regarding their induction. However, efforts to broadly stimulate CD4 T cell responses in general have been more thoroughly investigated. Adenovirus vectors, for example, have been shown to induce potent memory CD4 T cell responses [47]. Likewise, vaccination of rhesus macaques with a DNA-prime, adenovirus boost SIV-vaccine regimen resulted in the generation of durable, tissue-wide virus-specific CD4 T cell responses [48]. In the context of EBV, it was found that dendritic cells vaccinated with a Modified Vaccinia Ankara (MVA) vector could effectively induce both CD4 and CD8 T cell responses to antigen *in vitro* [49]. DNA vaccination efforts have also been successful at stimulating CD4 T cell responses. A test of an experimental anti-HIV-1 DNA vaccine in mice revealed that the promiscuous class II targeting epitopes encoded by the vaccine could induce broad CD4 T cell responses in the context of several different MHC class II molecules [50]. Though the molecular mechanisms of antigen processing that lead to efficient presentation of peptides on MHC class II molecules are still unclear, it has also been found that CD4+ responses to specific DNA vaccine epitopes can
be strengthened through the addition of lysosomal targeting sequences [51]. Lastly, rationally designed adjuvants are likely to be important for the efficient targeting of cytolytic CD4 T cell responses and or polyfunctional profiles. Adjuvants like toll like receptor ligands, cytokines, and costimulatory molecules can provide the proinflammatory and conditioning signals necessary to drive T cell differentiation towards a particular lineage [52]. As new immunogens are designed and as the pathways that lead to the generation of specific CD4 T cell phenotypes are uncovered, the specific stimulation of these responses *in vivo* is likely to move closer to reality.

As cytolytic CD4 T cells specific for HIV have become a particular area of relatively greater interest recently, it is likely that considerable advances will be soon be made in understanding this unique cell type. One specific unanswered question is precisely when HIV-specific CD4 T cells commit to a cytolytic program during their lineage development. It is currently unknown if cytolytic activity is simply a function acquired by terminally differentiated CD4 Th1, or if it represents a characteristic of a unique CD4 T cell subset. Combining careful *ex vivo* multiparametric analysis of the various CD4 T cell phenotypes that have shown cytolytic potential in combination with detailed transcriptional profiling will shed further light on this question and help to evaluate their role in HIV pathogenesis. A closely related question—and one especially relevant in the context of vaccine design—is how can cytolytic CD4 responses be induced and/or maintained *in vivo*? Here it will be important to understand which antigens—but also whether specific adjuvants—play a role in the directed stimulation of cytolytic CD4 T cell responses. Vaccine trials in animals will need to elucidate if the induction of such responses can bring about better results than the primary induction of antigen-specific cytolytic CD8 T cells. In terms of HIV-1 vaccine design, it will be critical to understand whether the induction of cytolytic
CD4 T cells is beneficial for viral control or rather leads to the generation of activated target cells and accelerates disease progression. Considering the greater similarity of these responses with cytolytic CD8 T cells, it will also be important to determine the circumstances under which one or the other pathway is preferentially elicited. This knowledge will be pivotal to guide rational, reverse vaccine design, especially against viral infections like HIV that have so far eluded efforts to create effective vaccines.

FINAL COMMENTS

HIV pathogenesis represents an interdependent relationship between a multitude of HIV-specific CD4 T cell effector functions in the background of a larger immune response and ongoing CD4 T cell depletion. The complex nature of these interactions has made the identification of the most protective CD4 T cell functions difficult and it remains unclear how CD4 T cells should be best harnessed as the HIV vaccine field moves forward. The work described here in this thesis points to a role for specific, highly functional CD4 T cells in contributing to viral control and suggests that a closer look should be given to unconventional CD4 T cell functions like cytolysis. Nonetheless, additional creative and multi-factorial approaches will be required for the elucidation of key unanswered questions and a better understanding of the most important CD4 effector functions going forward.
REFERENCES


