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Insights into the Cytotoxic Potential of Human CD8+ T Cells: Implications for Virologic Control of HIV

Abstract
CD8+ T cells are often referred to as cytotoxic T lymphocytes because of their ability to induce the apoptosis of cells infected with an intracellular pathogen, thereby limiting the spread of an infection to previously uninfected cells. CD8+ T cells produce many proteins - including perforin, various granzymes, and granulysin - that are responsible for inducing target cell cytolysis. These cytolytic proteins are found pre-packaged into secretory granules within many resting CD8+ T cells, but their de novo synthesis can also occur after activation. In the setting of HIV infection, a rare group of HIV-positive patients, termed elite controllers (EC), naturally control HIV viremia to virtually undetectable levels without the intervention of drug therapy. Some evidence has implicated HIV-specific CD8+ T cells in achieving or maintaining this virologic control; however, the mechanism(s) to explain these findings remains largely undefined. In this work, we report that HIV-specific CD8+ T cells from EC demonstrated a superior ability to express perforin and granzyme B after activation compared to patients with progressive disease. Therefore, HIV-specific CD8+ T cells from EC possessed a greater cytotoxic potential by expressing higher levels of two principal cytolytic mediators, which may at least partially explain the ability of EC to suppress the replication of HIV in vivo. One critical upstream regulator of effector functionality and differentiation is the T-box transcription factor T-bet. We demonstrated a clear link between levels of T-bet and the presence of perforin and granzyme B within human CD8+ T cells. Notably, HIV-specific CD8+ T cells from EC expressed higher amounts of T-bet than progressors, suggesting that therapeutic modulation of T-bet may restore the cytolytic potential that is deficient among patients with uncontrolled viremia. Collectively, our results imply that the underlying defect(s) in effector function by HIV-specific CD8+ T cells from progressors lie not in the cytolytic proteins themselves, but rather in the elements controlling their expression, including T-bet.

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INSIGHTS INTO THE CYTOTOXIC POTENTIAL OF HUMAN CD8$^+$ T CELLS: IMPLICATIONS FOR VIROLOGIC CONTROL OF HIV

Adam R. Hersperger

A DISSERTATION

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DEDICATION

I dedicate this work to my parents, Stephen and Edna, and my wife, Chelsea, for their continuous love and support during the course of my graduate studies. I am eternally grateful for their presence in my life.

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ABSTRACT

INSIGHTS INTO THE CYTOTOXIC POTENTIAL OF HUMAN CD8$^+$ T CELLS: IMPLICATIONS FOR VIROLOGIC CONTROL OF HIV

Adam R. Hersperger

Supervisor: Michael R. Betts

CD8$^+$ T cells are often referred to as cytotoxic T lymphocytes because of their ability to induce the apoptosis of cells infected with an intracellular pathogen, thereby limiting the spread of an infection to previously uninfected cells. CD8$^+$ T cells produce many proteins - including perforin, various granzymes, and granulysin - that are responsible for inducing target cell cytolysis. These cytolytic proteins are found pre-packaged into secretory granules within many resting CD8$^+$ T cells, but their *de novo* synthesis can also occur after activation. In the setting of HIV infection, a rare group of HIV-positive patients, termed elite controllers (EC), naturally control HIV viremia to virtually undetectable levels without the intervention of drug therapy. Some evidence has implicated HIV-specific CD8$^+$ T cells in achieving or maintaining this virologic control; however, the mechanism(s) to explain these findings remains largely undefined. In this work, we report that HIV-specific CD8$^+$ T cells from EC demonstrated a superior ability to express perforin and granzyme B after activation compared to patients with progressive disease. Therefore, HIV-specific CD8$^+$ T cells from EC possessed a greater cytotoxic potential by expressing higher levels of two principal cytolytic mediators, which may at least partially explain the ability of EC to suppress the replication of HIV *in*

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CHAPTER 1

INTRODUCTION

Overview

Human Immunodeficiency Virus (HIV) currently infects millions of people worldwide causing high rates of morbidity and mortality. While highly active anti-retroviral therapy (HAART) has proven successful at inhibiting viral replication and improving disease outcomes, it cannot eradicate the virus from an infected individual and is prohibitively expensive in many instances, especially in developing nations. Therefore, the ability to develop an effective HIV vaccine to either prevent infection or improve disease prognosis is of extreme importance.

There are a small subset of HIV-infected individuals, termed elite controllers, who naturally control viral replication to extremely low levels without drug therapy. There is intense interest in studying these patients to potentially understand the mechanism(s) underlying their ability to curb the replication of HIV. Furthermore, insights garnered from elite controllers can potentially be used to create vaccine or treatment interventions aimed at combating HIV. There is an increasing body of evidence suggesting that $CD8^+$ T cells, which have the ability to directly eliminate infected cells within the host, play an important role within elite controllers by suppressing HIV replication. In this work, we conducted an in-depth examination of the cytolytic proteins that $CD8^+$ T cells use to eliminate infected target cells and report on the implications for long-term control of HIV replication.

Biology and functionality of CD8$^+$ T cells

CD8$^+$ T cells are primarily responsible for recognizing host cells that are either abnormal in some fashion (e.g. tumor cell) or infected by an invading intracellular pathogen (e.g. virus, bacteria, etc). CD8$^+$ T cells originate from pluripotent hematopoietic stem cells in the bone marrow before maturing in the thymus. Once T cells have completed their development in the thymus, they enter the bloodstream and survey the host for transformed or infected cells. Mature, recirculating T cells that have not yet come into contact with foreign antigen are referred to as naïve T cells, which are largely quiescent and exhibit little if any effector functionality.

Mature CD8$^+$ T cells are highly specific for a limited number of peptide epitopes that are found on the surface of professional antigen presenting cells or cells infected by an intracellular pathogen. These peptides are typically 8-11 amino acids in length and are presented to CD8$^+$ T cells on major histocompatibility class I (MHC-I) molecules. In the context of viral or bacterial infection, these epitopes result from proteasomal degradation of pathogen-derived proteins that are defective in some manner or do not otherwise escape the cellular quality control machinery (Eisenlohr et al., 2007; Yewdell and Haeryfar, 2005). Upon recognition of target cells bearing specific peptide:MHC-I complexes in combination with the necessary activation signals, naïve T cells commence upon a program of expansion and differentiation that allows for the acquisition of effector functionality (Kaech and Ahmed, 2001). During the 2 to 3 weeks following the peak of CD8$^+$ T cell expansion, the majority (greater than 90%) of the activated effector cells die by apoptosis. The remaining antigen-specific CD8$^+$ T cells eventually populate a pool of long-lived memory cells (Wherry and Ahmed, 2004). The primary goals of effector CD8$^+$

T cells are to limit the spread of a replicating pathogen to uninfected cells and to clear the invading pathogen from the host. Memory T cells are important for future encounters with the same pathogen as they allow for a rapid and robust recall response upon re-infection (Wherry and Ahmed, 2004; Wherry et al., 2003).

Effector and memory $CD8^+$ T cells possess the ability to produce and secrete various proteins that can be critical to the control of an invading pathogen. For example, $CD8^+$ T cells can secrete effector cytokines (e.g. IFN-γ, TNFα, and IL-2) and chemokines (e.g. MIP1α, MIP1β, and RANTES) that elicit immunomodulatory effects during an active infection. The secretion of chemokines by T cells attract other immune cells, such as macrophages or other T cells, that bear the appropriate receptor(s) for each respective chemokine. The recruitment of many different cell types to the site of inflammation by chemokines allows for the containment and possible clearance of infected cells within the local microenvironment. The cytokine IFN-γ is routinely used as a marker for antigen-specific T cell activity but is also a key anti-pathogen factor. Mice that lack the receptor for IFN-γ through genetic manipulation demonstrate deficiencies in natural resistance to several intracellular pathogens, including Listeria monocytogenes (Huang et al., 1993). IFN-γ signaling has been shown to increase the sensitivity of infected cells to apoptosis (Tsujimoto et al., 1986; Xu et al., 1998). Additionally, the actions of IFN-γ favor more efficient presentation of the antigens associated with intracellular pathogens by stimulating the immunoproteasome and the synthesis of MHC-I and other molecules that are associated with antigen processing (Schroder et al., 2004). The cytokine IL-2 is an important growth factor that is a critical regulator of the proliferation and differentiation of antigen-specific $CD8^+$ T cells (Malek, 2008; Pipkin et al., 2010b). It has also been

shown to promote a robust secondary expansion of memory $CD8^+$ T cells upon re-exposure to a pathogen (Schoenberger and Janssen, 2006). Finally, TNFα is a major proinflammatory mediator that amplifies the Th1 response directed at intracellular pathogens by inducing the synthesis of many key cytokines (Bazzoni and Beutler, 1996; Kim and Solomon, 2010). TNFα can also directly influence pathogen clearance through its ability to induce apoptosis in infected cells that express the necessary cognate receptor, which contains an intracellular pro-apoptotic "death domain" (Pfeffer, 2003).

Besides the production and secretion of various chemokines and cytokines, many $CD8^+$ T cells also possess the ability to directly kill infected target cells via apoptosis. They can lyse their cellular targets by either granule- or receptor-mediated mechanisms. The predominant killing pathway makes use of preformed granules containing various apoptosis-inducing proteins that are secreted via exocytosis following target cell recognition (Shiver and Henkart, 1991; Shiver et al., 1992). After cell-to-cell contact is established, these cytotoxic granules track along microtubules and are released into the space - referred to as the immunological synapse - between the T cell and its target (Stinchcombe et al., 2001; Stinchcombe and Griffiths, 2007). The secondary pathway involves the engagement of receptors on target cells that induce classical caspase-dependent apoptosis, such as those specific for Fas ligand (Nagata and Golstein, 1995) or TNFα (as mentioned above). Importantly, the induction of apoptosis in target cells inhibits the spread of a pathogen to uninfected cells and is thus beneficial to the host.

Cytotoxic granule-resident proteins and their regulation

Cytotoxic granules are specialized secretory lysosomes that contain many proteins, including perforin, a group of serine proteases called granzymes, and granulysin, within a matrix of proteoglycans (Peters et al., 1991; Russell and Ley, 2002). Granulysin is a lipid-binding protein of the saposin-like protein family and may be important for the control of a wide variety of pathogenic bacteria, fungi, and parasites through its ability to disrupt membrane integrity (Clayberger and Krensky, 2003; Kolter et al., 2005; Krensky and Clayberger, 2009; Stenger et al., 1998). Granzymes A and B, which are the most abundant of the granzymes, induce an apoptotic cascade once inside a target cell (Gershenfeld and Weissman, 1986; Lobe et al., 1986; Pasternack and Eisen, 1985); some granzymes, including granzyme A, may also possess inflammatory and other immunomodulatory properties (Froelich et al., 2009; Metkar et al., 2008; Pardo et al., 2009a; Pardo et al., 2009b). The pore-forming molecule perforin is perhaps the most crucial component of the granule because it is responsible for allowing the entry of granzymes into a target cell (Bolitho et al., 2007; Shiver et al., 1992); however, the precise mechanism of this process remains unclear (Pipkin and Lieberman, 2007). Nevertheless, abrogation of perforin activity through genetic mutation or deletion results in profoundly impaired cellular cytotoxicity and subsequent immunodeficiency (Kagi et al., 1994; Molleran Lee et al., 2004; Smyth et al., 1999; Stepp et al., 1999; van den Broek et al., 1996).

The study of the transcriptional control of these cytolytic proteins has been an area of great interest with many important recent developments (Glimcher et al., 2004; Pipkin et al., 2010a). The majority of published work thus far has focused on

understanding the regulation of perforin with some reports also examining the elements that influence granzyme B (grz B) expression. There is a paucity of published material concerning the genetic regulation of granzyme A (grz A) or granulysin. As one might imagine, the regulation of perforin and grz B involves a complex array of control elements and signaling pathways that carefully direct the expression of these cytolytic molecules in time-, context-, and tissue-specific manners. Without such strict control, the aberrant expression of these proteins could be detrimental to the host due to their potent ability to induce apoptosis.

The hypothesis that target cells could be eliminated via secreted cytotoxic granules became fully developed in the early 1980s (Henkart et al., 1984; Millard et al., 1984; Podack and Konigsberg, 1984). The particular molecule(s) that mediated target lysis remained elusive until a series of papers were published that identified and biochemically characterized a protein, termed perforin or cytolysin, purified from the granules of both murine NK cells and $CD8^+$ T cells that was responsible for target cell lysis (Liu et al., 1986; Masson and Tschopp, 1985; Podack et al., 1985; Young et al., 1986b; Young et al., 1986d). Later analysis of human perforin yielded findings consistent with what was previously discovered concerning mouse perforin (Lichtenheld et al., 1988). Interestingly, perforin was shown to share structural and functional similarities to complement component C9 most likely due to the ability of both proteins to polymerize and cause pore formation in membranes (Tschopp et al., 1986; Young et al., 1986a; Young et al., 1986c; Zalman et al., 1986).

Perforin is abundantly expressed in NK cells and gamma-delta T cells but also found in NKT cells, $CD8^+$ T cells, and a small fraction of $CD4^+$ T cells. $CD8^+$ T cells are

even sometimes referred to as “cytotoxic T lymphocytes” because they can express high levels of perforin depending on their maturation and differentiation state (Chattopadhyay et al., 2009; Takata and Takiguchi, 2006). Naïve mouse or human $CD8^+$ T cells, which have never encountered their cognate antigens, do not express any detectable perforin protein (Pipkin et al., 2010b; Takata and Takiguchi, 2006). However, upon recognition of their specific targets under the appropriate conditions, naïve $CD8^+$ T cells commence upon a program of expansion and differentiation that allows for the acquisition of cytolytic gene expression (Kaech et al., 2002a; Kaech et al., 2002b; Oehen and Brduscha-Riem, 1998; Peixoto et al., 2007; Wherry et al., 2003). Central memory cells typically do not demonstrate any perforin expression but increasing amounts of this cytolytic protein are observed in effector memory and effector $CD8^+$ T cells (Chattopadhyay et al., 2009; Takata and Takiguchi, 2006). In fact, the presence of perforin in these latter subsets explains their enhanced ability compared to central memory $CD8^+$ T cells to mediate target cell lysis (Hamann et al., 1997; Sallusto et al., 1999; Wolint et al., 2004).

Most of what is known about the regulation of perforin expression comes from the mouse model - although some work has been done in humans as well (Pipkin et al., 2010a). A 5 kb region upstream of the murine perforin gene was shown to contain promoter elements sufficient to confer tissue-specific expression (Lichtenheld and Podack, 1992; Zhang and Lichtenheld, 1997). Additionally, using microcell-mediated chromosome transfer, Pipkin and colleagues identified the locus control region of human perforin, which conferred both cell lineage-specific and T cell receptor (TCR) inducible gene expression (Pipkin et al., 2007). Many factors, including epigenetic modifications and various cytokines, have been reported to influence perforin expression in distinct cell

types. Levels of DNA methylation (Lu et al., 2003) and histone acetylation (Araki et al., 2008) can influence the chromatin structure and subsequent expression of perforin. For example, the perforin promoter in fibroblasts, which have no detectable levels of this protein, was found to be heavily methylated and in a state consistent with closed chromatin; conversely, the perforin promoter in NK cells, which display constitutive expression of perforin, was largely devoid of methylation (Lu et al., 2003). The cytokine milieu in the local environment of various cell types can also influence cytolytic gene expression (Agnello et al., 2003; Morishima et al., 2005). Moreover, the cytokine IL-2 has been particularly implicated in increasing perforin expression in some settings (DeBlaker-Hohe et al., 1995; Pipkin et al., 2010b; Zhang et al., 1999), but IL-2 independent pathways of perforin induction have also been described (Lu et al., 1992).

Another layer of regulation of the perforin gene is through the action of transcription factors. Indeed, DNase I footprinting analysis of the perforin promoter identified several transcription factors and their binding sites, including AP1, SP1, NFAT, MEF, and various STAT proteins (Glimcher et al., 2004; Lacorazza et al., 2002; Zhang et al., 1999; Zhang and Lichtenheld, 1997). Additionally, recent data have implicated two members of the T-box transcription factor family, T-bet and Eomesodermin (Eomes). While many different classes of transcription factors have been shown to influence perforin expression, T-bet and Eomes seem to be the most critical and are also intimately involved in effector lineage commitment (Glimcher et al., 2004).

Members of the T-box family are found in a wide variety of tissues and are required for diverse cell fate decisions in vertebrate development (Naiche et al., 2005). The T-box family itself is defined by a highly conserved 200 amino acid DNA-binding

domain, which is termed the "T-box" (Papaioannou, 1997; Smith, 1997). In keeping with the role played by various T-box family members in directing cell lineage decisions, T-bet (or T-box expressed in T cells), which was first cloned in 2000 (Zhang and Yang, 2000), was found to be the master regulator in directing Th1 lineage commitment in $CD4^+$ T cells (Szabo et al., 2000). Interestingly, T-bet is the only known T-box gene specifically expressed in the lymphoid system. During Th1 helper cell development, T-bet expression itself appeared to be initiated and/or subsequently controlled by signaling through the TCR, IFN-γR/STAT1, and IL-12/STAT4 (Afkarian et al., 2002; Lighvani et al., 2001; Takemoto et al., 2006).

While initially characterized within $CD4^+$ T cells, subsequent work has demonstrated that T-bet is also important for the effector programming of both murine and human $CD8^+$ T cells (Chtanova et al., 2005; Intlekofer et al., 2007; Joshi et al., 2007; Sullivan et al., 2003; Takemoto et al., 2006). However, T-bet did not appear to be as important for the production of IFN-γ in $CD8^+$ T cells (Szabo et al., 2002) as it was for IFN-γ expression in $CD4^+$ T cells (Szabo et al., 2000). Subsequent work showed that another T-box transcription factor, Eomes, was also important for $CD8^+$ T cell effector function and IFN-γ production (Pearce et al., 2003). Therefore, it appears that both T-bet and Eomes possibly have complementary and/or redundant roles in $CD8^+$ T cell effector differentiation. Indeed, one group reported that $CD8^+$ T cells deficient in both of these transcription factors failed to differentiate into functional cells capable of target cell lysis following viral infection, but instead differentiated into an IL-17-secreting lineage that offered no protection (Intlekofer et al., 2008).

In terms of directing perforin expression and target cell cytolysis, both T-bet and Eomes have been shown to be important - with one demonstrating a more pronounced effect over the other depending on the experimental conditions or cell types involved (Cruz-Guilloty et al., 2009; Intlekofer et al., 2005; Pearce et al., 2003; Sullivan et al., 2003; Taqueti et al., 2006). Both transcription factors have been reported to bind to the perforin promoter in both human and mouse NK cells and $CD8^+$ T cells (Pipkin et al., 2010b; Townsend et al., 2004). Moreover, the deletion of both T-bet and Eomes virtually ablates perforin expression and the cytotoxic capacity of $CD8^+$ T cells (Intlekofer et al., 2008; Intlekofer et al., 2005). It is known that many T-box family members can recruit histone-modifying enzymes, providing a potential mechanism to explain the ability of T-bet and Eomes to critically regulate perforin expression and control lineage commitment (Lewis et al., 2007; Miller et al., 2008; Miller and Weinmann, 2009a, b).

In terms of clinical significance, many murine experimental disease models have further uncovered the ability of T-box transcription factors to influence effector $CD8^+$ T cells. Because germline deletion of Eomes results in early embryonic lethality (Russ et al., 2000), the majority of published work in this area has used T-bet knock-out mice. Interestingly, T-bet has been shown to be essential for the development of many experimental autoimmune diseases, including type 1 diabetes and myocarditis in transgenic mice (Juedes et al., 2004; Taqueti et al., 2006); in both of these models, pathogenic $CD8^+$ T cells are thought to be causally associated with the onset of disease, which is mitigated or prevented in the absence of T-bet. Additionally, T-bet plays a key role in NK cell-mediated control of melanoma metastatic disease by inhibiting the systemic dissemination of cancer cells in this model (Werneck et al., 2008).

Besides perforin, the other critical component of the cytotoxic granule is a group of serine proteases called granzymes (granule enzymes). Both work synergistically to induce apoptosis in a target cell after recognition by NK cells or cytotoxic $CD8^+$ T cells (Lieberman, 2003). There are numerous granzymes: granzymes A and B are the most well studied in mice and humans whereas much less in known about granzymes C, D, E, F, G (mouse), H (human), K (both species), and M (human). Granzymes A and B were the first to be discovered, which occurred shortly after the initial description of perforin. They were isolated from cytotoxic granules and shown to possess enzymatic activity consistent with other known serine proteases (Gershenfeld and Weissman, 1986; Lobe et al., 1986; Pasternack and Eisen, 1985). The expression of granzymes A and B occurs in many immune cells, especially NK cells, a subset of $CD8^+$ T cells, and a small fraction of $CD4^+$ T cells. Among $CD8^+$ T cells, Grz A is the most ubiquitously expressed granzyme, being found in effectors, effector memory, and even some central memory cells (Chattopadhyay et al., 2009; Takata and Takiguchi, 2006). Grz B often tracks closely with perforin and is expressed at the highest levels in differentiated effector $CD8^+$ T cells (Chattopadhyay et al., 2009; Makedonas et al., 2010; Takata and Takiguchi, 2006). Interestingly, granzymes appear to be differentially regulated during lymphocyte development as a distinct repertoire is expressed by individual $CD8^+$ T cells (Grossman et al., 2004; Kelso et al., 2002).

Like perforin, grz B is not found in naïve $CD8^+$ T cells, but its expression is induced following stimulation and subsequent effector differentiation (Kaech et al., 2002a; Kaech et al., 2002b; Oehen and Brduscha-Riem, 1998; Peixoto et al., 2007; Wherry et al., 2003). The regulatory elements of the grz B gene have been mapped to a

200 base pair region of the promoter, which contain binding sites for NFAT, AP1, and other transcription factors that have variable effects on its expression (Babichuk and Bleackley, 1997; Babichuk et al., 1996; Haddad et al., 1993; Hanson et al., 1993; Heusel et al., 1991; Wargnier et al., 1998; Wargnier et al., 1995). The expression of grz B has also been shown to be upregulated by IL-2, IL-12, and IL-15, which parallels with the activation of perforin by these cytokines (DeBlaker-Hohe et al., 1995; Manyak et al., 1989; Ye et al., 1996). Finally, both T-bet and Eomes have been implicated in the regulation of grz B. For example, T-bet was reported to bind to the grz B promoter in both humans and mice, and T-bet knock-out NK cells were shown by the same group to have significantly decreased levels of grz B (Townsend et al., 2004). Additionally, overexpression of Eomes within Th2 cells resulted in the upregulation of grz B (Pearce et al., 2003). Therefore, similar to the importance of T-bet and Eomes in driving perforin expression, these transcription factors are also critical for optimal grz B levels and effector functionality in general (Cruz-Guilloty et al., 2009; Pearce et al., 2003; Pipkin et al., 2010b; Taqueti et al., 2006; Townsend et al., 2004).

Our understanding of the regulation of the cytolytic molecules, perforin and grz B, has advanced greatly in the past decade. While many elements are involved in controlling their expression, we now know that only a few factors, such as T-bet and Eomes, are critical and necessary for the expression of perforin and grz B in addition to proper effector $CD8^+$ T cell development. Given the important role played by $CD8^+$ T cells in fighting infection by viruses and other intracellular pathogens, it is tempting to think that therapeutic platforms aimed at modulating or promoting the expression of T-bet or Eomes may be clinically beneficial in some settings. Additionally, we need to

understand in more detail what elements or factors control the expression of T-bet and Eomes themselves during effector differentiation (Minter et al., 2005; Taylor et al., 2010).

Biology and pathogenesis of Human Immunodeficiency Virus

Starting in the late 1970s, a growing number of previously healthy individuals began showing symptoms of immunologic dysfunction. This new syndrome, which was eventually called Acquired Immune Deficiency Syndrome (AIDS), was characterized by generalized lymphadenopathy, opportunistic infections (e.g. cytomegalovirus-associated retinitis and *Toxoplasma gondii* encephalitis), and a variety of cancers (e.g. non-Hodgkin's lymphoma). Interestingly, a common accompanying feature was marked depletion of $CD4^+$ T cells from the peripheral blood in affected patients. In 1983, researchers recovered an infectious agent from the lymph nodes of a patient who presented with generalized lymphadenopathy of unknown origin. Subsequent study of the infectious agent revealed that it displayed many characteristics of retroviruses (Barre-Sinoussi et al., 1983). However, unlike the commonly studied retroviruses known at that time, the virus cultured from AIDS patients was cytopathic in human peripheral blood mononuclear cells (PBMC) with specific killing of $CD4^+$ T cells (Klatzmann et al., 1984a). Subsequent morphologic and genetic study of this new retrovirus revealed characteristics typical of the *Lentivirus* genus; the virus was finally named human immunodeficiency virus, or HIV (Coffin et al., 1986).

It was originally thought that HIV, like previously studied retroviruses, would be relatively genetically homogenous. However, as proviral DNAs corresponding to various

HIV isolates became available and were compared, their extensive genetic heterogeneity between isolates became apparent (Benn et al., 1985). In fact, even HIV isolates recovered from a single infected person exhibited significant sequence diversity (Saag et al., 1988). The sequence diversity of HIV between and within individuals is largely the result of its error-prone reverse transcriptase enzyme and the ability of multiple strains to undergo recombination upon co-infection of a single cell. Unfortunately, this ability to undergo rapid genetic mutation has hindered attempts to combat HIV through vaccine or drug treatment efforts. Indeed, HIV is poised to escape the myriad selective pressures placed upon it by the human immune system or small molecule inhibitors.

The principal determinant of HIV tropism resides in the surface envelope (Env) glycoprotein. Consistent with the preferential loss of peripheral blood $CD4^+$ T cells from HIV-infected patients, the CD4 molecule, found mainly on a subset of T cells and macrophages, was identified as the major cell-surface receptor for HIV (Bour et al., 1995; Klatzmann et al., 1984b). However, soon after the identification of CD4 as the receptor for HIV, it was recognized that this protein alone was not sufficient for virus entry into an uninfected cell. For example, mouse cells expressing just human CD4 were not able to be infected (Maddon et al., 1986; Weiner et al., 1991), whereas $CD4^+$ mouse-human cell hybrids could be induced to fuse upon expression of HIV Env (Broder et al., 1993; Dragic et al., 1992). These results suggested that at least one secondary receptor, or coreceptor, was necessary for virus entry. Subsequent studies from multiple laboratories revealed that the chemokine receptors CCR5 and CXCR4 were the principal coreceptors that HIV used to enter cells after initial binding to CD4 (Alkhatib et al., 1996; Choe et al., 1996; Cocchi et al., 1995; Deng et al., 1996; Feng et al., 1996).

The disease course in the majority of HIV-infected patients can be divided into three main stages: (1) acute, (2) asymptomatic, and (3) AIDS manifestation (Rowland-Jones, 2003). The acute phase, which occurs within the first several weeks after initial infection, is characterized by massive levels of viral replication in the mucosa and peripheral blood with a concomitant depletion of $CD4^+$ T cells in both of these compartments (Brenchley et al., 2004; Daar et al., 1991). After the initial peak of viral replication, HIV viral load usually lowers during the asymptomatic phase to a "set point" of approximately 10^3 to 10^4 copies of HIV RNA per mL of plasma. During this period, which can last for up to 5-10 years on average, the levels of HIV replication remain relatively stable. Notably, it was shown that the degree of set point viral load is inversely correlated with the rate of disease progression; in other words, a high set point viral load is associated with faster progression to AIDS and vice versa (Mellors et al., 1995). While the asymptomatic phase may be clinically latent in the sense that infected patients appear relatively healthy, there is ongoing replication of HIV and continuous turnover of $CD4^+$ T cells that are destroyed either directly or indirectly by the virus (Ho et al., 1995; Pantaleo et al., 1993; Piatak et al., 1993; Wei et al., 1995). Finally, after roughly 10 years of infection with HIV, untreated individuals will succumb to an opportunistic infection, which results from a failure of the immune system to further combat invading pathogens. Additionally, there is a large spike in HIV viral load observed during this final phase of the disease course. The continual loss of $CD4^+$ T cells, which play many important roles in pathogen defense, throughout the disease course of HIV infection most likely underlies the ultimate failure of the immune system to control HIV replication and the vulnerability to opportunistic infections (Kalams and Walker, 1998).

Current insights into HIV elite controllers

While the vast majority of individuals are unable to control HIV replication, a rare subset of less than 1% of infected patients maintain extremely low viral loads (<50 copies of HIV RNA/mL of plasma) for extended periods of time without the intervention of anti-retroviral drug therapy (Grabar et al., 2009; Hubert et al., 2000; Lambotte et al., 2005; Lefrere et al., 1999). The majority of these infected individuals display very low rates of $CD4^+$ T cell decline in the peripheral blood (Sedaghat et al., 2009) and rarely progress to AIDS despite being HIV-positive for many years (Migueles and Connors, 2010; Sajadi et al., 2009). Primarily for these reasons, there is intense interest in studying these patients to understand the mechanism(s) underlying their ability to suppress HIV with the goal of using this information to aid in the development of vaccines and therapeutics to combat HIV. While these rare HIV-infected individuals are referred to by many different labels (e.g. elite controllers, elite long-term nonprogressors, or elite suppressors), they will be termed elite controllers (EC) for the remainder of this work.

Role of virologic factors

Currently, no known epidemiological factors, including sex, race, ethnicity, or mode of HIV acquisition, predispose an individual to become an EC (Migueles and Connors, 2010). There are many remaining possibilities that could explain the ability of EC to suppress the replication of HIV, which fall into two broad categories: virologic or host factors. In terms of virologic factors, one major hypothesis is that EC are infected with defective strains of HIV. Indeed, there have been a number of studies and case reports which suggest that virus from EC is less fit than strains of HIV taken from progressors due to deletions or genetic mutations (Alexander et al., 2002; Alexander et

al., 2000; Calugi et al., 2006; Deacon et al., 1995; Hassaine et al., 2000; Huang et al., 1998; Iversen et al., 1995; Kirchhoff et al., 1995; Mariani et al., 1996; Michael et al., 1995; Salvi et al., 1998; Wang et al., 1996; Wang et al., 2003; Yamada and Iwamoto, 2000; Zhang et al., 1997). It was recently reported that EC-derived HIV envelopes exhibited decreased entry efficiency into target cells compared to envelopes isolated from chronically-infected progressors (Lassen et al., 2009). Additionally, another group constructed chimeric viruses using patient-derived Gag and Pol sequences and found that viruses generated from EC displayed lower replicative capacities on average than viral constructs from progressors (Miura et al., 2009a).

The development of extremely sensitive assays able to measure viral loads at significantly lower limits of detection than standard assays has broadened the understanding of viral dynamics within EC. These novel techniques have revealed the presence of persistent low-level HIV viremia in EC (Hatano et al., 2009; O'Connell et al.; Pereyra et al., 2009). Consequently, there is evidence of ongoing viral evolution, even within $CD8^+$ T cell epitopes (Miura et al., 2009c); however, EC have been shown to maintain suppression of HIV even under such circumstances (Bailey et al., 2006b; O'Connell et al., 2010). Moreover, recent reports from several independent groups suggest that few, if any, virologic defects or genetic abnormalities exist among HIV strains from many EC (Blankson et al., 2007; Lamine et al., 2007; Miura et al., 2008). While it can be difficult to observe autologous viral outgrowth during *in vitro* culture of activated $CD4^+$ T cells purified from EC, proviral DNA levels are significantly lower in EC compared to progressors (Julg et al., 2010). Therefore, it may be difficult to recover virus from cultures of $CD4^+$ T cells from EC due to the low frequencies of infected cells

in the peripheral blood of these individuals prior to *in vitro* culture. Nevertheless, virus that can ultimately be isolated from $CD4^+$ T cells among EC have been shown to replicate as efficiently in culture as laboratory reference strains (Blankson et al., 2007).

There are several case studies and reports that provide further evidence suggesting that most EC maintain low HIV viral loads via mechanisms unrelated to the replicative fitness of the infecting virus. One study showed that virologic breakthrough occurred in an EC after a period of virologic control, suggesting that the patient was not infected with a defective virus (Bailey et al., 2007). There are at least two published case studies demonstrating maintenance of viral control in separate EC despite superinfection with a second, divergent strain of HIV that appeared to be otherwise fully pathogenic (Casado et al., 2007; Rachinger et al., 2008). Of interest, another group studied a transmission pair in which HIV was transmitted from a patient who developed AIDS to an individual who went on to become an EC (Bailey et al., 2008). This latter case is a clear example that some EC can effectively control a strain of virus that was fully pathogenic in another person. In a final study that should be highlighted, it was shown that primary isolates from EC are as effective as those derived from progressors at causing downmodulation of HLA-A2 and HLA-B57 from the surface of primary $CD4^+$ T cells (Nou et al., 2009). This study indicates that viral isolates from EC most likely have the ability to evade HIV-specific immune detection to a similar degree as progressors. In summary, while it is certainly feasible that infection with defective virus can lead to virologic control, there is ample reason to believe that many EC are infected with fully replication competent and pathogenic strains of HIV. Therefore, infection with HIV of attenuated fitness cannot be a general factor to explain undetectable viremia in EC.

Role of host genetic or anti-viral factors

If virologic factors do not seem to contribute to the control of HIV replication in many EC, this suggests that one or more host factors underlie their controller status. Numerous studies have shown normal *in vitro* replication of heterologous or autologous isolates of HIV in activated $CD4^+$ T cells purified from EC (Blankson et al., 2007; Migueles et al., 2002; Migueles et al., 2008; Saez-Cirion et al., 2007). Similarly, another group found that $CD4^+$ T cells isolated from EC and HIV-negative subjects supported equivalent levels of viral replication of both CCR5 and CXCR4 tropic strains (Julg et al., 2010). Therefore, $CD4^+$ T cells in EC are as equally infectable as those from progressors and do not appear to contain a universal host restriction factor that can explain virologic control. Nevertheless, some EC have been reported to possess polymorphisms in the chemokine receptor CCR5, in particular the delta 32 mutation, that render these patients resistant to efficient infection by HIV and are associated with delayed disease progression (Dean et al., 1996; Meyer et al., 1997; Stewart et al., 1997). However, these polymorphisms are only found in a fraction of all identified EC (Pereyra et al., 2008).

Other groups have focused on the host anti-viral family of APOBEC cytidine deaminases (Chiu and Greene, 2008). One report showed that there was no difference in the frequency of hypermutated proviral clones isolated from EC or HAART-suppressed progressors (Gandhi et al., 2008). In the same study, the Vif gene of HIV, which directly counteracts APOBEC proteins, appeared to be normal in EC, suggesting that APOBEC activity is equivalent between EC and progressors (Gandhi et al., 2008). In summary, no host genetic or anti-viral factors have been identified that explain the low levels of HIV replication in the majority of EC. In light of these findings, many researchers then

suspected that one or more components of the immune system were able to suppress the ability of HIV to replicate in EC.

Role of innate immunity

Some groups turned their attention to the study of the innate immune system, which is the first line of defense against an invading pathogen. One important element of innate immunity is a group of molecules, called toll-like receptors (TLRs), that detect pathogen-associated molecular patterns and initiate inflammatory signals. To date, though, no published studies have linked any TLR polymorphism with the ability to control HIV. Natural killer (NK) cells are another important arm of the innate immune system that help combat viral infection. It has been reported that individuals who demonstrate delayed progression to AIDS are enriched for certain genotypes of NK cell receptors - in particular, the killer immunoglobulin receptors (KIRs) - suggesting that NK cells may play a role in influencing disease outcome in some infected persons (Alter and Altfeld, 2009; Martin et al., 2002; Martin et al., 2007). On the other hand, one study of a small cohort of African American EC showed that previously identified KIRs known to be associated with improved clinical outcome were not overrepresented in the cohort (O'Connell et al., 2009b). Clearly, more work needs to be done to elucidate any potential protective effects of certain KIR alleles expressed by NK cells and the ability to suppress the replication of HIV in EC.

A small number of studies have examined the phenotypic characteristics of circulating NK cells in EC. It has been shown that high HIV viral loads are associated with an increased presence of dysfunctional NK cells lacking surface expression of CD56 but retaining CD16 positivity (Alter et al., 2005; Mavilio et al., 2005). Another study

compared levels of various NK cell subsets, including $CD56^{dim}CD16^{+}$ and $CD56^{-}CD16^{+}$ populations, among EC, progressors, and HAART-suppressed patients (Barker et al., 2007). [Note: The $CD56^{dim}CD16^{+}$ subset has demonstrated the strongest ability to mediate target cell cytotoxicity (Alter et al., 2005; Mavilio et al., 2003).] Interestingly, there was no consistent NK cell phenotypic "signature" to distinguish EC from individuals who had viral load suppressed with HAART (Barker et al., 2007). These results suggest that the magnitude of viral replication may itself cause alterations, either directly or indirectly, in NK cell phenotypic patterns or functional abilities (Alter et al., 2004). Moreover, two functional studies of NK cells showed limited ability to lyse HIV-infected autologous targets in culture, implying that NK cell cytotoxic activity is not an explanation for viral control (Bonaparte and Barker, 2003; O'Connell et al., 2009b). Therefore, while NK cells may play a role in suppressing viral replication in some cases, there is no convincing evidence to link any particular component of innate immunity with EC status.

Role of adaptive immunity

The other major arm of the host response to pathogen challenge is the adaptive immune system. Adaptive immunity typically develops several days or weeks after initial infection and is characterized by the presence of cell types, including B cells, $CD4^{+}$ T cells, and $CD8^{+}$ T cells, that are highly antigen-specific in nature. Various groups have examined each of these cell types in the context of HIV elite control.

Role of B cells and humoral immunity

B cells produce antibodies combat invading pathogens by many mechanisms, including antibody-dependent cell-mediated cytotoxicity (ADCC) or by sterically

hindering the interaction of a pathogen with an uninfected cell in a process called neutralization. Many currently licensed vaccines that prevent viral or bacterial infection rely greatly on the ability of antibodies to neutralize invading pathogens. Unfortunately, it has proved extremely difficult to elicit effective HIV-specific neutralizing antibodies (Mascola and Montefiori, 2010). The envelope (Env) surface protein on HIV virions, which is the target of neutralizing antibodies, is highly variable and also covered by a dense coat of carbohydrate moieties. Therefore, the very nature of the Env glycoprotein and the has hindered efforts to elicit neutralizing antibodies via vaccination.

Recently, investigators have studied the role of neutralizing antibodies in EC. Interestingly, several groups have shown that EC either demonstrate similar or significantly lower titers of neutralizing antibodies against autologous virus, laboratory strains of HIV, or viral isolates from across multiple clades (Bailey et al., 2006a; Doria-Rose et al., 2010; Doria-Rose et al., 2009; Li et al., 2007; Li et al., 2009; Pereyra et al., 2008). Additionally, another group reported that levels of antibody against the CD4 binding site of gp120, the Env transmembrane segment gp41, and Env epitopes near those previously identified for broadly neutralizing antibodies were not different between EC and progressors (Lambotte et al., 2009). In summary, the majority of EC fail to develop broadly neutralizing antibodies, suggesting that they do no play a major role in these individuals to control viral replication. In fact, development of these antibodies may actually be a consequence of viral replication; at least one study has shown a positive correlation between viral load and titers of neutralizing antibodies to a panel of heterologous viral strains (Deeks et al., 2006).

Role of $CD4^+$ T cell responses

$CD4^+$ T cells are a key player in adaptive immunity and are often referred to as helper T cells. They are known to promote effective B cell and $CD8^+$ T cell responses in the setting of infection. However, $CD4^+$ T cells are themselves the major target of HIV. In fact, the gradual loss of these cells due to direct infection or chronic immune activation during HIV infection is likely the primary explanation for the vulnerability of AIDS patients to opportunistic infections (Kalams and Walker, 1998). The examination of HIV-specific $CD4^+$ T cells has been an area of intense study. One group reported that there was no correlation between HIV viral load and total or Gag-specific $CD4^+$ T cell responses. Therefore, many investigators searched for qualitative differences in HIV-specific $CD4^+$ T cells between controllers and progressors.

To date, there have been several studies that have indeed revealed some qualitative and functional differences in $CD4^+$ T cells among HIV-infected individuals that differentially control viral replication. Compared to progressors, HIV-specific $CD4^+$ T cells from EC or patients with nonprogressive infection have been shown to possess higher proliferative capabilities (Boaz et al., 2002; Dyer et al., 2008; Rosenberg et al., 1997; Younes et al., 2003). Some evidence has suggested that the increased ability of HIV-specific $CD4^+$ T cells from EC to proliferate is most likely a consequence of the low viral loads in these subjects (Iyasere et al., 2003; McNeil et al., 2001; Tilton et al., 2007). In terms of functional responses, various groups have reported that a significantly higher percentage of HIV-specific $CD4^+$ T cells from EC are able to simultaneously produce IL-2 and IFN-γ than patients with progressive disease (Emu et al., 2005; Pereyra et al., 2008; Younes et al., 2003). As with the proliferative capacity of HIV-specific $CD4^+$ T cells

described above, at least one study has indicated the ability of HIV-specific $CD4^+$ T cells to secrete IL-2 is linked to viremia. The authors found no difference in IL-2 production in response to HIV antigens in $CD4^+$ T cells from EC compared to patients with undetectable viral load due to HAART (Tilton et al., 2007). Further experiments need to be carried out to replicate these findings in additional cohorts. However, available evidence indicates that the increased proliferative and functional capacities of HIV-specific $CD4^+$ T cells in EC is likely a consequence and not a cause of virologic control.

Role of $CD8^+$ T cell responses

Another important aspect of adaptive immunity, as discussed previously, is the ability of $CD8^+$ T cells to eliminate host cells infected with an intracellular pathogen, such as HIV. In the early years of the AIDS epidemic, it became apparent that HIV-specific $CD8^+$ T cells were present in seropositive individuals but their potential role in combating disease progression was entirely unclear (Walker et al., 1987). Beginning in the mid-1990s, increasing amounts of evidence suggested that HIV-specific $CD8^+$ T cells played an important role in the setting of HIV infection and disease progression.

$CD8^+$ T cells were first shown to influence HIV replication in two studies published in 1994. These studies showed that reduction in peak viremia was temporally associated with the appearance of HIV-specific $CD8^+$ T cells (Borrow et al., 1994; Koup et al., 1994). To confirm and extend these findings, experimental manipulation was carried out in rhesus macaques using the simian immunodeficiency virus (SIV) model. In these studies, anti-CD8 monoclonal antibodies were used to deplete $CD8^+$ T cells *in vivo*. Animals that did not have circulating $CD8^+$ T cells experienced significantly higher viral loads than control monkeys (Jin et al., 1999; Schmitz et al., 1999). Furthermore, in a

small cohort of rhesus macaques that naturally controlled SIVmac239 to low levels, the depletion of $CD8^+$ T cells during the chronic phase of infection resulted in 100- to 10,000-fold increases in viremia (Friedrich et al., 2007). Importantly, when $CD8^+$ T cells eventually returned, virologic control was subsequently reestablished.

A single individual can possess up to six different class I alleles: two from each of the classical MHC class I loci, namely HLA-A, HLA-B, and HLA-C. HIV-positive individuals who are homozygous at any of the three HLA class I loci typically progress more rapidly to AIDS (Carrington et al., 1999). This implies that not only are $CD8^+$ T cell responses important in the containment of HIV but also the breadth of recognized viral epitopes. However, in certain situations, a narrowly focused HIV-specific $CD8^+$ T cell activity can be associated with effective control of viremia over a long period of time (Goulder et al., 1997). EC are also consistently enriched for certain HLA alleles, including HLA-B27 and B57, which further implicates $CD8^+$ T cells in the control of HIV viremia (Emu et al., 2008; Kaslow et al., 1996; Kiepiela et al., 2004; Lambotte et al., 2005; Migueles et al., 2000; Pereyra et al., 2008; Sajadi et al., 2009). Moreover, recent studies by several independent groups have reported that single nucleotide polymorphisms (SNPs) within the MHC locus are consistently associated with lower viral loads (Catano et al., 2008; Dalmasso et al., 2008; Fellay et al., 2007; Limou et al., 2009; Shrestha et al., 2009). Due to the fact that HLA class I molecules present pathogen-associated epitopes to $CD8^+$ T cells, this evidence concerning the association between certain MHC molecules and viral control supports a role for $CD8^+$ T cells in the setting of HIV infection.

The other major piece of evidence concerning the significance of HIV-specific $CD8^+$ T cells relates to the ability of the virus to escape from immune-mediated pressure. The first unambiguous examples of $CD8^+$ T cell escape in natural infection with HIV were described in 1997 (Borrow et al., 1997; Goulder et al., 1997; Price et al., 1997). A large body of literature has since shown that mutations in the viral genome during infection are concentrated within $CD8^+$ T cell epitopes (Evans et al., 1999; Goulder and Watkins, 2004). Notably, HIV-specific $CD8^+$ T cells begin to place pressure on HIV immediately after their appearance during acute infection (Fischer et al., 2010; Goonetilleke et al., 2009). Many of these escape mutations confer negative fitness costs on HIV (Troyer et al., 2009) and have been shown to revert to wild-type when transmitted to HLA mismatched persons (Leslie et al., 2004). Therefore, HIV continually attempts to escape from $CD8^+$ T cell-mediated pressure, even with large fitness costs in some instances, further suggesting a critical role for $CD8^+$ T cells in combating HIV.

Many studies to date have shown that the magnitude of HIV-specific $CD8^+$ T cell responses is largely the same in EC compared to patients with detectable levels of viremia (Addo et al., 2003; Betts et al., 2001; Emu et al., 2008; Gea-Banacloche et al., 2000; Hersperger et al., 2010; Migueles et al., 2002). Therefore, it is clear that the total frequency of HIV-specific $CD8^+$ T cells within EC and progressors cannot explain the disparity in viral control between these groups. In fact, a growing number of recent studies have discovered important qualitative features of HIV-specific $CD8^+$ T cells that distinguish EC from progressors.

EC have been shown to contain a higher fraction of HIV-specific $CD8^+$ T cells that can degranulate, produce multiple cytokines and chemokines in addition to

displaying markedly better proliferative potential upon HIV peptide stimulation than individuals with progressive disease (Almeida et al., 2007; Betts et al., 2006; Boaz et al., 2002; Emu et al., 2008; Horton et al., 2006; Migueles et al., 2002; Pereyra et al., 2008; Zimmerli et al., 2005). While all of these studies examined cells taken from peripheral blood, HIV-specific $CD8^+$ T cells isolated from mucosal tissues were also shown to be more polyfunctional in EC than progressors (Ferre et al., 2009). Additionally, a series of recently published studies have shown that HIV-specific $CD8^+$ T cells from EC demonstrate enhanced cytotoxic functionality compared to progressors: $CD8^+$ T cells from EC have a superior ability to suppress the replication of HIV in autologous $CD4^+$ T cells during extended culture (O'Connell et al., 2009b; Saez-Cirion et al., 2007). $CD8^+$ T cells from EC expanded *in vitro* following HIV-specific stimulation demonstrate enhanced upregulation of perforin and granzyme B after proliferation (Migueles et al., 2002; Migueles et al., 2008), which translates into a greater capacity to induce target cell death on a per cell basis (Migueles et al., 2008). EC also express higher levels of perforin immediately after antigen recognition, resulting in a greater *ex vivo* cytotoxic potential (Hersperger et al., 2010). [The details of this study are the focus of Chapter 3.] Collectively, these findings suggest that $CD8^+$ T cells can play a key role in the control of HIV replication, particularly within EC.

The superior functional and cytotoxic capabilities of HIV-specific $CD8^+$ T cells among EC most likely affect viral fitness within these individuals through the suppression of viral replication and the desire of HIV to escape this pressure. This may explain why some groups have previously found defective or less fit virus from EC compared to progressors (Lassen et al., 2009; Miura et al., 2009a; Miura et al., 2009b).

Indeed, viral isolates from a small number of EC that were identified during primary infection showed lower replication capacities than isolates from persons who failed to control acute viremia (Miura et al., 2010). The authors showed that the reduced replication capacity of the isolates from EC was associated with transmitted or acquired $CD8^+$ T cell escape mutations in many of the controllers.

While many studies suggest that HIV-specific $CD8^+$ T cells play a critical role in suppressing HIV replication, it is also clear that they do not completely explain elite control in every situation. For example, some EC have very low frequencies of HIV-specific $CD8^+$ T cells or otherwise fail to display any functional ability previously identified as a correlate of control, such as cytotoxic potential or polyfunctionality (Emu et al., 2008; Hersperger et al., 2010; Saez-Cirion et al., 2009). More work needs to be carried out on acutely-infected individuals that are followed longitudinally. The earliest events during primary infection most likely play a pivotal role in determining later levels of viral replication. Nevertheless, it is apparent that HIV-specific $CD8^+$ T cells can play a pivotal role in controlling HIV during the chronic phase of infection.

Measuring immune responses using multiparametric flow cytometry

Flow cytometry is one of the most powerful and widely used technologies in the field of Immunology. This technology allows for the rapid analysis of numerous cells on a cell-by-cell basis and even permits viable cells to be separated (termed "cell sorting") from a mixture of many cell types into a highly purified population. Flow cytometry was first developed in the late 1960s at Stanford University (Hulett et al., 1969). Initial instruments measured just three parameters (one fluorescence signal and two signals

based on a cell's light scatter properties); however, technological advances eventually allowed for the simultaneous detection of six parameters by the 1980s and now approximately 19 fluorescent and physical parameters at present, depending on the configuration of the cytometer (De Rosa et al., 2001; Perfetto et al., 2004).

Currently, the technique of labeling and characterizing populations of cells by flow cytometry is limited to the visible spectrum of electromagnetic radiation. The technique involves labeling cells with both synthetically and naturally occurring molecules that emit light at a defined wavelength after excitement by the energy from a laser. Importantly, these fluorescent molecules can be covalently conjugated to monoclonal antibodies that specifically bind to and label molecules of interest (i.e. CD3, CD4, CD8, etc.). The ability of multiparametric (otherwise known as polychromatic) flow cytometry to measure up to 19 parameters relied upon advancements in the development of various fluorescent molecules. For example, "quantum dots", which are inorganic crystals of cadmium selenide, have exceptionally narrow and symmetrical emission spectra, which means they can be multiplexed with minimal compensation concerns that arise from spectral overlap (Chattopadhyay et al., 2006; Roederer, 2002).

Until the advent of multiparametric flow cytometry, most researches were limited in their ability to assess immune cell function and phenotype in any particular experiment. For example, researchers could quantify antigen-specific T cells using limiting dilution assays or the secretion of IFN-γ by means of an ELISPOT, but they were largely unable to simultaneously measure numerous parameters in the same experiment. Polychromatic flow cytometry has made strides to overcome this obstacle and been able to reveal the complexity of the immune system in a level of detail not previously

achieved (De Rosa and Roederer, 2001). One of the most important contributions of this technology has been in the measurement of immune responses against various pathogens (Betts et al., 2006; Makedonas and Betts, 2006) and in the assessment of vaccination strategies (Roederer et al., 2004; Seder et al., 2008).

As stated previously, the cytokine IFN-γ has historically been used as the sole marker for antigen-specific T cell activity. However, with the development of multiparametric flow cytometry, many researchers began to postulate that more information might be garnered about an immune response by measuring more than just IFN-γ secretion. At the same time, it was necessary to block the release of newly produced cytokines after cellular activation so that each cytokine-producing cell could be identified by flow cytometry (Jung et al., 1993). In these intracellular cytokine staining (ICS) assays, two main chemical inhibitors, monensin (Mellman et al., 1986; Tartakoff, 1983) and Brefeldin A (BFA) (Klausner et al., 1992) were employed to inhibit the secretion of a substantial fraction of newly produced proteins. The use of ICS assays in conjunction with polychromatic flow cytometry has uncovered a large degree of functional complexity that exists for a range of immune responses (Picker et al., 1995).

An illustration of the functional complexity revealed by polychromatic flow cytometry is shown in **Figure 1**. In this example, human peripheral blood mononuclear cells (PBMC) were stimulated in the context of a standard ICS assay with Staphylococcal enterotoxin B (SEB) for six hours in the presence of BFA. As shown in **Figure 1A**, 12% of $CD8^+$ T cell in this subject produced the cytokine IFN-γ after activation by SEB. As a comparison, less than 0.5% of total $CD8^+$ T cells were positive for IFN-γ in the no stimulation control under the same conditions (data not shown). The levels of three other

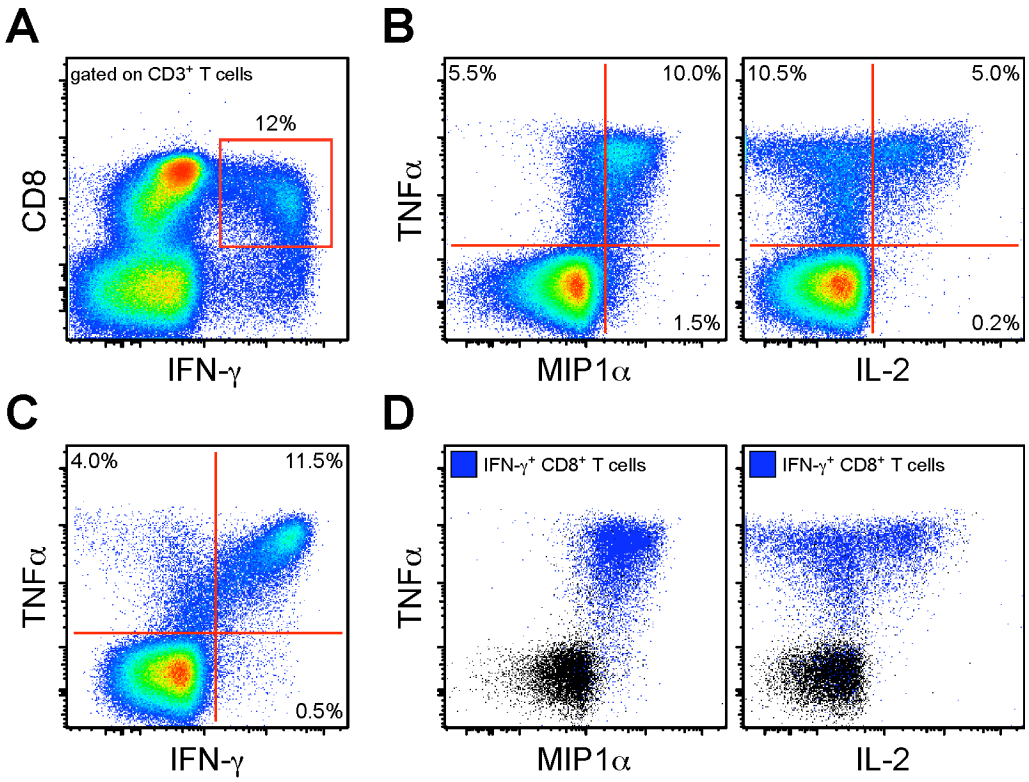
functional markers, TNFα, IL-2 and MIP1α, were also measured within the same experiment using polychromatic flow cytometry. These molecules were induced to varying degrees by SEB (**Fig. 1B**). Interestingly, 15.5% of $CD8^+$ T cells were induced to produce TNFα while only 5.2% upregulated IL-2. Therefore, the total magnitude of the SEB-induced $CD8^+$ T cell response would have been underestimated had IFN-γ or IL-2 been the only functional markers examined. These data also reveal that most T cell responses are quite complex. For instance, not all $TNF\alpha^+$ cells were also positive for IFN-γ and vice versa (**Fig. 1C**). Additionally, even though virtually all $IFN\text{-}\gamma^+$ cells co-expressed MIP1α, the appearance of IL-2 within $IFN\text{-}\gamma^+$ cells was quite variable (**Fig. 1D**). In summary, multiparametric flow cytometry allowed for the simultaneous detection of numerous functional parameters, which revealed that T cell responses can be quite heterogeneous and that many sub-populations of functional cells exist.

A number of flow cytometric-based assays have also been developed that can detect many other immune cell processes and functional capabilities besides the production of cytokines or chemokines. For example, researchers can now quantify *in vitro* target cell killing (Casazza et al., 2006; Lecoeur et al., 2001), directly label antigen-specific $CD8^+$ T cells by peptide:MHC-I tetramers (Altman et al., 1996), or directly assess degranulation by measuring surface expression of CD107a following activation (Betts et al., 2003). Together with this array of novel assays, polychromatic flow cytometry has proved beneficial in many clinically relevant settings, including oncology (Lee et al., 1999) and vaccine development (De Rosa et al., 2004; Precopio et al., 2007). The utility of multiparametric flow cytometry has also allowed researchers to identify correlates of immune protection in the setting of infection, assess therapeutic efficacy,

and facilitate clinical diagnoses. Therefore, this important technology has progressed a great deal since its initial development four decades ago and will undoubtedly continue to impact basic research and human health for decades to come.

Conclusion and Project Aims

While EC are a rare subset of all infected individuals, they do provide hope that durable immune-mediated control of HIV viremia can be achieved through vaccination. Not only do these patients control HIV viral loads to extremely low levels but they also rarely progress to AIDS even after many years of infection. A growing body of evidence points to HIV-specific $CD8^+$ T cells as the foremost means by which HIV replication is curtailed in EC. Before this project began, there was no *ex vivo* functional parameter with known cytolytic ability that could distinguish HIV-specific $CD8^+$ T cells in EC from individuals with progressive disease. Therefore, the primary goal of the project was to perform a detailed characterization of granule-resident cytolytic protein expression within *ex vivo* HIV-specific $CD8^+$ T cells between patient groups that differentially control viral replication off therapy. A secondary aim of this work was to study the expression of the transcription factor T-bet, which has been shown previously to influence effector differentiation and the expression of perforin and granzyme B, within HIV-specific $CD8^+$ T cells between EC and progressors. The hope is that the results from this work will increase our understanding of the effector capacities of human anti-viral $CD8^+$ T cells and perhaps also be beneficial in the monitoring of vaccine platforms to combat HIV or other pathogens.



<u>Figure 1</u>: T cell responses are heterogeneous and multi-faceted. PBMC from a healthy HIV-negative subject were stimulated for 6 hours with SEB in a standard ICS assay. BFA was added just prior to stimulation in order to inhibit secretion of newly synthesized proteins. **(A)** Events shown have been gated on all $CD3^+$ T cells. As indicated, 12% of $CD8^+$ T cells produced IFN-γ after stimulation. **(B and C)** Events shown have been gated on $CD3^+CD8^+$ T cells. Percentages represent the fraction of total $CD8^+$ T cells within each quadrant. **(D)** All IFN-γ^+ $CD8^+$ T cells (blue events) as seen in (A) were overlaid onto a dot plot (black events) of all $CD8^+$ T cells.

CHAPTER 2

IDENTIFICATION AND ASSESSMENT OF *DE NOVO* PERFORIN PRODUCTION BY HUMAN CD8$^+$ T CELLS USING POLYCHROMATIC FLOW CYTOMETRY

Summary

Perforin and granzymes work synergistically to induce apoptosis in target cells recognized by CD8$^+$ T cells. While perforin protein is readily detectable in resting CD8$^+$ T cells, its upregulation in activated cells has been reported to require cellular division. However, perforin undergoes numerous conformational changes during its maturation, which may affect the ability of conventional antibodies to recognize newly synthesized perforin. Here, polychromatic flow cytometry and standard intracellular cytokine staining assays were used to detect perforin and cytokine production following stimulation of *ex vivo* human CD8$^+$ T cells. Two different anti-perforin antibodies, clones B-D48 and δG9, were used to discriminate various forms of perforin after cellular activation. We provide evidence that antigen-specific CD8$^+$ T cells can rapidly upregulate perforin protein within hours of stimulation, thus defining a novel cytotoxic capability of human CD8$^+$ T cells. The δG9 clone most likely recognizes only the granule-associated conformation of perforin, while the B-D48 clone is able to detect perforin in multiple forms. Collectively, our results show that human CD8$^+$ T cells are capable of rapid perforin production and define a novel flow cytometric procedure that can be used to more completely assess the cytotoxic potential of human CD8$^+$ T cells.

Introduction

Perforin is synthesized as a 70 kDa precursor with two glycosylation sites. It is equipped with a C-terminal C2 domain capable of binding phospholipid membranes in a calcium-dependent manner (Voskoboinik et al., 2005). The precursor is later cleaved at the carboxy terminus, which exposes the C2 domain, to yield the active 60 kDa form of perforin (Uellner et al., 1997). The low pH of the cytotoxic granule, along with a dearth of available calcium ions, favors stable interactions with resident proteoglycans, thereby neutralizing the action of perforin (Masson et al., 1990). In fact, the long-term stability of perforin in the granules seems to require an acidic environment (Kataoka et al., 1994; Kataoka et al., 1997). Overall, it is important to consider that the conformation of perforin is very different in the endoplasmic reticulum (ER) shortly after synthesis compared to when it resides in its mature form in the cytotoxic granule.

While most of our understanding of perforin is derived from experiments designed to study its release and aftereffects, very little is known about perforin upregulation and regeneration after activation. Granule-mediated killing occurs within minutes to hours of target cell recognition, but following activation the reconstitution or upregulation of intracellular perforin has only been detected after cellular proliferation (Meng et al., 2006; Migueles et al., 2002; Sandberg et al., 2001). Therefore, the measurement of perforin as a marker of the antiviral capacity of $CD8^+$ T cells has largely been limited to staining for baseline levels in unstimulated cells (Zhang et al., 2003). Historically, human perforin has been detected by means of an antibody (clone δG9) that was made by using purified $CD8^+$ T cell granules as an immunogen in mice (Hameed et al., 1992). By virtue of an antibody (clone B-D48) that was raised against recombinant

perforin, its *de novo* synthesis can, in fact, be detected by flow cytometry using six hour intracellular cytokine-staining (ICS) assays. Our results demonstrate for the first time that $CD8^+$ T cells can rapidly upregulate perforin following target cell recognition without the need for cellular proliferation.

Materials and Methods

<u>*Human Subjects*</u>: Donor peripheral blood mononuclear cells (PBMC) were obtained from the University of Pennsylvania's Center for AIDS Research Human Immunology Core and from the lab of Dr. Guido Ferrari of Duke University, in compliance with the guidelines set by the internal review boards of both institutions. We identified two donors who exhibited strong responses to human cytomegalovirus (HCMV) antigens. ND172 responded to the HLA-B7-restricted pp65 epitope TPRVTGGGAM (TM10; 4200 spot forming cells, SFC, per 10^6 PBMC). ND171 also responded to peptide TM10 (1500 SFC/10^6 PBMC) in addition to the HLA-B18-restricted IE1 epitope SDEEEAIVAYTL (SL12; 3000 SFC/10^6 PBMC). PBMC from donors ND168, ND191, ND200, and BC75 were also used in this study but were not epitope mapped. PBMC from all subjects were cryopreserved in fetal bovine serum (FBS; ICS Hyclone, Logan, Utah) containing 10% dimethyl sulfoxide (DMSO; Fisher Scientific, Pittsburgh, Pennsylvania) for later use.

<u>*Antibodies*</u>: The following antibodies were used in these studies: anti-CD4 PE Cy5.5, anti-CD19 Pacific Blue (Invitrogen, Carlsbad, California), anti-CD14 Pacific Blue, anti-CD16 Pacific Blue, anti-CD107a FITC (BD Biosciences, San Jose, California), anti-Granzyme B PE (eBioscience, San Diego, California), anti-TNFα PE Cy7, anti-IFN-γ

Alexa 700, anti-Granzyme B Texas Red PE (BD Pharmingen), anti-CD3 Qdot 585, and anti-CD8$^+$ Qdot 655 (custom). Custom conjugations to Quantum (Q) dot nanocrystals were performed in our laboratory with reagents purchased from Invitrogen. Anti-human perforin antibodies were purchased from Diaclone (clone B-D48, Besancon, France) and BD Biosciences (clone δG9). It should be noted that for the remainder of this chapter we will refer to the perforin clone purchased from Diaclone as "D48" so as not to cause confusion with the δG9 clone purchased from BD Biosciences.

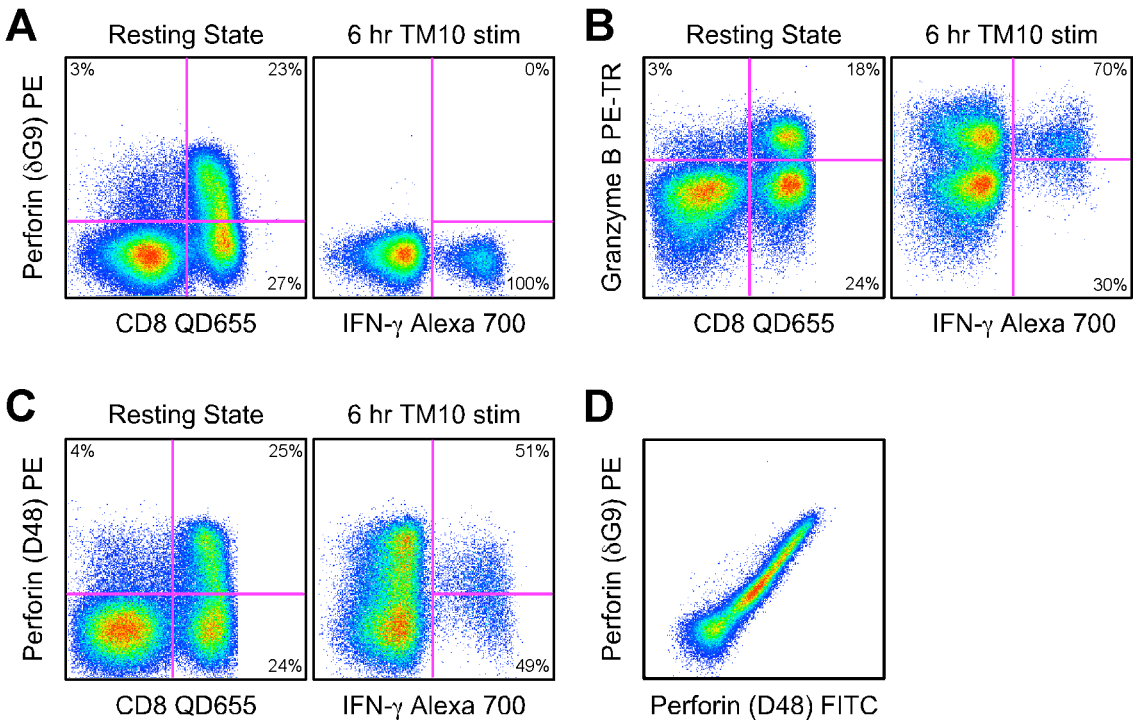
Flow cytometric staining assays: Standard intracellular cytokine stimulation, CD107a assay, and staining procedures were performed as published previously (Betts et al., 2003; Betts et al., 2006; Chattopadhyay et al., 2006). During some experiments, we stained cells with APC-labeled HLA-B7/TM10 tetramers in calcium-free PBS for 15 minutes on ice and in the dark. Monensin (1 μg/mL final concentration) was used for inhibition of cytotoxic granule acidification where indicated.

Flow cytometric analysis: For each tube, between 500,000 and 1,000,000 total events were acquired on a modified flow cytometer (LSRII; BD Immunocytometry Systems, San Jose, California) equipped for the detection of 18 fluorescent parameters. Antibody capture beads (BD Biosciences) were used to prepare individual compensation tubes for each antibody. Data analysis was performed using FlowJo (version 8.5.2; TreeStar, Ashland, Oregon). Spice (version 4.1.6, Dr. Mario Roederer, NIH, Bethesda, Maryland) was used to examine the functional profiles of certain CD8$^+$ T cell responses. Reported data have been corrected for background (no stim condition) when appropriate.

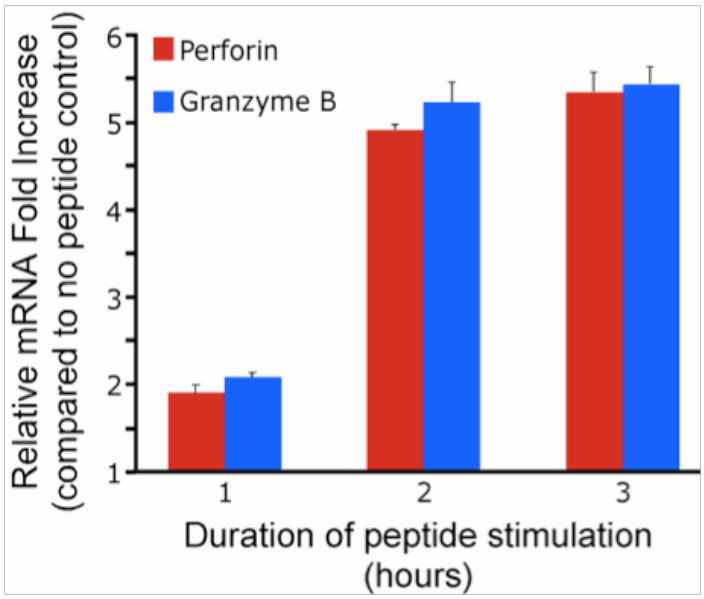
Results

Activated CD8$^+$ T cells rapidly upregulate perforin and granzyme B mRNA and protein

It is well established that CD8$^+$ T cells release perforin-containing granules toward the immunological synapse upon recognition of target cells (Stinchcombe et al., 2001). Consequently, activated CD8$^+$ T cells producing cytokine appear to be perforin-negative after six hours of stimulation in a standard ICS assay (**Fig. 2A)**, suggesting that CD8$^+$ T cells cannot immediately upregulate perforin after their preformed stores are lost after stimulation. This finding has been further substantiated by studies showing upregulation of new perforin only after cell division (Meng et al., 2006; Migueles et al., 2002; Sandberg et al., 2001). Interestingly, it has been previously reported that increased levels of perforin mRNA can be detected shortly after nonspecific activation of human CD8$^+$ T cells (Lu et al., 1992). To determine whether this occurs in an antigen-specific manner, we stimulated PBMC from ND172 with the TM10 peptide and analyzed perforin and granzyme B transcript levels by real time RT-PCR. As shown in **Figure 3**, relative amounts of both perforin and granzyme B mRNA increased roughly five-fold after only two hours of stimulation with TM10. Consistent with the upregulation of granzyme B mRNA, we observed that granzyme B protein expression could be detected in responding cells following six hours of TM10 stimulation (**Fig. 2B**).



<u>Figure 2</u>: The D48 anti-perforin clone detects *de novo* perforin synthesis. (A-C) *Left*: Resting PBMC from ND172 were stained with anti-perforin or granzyme B antibodies. Events shown are gated upon $CD3^+$ cells, and percentages represent the frequency of total $CD3^+$ cells. *Right*: PBMC were stimulated with peptide TM10 for six hours in the presence of BFA and monensin. Events shown are gated upon $CD3^+CD8^+$ cells, and percentages represent the proportion of IFN-γ^+ cells that stain either positive or negative for perforin or granzyme B. **(D)** Both anti-perforin monoclonal antibodies detect the same amount of perforin in resting $CD8^+$ T cells. Resting PBMC from ND172 were co-stained using both antibodies. The δG9 clone was conjugated to PE and D48 to FITC. Events shown are gated upon $CD3^+CD8^+$ cells.



<u>Figure 3</u>: Perforin and granzyme B transcript levels are increased following antigen-specific stimulation of human CD8^{+} T cells. PBMC from ND172 were stimulated with TM10 peptide for 1, 2 or 3 hours. Total RNA was isolated at each time point using the RNAqueous-4PCR kit (Ambion, Austin, Texas) according to the manufacturer's instructions. Reverse transcription of total RNA into cDNA was accomplished using the RETROscript kit (Ambion) with random decamers and MMLV-RT enzyme. Primers to identify and amplify perforin and granzyme B transcripts were designed using Primer Express (Perkin-Elmer software) and received from Applied Biosystems [forward perforin primer: ACGGTGGAGTGCCGCTTCTA; reverse perforin primer: GCCCTCTTGAAGTCAGGGTG; forward granzyme B primer: GGCTCCTGTTCCTTGATATTGTG; reverse granzyme B primer: CGTGCTGACAGCTGCTCACT]. Transcripts were measured on a 7500 Fast Real-time PCR system (Applied Biosystems) through use of TaqMan probes [perforin probe: ATTTCCATGTGGTACACACTCCCC; granzyme B probe: CAAGGTGACATTTATGGAGCTTCCCCAA]. For each sample, mRNA abundance was normalized to GAPDH and expressed as relative fold change from the no peptide control using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Each condition was run in triplicate, and the Dunnett's t-test was used to compare them to the no peptide control.

Therefore, we could detect *de novo* synthesis of granzyme B protein but not perforin, even though mRNA for both proteins was upregulated to a similar extent immediately following activation (**Fig. 3**). Although a block in the translation of perforin mRNA is a possibility, Isaaz and colleagues previously demonstrated that new perforin is secreted by $CD8^+$ T cell clones after a short stimulation period (Isaaz et al., 1995). Furthermore, perforin undergoes several post-translational modifications, including glycosylation and cleavage, prior to and after sequestration within the cytotoxic granule (Russell and Ley, 2002; Uellner et al., 1997). Importantly, the antibody we were employing to detect perforin (clone δG9) was raised against granule-associated perforin (Hameed et al., 1992).

Therefore, we hypothesized that the inability to detect perforin upregulation by flow cytometry may be an artifact that arises because newly produced perforin - trapped in an ER/Golgi compartment by secretion inhibitors in ICS assays - fails to achieve the conformation characteristic of granule-associated perforin. We tested a novel perforin antibody [clone D48 (Diaclone; Besancon, France)] that was raised against a recombinant form of perforin. Using this antibody, we could readily detect perforin expression within activated, IFN-γ producing $CD8^+$ T cells after six hours of peptide TM10 stimulation under identical assay conditions (**Fig. 2C**). Additionally, co-staining with both perforin clones resulted in a one-to-one relationship in resting $CD8^+$ T cells (**Fig. 2D**), suggesting that both clones can equally recognize perforin in the resting state but not after $CD8^+$ T cells have been activated. These results demonstrated that human $CD8^+$ T cells can indeed upregulate new perforin protein upon T cell receptor (TCR) stimulation, which can be detected by flow cytometry.

Kinetics of perforin upregulation in activated CD8$^+$ T cells

In order to define the temporal aspects of perforin upregulation, we stimulated PBMC from ND172 with peptide TM10 for various periods of time and subsequently stained for perforin using the D48 clone together with TNFα and IFN-γ (**Fig. 4A**). Concomitant to cytokine production, perforin upregulation was noted within some activated cells and peaked after eight hours. It can be seen that not all cytokine producing cells upregulate perforin to an equal extent, which is most likely attributed to the differential ability of sub-populations of responding cells to upregulate perforin. To demonstrate that we were measuring newly produced protein rather than residual, unreleased perforin within cytokine producing cells, we also stained the cells during stimulation with anti-CD107a antibodies to assess degranulation (Betts et al., 2003). After one hour of stimulation, the majority of degranulating cells were perforin-negative (**Fig. 4A**). These cells have presumably released their preformed perforin but have not yet begun new perforin production. Notably, perforin gradually appeared in the degranulating population of CD8$^+$ T cells over time, peaking at approximately eight hours. The increase in the proportion of degranulating cells that also co-expressed perforin indicates that some of the responding CD8$^+$ T cells were upregulating perforin. This concept is also depicted in **Figure 4B**, which shows degranulation and total perforin levels at various times after TM10 stimulation in bulk CD8$^+$ T cells. Initially, as responding cells degranulated, the amount of perforin in the total CD8$^+$ T cell population decreases. However, the level of perforin gradually returns to baseline over time while the frequency of degranulating cells remains relatively constant. Taken together, these data indicate that human CD8$^+$ T cells are capable of rapidly upregulating perforin in activated cells.

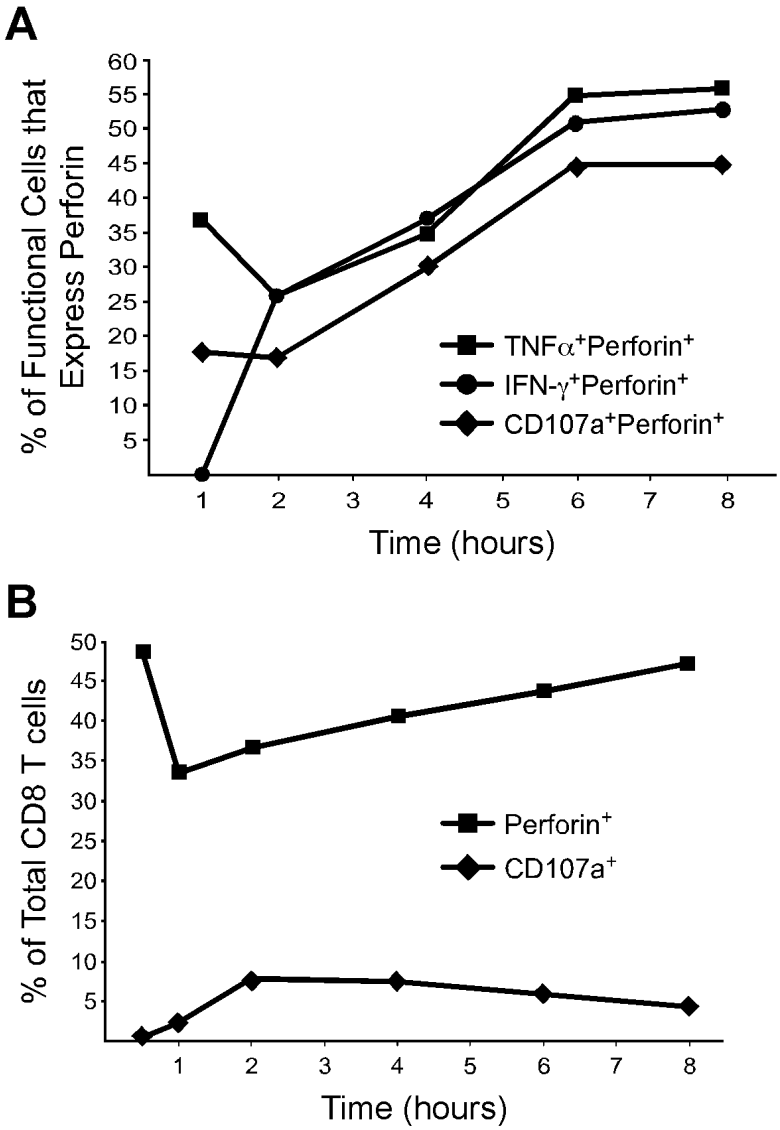
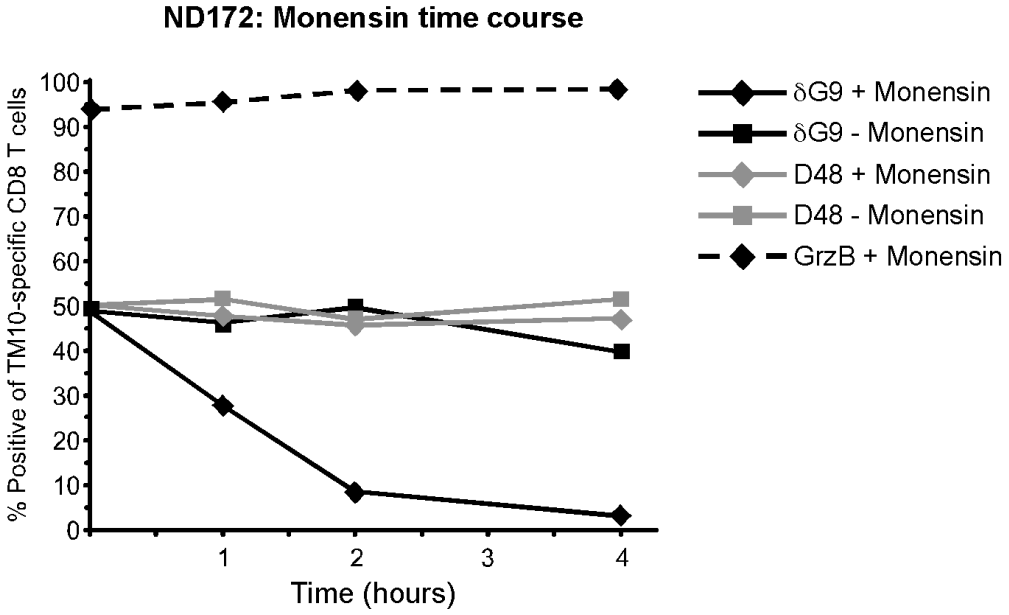


Figure 4: Activated CD8$^+$ T cells rapidly upregulate perforin simultaneous with cytokine production and degranulation. (A) Evolution of the antigen-specific perforin response over time. PBMC from ND172 were stimulated with peptide TM10 in the presence of BFA and monensin for various periods of time and stained for perforin (D48 clone) in conjunction with TNFα (squares), IFN-γ (circles), and CD107a (diamonds). The proportions of TNFα$^+$, IFN-γ$^+$, or CD107a$^+$ cells (CD3$^+$CD8$^+$) that also stained positive for perforin were plotted over time. **(B)** Following activation, total perforin levels recover after an initial decline. PBMC from ND172 were stimulated with peptide TM10 in the presence of BFA and monensin for various periods of time and stained for perforin (D48 clone; squares) and CD107a (diamonds). The graph depicts the frequency of positivity for each molecule in the total CD8$^+$ T cell population.

The D48 clone recognizes more conformations of perforin than δG9

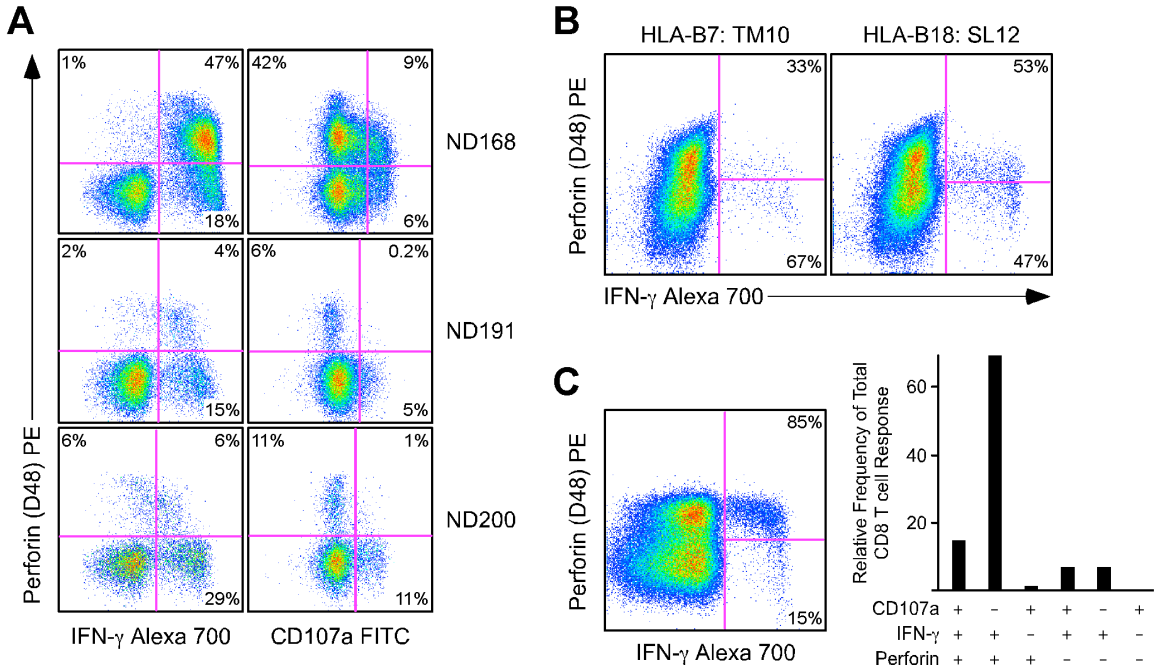
To further characterize the differences between the antibody clones, we made use of an inhibitor that prevents granule acidification. It is known that perforin is inactive at the acidic pH of the cytotoxic granule (Pipkin and Lieberman, 2007). Presumably, the conformation of perforin can vary substantially depending upon the pH of its surrounding environment. Monensin, a carboxylic ionophore specific for monovalent cations, elevates vacuolar pH by intercalating into membranes and exchanging protons for potassium ions (Mellman et al., 1986; Tartakoff, 1983). **Figure 5** shows the effect of monensin on the ability of the two clones to recognize perforin in the resting, TM10-specific population of $CD8^+$ T cells in ND172 as identified by tetramer labeling. After just two hours of monensin treatment, there was a considerable reduction in the amount of detectable perforin using the δG9 clone. However, perforin was actually still present in these cells because staining by the D48 clone remained constant, suggesting that the δG9 clone recognizes a conformationally dependent epitope that is altered by a pH increase. Additionally, this phenomenon likely accounts for the disappearance of perforin in the non-responding population of $CD8^+$ T cells in **Figure 2A**, as monensin was included there. Indeed, if BFA alone is used in an ICS assay using the δG9 clone, only limited numbers of IFN-γ producing cells are perforin-positive but the presence of perforin is maintained in the non-responding population of $CD8^+$ T cells (data not shown).



<u>Figure 5</u>: The D48 clone recognizes more conformations of perforin than δG9. PBMC from ND172 were either left untreated (squares) or incubated with respective inhibitor (diamonds) for various lengths of time. Cells were subsequently stained for granzyme B (GrzB, dashed black) or perforin using either the D48 (solid gray) or δG9 clone (solid black) at each time point. The graph depicts the frequency of positivity for each molecule in the TM10-specific population identified by tetramer staining.

Inter- and intra-subject variability in the ability of CD8$^+$ T cells to upregulate perforin

We next assessed whether perforin upregulation is a common feature of all CD8$^+$ T cell responses. **Figure 6A** demonstrates that the ability of CD8$^+$ T cells to upregulate perforin following stimulation with PMA/Ionomycin is highly variable. PBMC from three different normal donors were stimulated for six hours and perforin upregulation was measured in conjunction with other functional parameters. A significant fraction of responding, IFN-γ-positive cells upregulated perforin in ND168, whereas smaller amounts of new perforin were measured in ND191 and ND200. There can also be variability in antigen-specific perforin upregulation within an individual donor. As shown in **Figure 6B**, donor ND171, who responds to two different HCMV epitopes, displayed a differential ability to upregulate perforin. The TM10 peptide induced 33% of the responding cells to upregulate perforin, whereas the SL12 peptide induced 53% of IFN-γ producing cells to express perforin. Additionally, we identified one subject that displayed a considerable ability to upregulate perforin after stimulation with a pool of HCMV, EBV, and Flu peptides (**Fig. 6C, *left***). However, a closer inspection of this response revealed that more than 60% of the activated cells failed to degranulate (**Fig. 6C, *right***), suggesting the seemingly large upregulation of perforin was actually a result of many of the activated cells not releasing their preformed perforin via degranulation. Nevertheless, some of the cells stained positive for CD107a while remaining perforin-positive after six hours, suggesting that this sub-population of cells did express new perforin after stimulation. This points to the importance of measuring degranulation concurrently with perforin in order to more accurately assess *de novo* perforin synthesis.



<u>Figure 6</u>: CD8$^+$ T cells demonstrate variable capacity to upregulate perforin. (A) There is inter-subject variability in the ability of CD8$^+$ T cells to upregulate perforin. PBMC from three separate healthy donors were stimulated with PMA/Ionomycin for six hours in the presence of BFA and monensin. Events shown are gated upon CD3$^+$CD8$^+$ cells. Percentages represent the frequency of total CD8$^+$ T cells. **(B)** There is intra-subject variability in the ability of CD8$^+$ T cells to upregulate perforin. PBMC from ND171 were stimulated with peptides TM10 (*left*) or SL12 (*right*) for six hours in the presence of BFA. Events shown are gated upon CD3$^+$CD8$^+$ cells. Percentages represent the proportion of IFN-γ$^+$ cells that stain either positive or negative for perforin. **(C)** Some antigen-specific responses appear to upregulate large amounts of perforin. PBMC from normal donor BC75 were stimulated with a pool of HCMV, EBV, and Flu peptides for six hours in the presence of BFA and monensin. *Left*: Events shown are gated upon CD3$^+$CD8$^+$ cells. Percentages represent the proportion of IFN-γ$^+$ cells that stain either positive or negative for perforin. *Right*: The entire response, based on the markers CD107a, IFN-γ, and perforin was broken down into the relative contribution of each functional combination. The triple-negative and perforin single-positive combinations were ignored.

Discussion

$CD8^+$ T cells play a crucial role in the elimination of tumors or host cells infected by intracellular pathogens. Perforin is the key player in the arsenal that $CD8^+$ T cells use to eliminate target cells; it is indispensable for the delivery of granzymes to their pro-apoptotic substrates within targeted cells. The mechanism for this perforin-mediated killing has been solely attributed to the exocytosis of cytotoxic granules present within $CD8^+$ T cells. Based on previous results, however, it has been thought that $CD8^+$ T cells replenish their store of perforin only after cellular proliferation (Meng et al., 2006; Migueles et al., 2002; Sandberg et al., 2001). Here we redefine this mechanism, demonstrating that antigen-specific $CD8^+$ T cells can rapidly upregulate perforin after activation, which we have also shown potentiates the ability of $CD8^+$ T cells to recognize and kill targets after the initial depletion of preformed granules (Makedonas et al., 2009). If not for the ability to rapidly upregulate cytolytic effector molecules, $CD8^+$ T cells would presumably have a substantial period of time after initial recognition of targets before they could recover the ability to kill additional infected cells.

Although increased perforin gene expression has been observed previously following activation (Lu et al., 1992), there has been a consistent failure to detect *de novo* synthesis of perforin by flow cytometry in human $CD8^+$ T cells. However, previous studies of perforin expression in humans have employed an antibody (δG9 clone) that was raised against intact, cytotoxic granules (Hameed et al., 1992), thereby unknowingly restricting the study of perforin to its granule-associated form. Using an anti-perforin antibody (D48 clone), which was developed using recombinant human perforin, we can now directly identify cells that have synthesized new perforin. We find that *ex vivo* $CD8^+$

T cells can initiate perforin production shortly after TCR stimulation. The kinetics of perforin upregulation parallel that of various cytokines (IFN-γ and TNFα) produced by responding $CD8^+$ T cells. By measuring the surface expression of CD107a as a marker of degranulation (Betts et al., 2003), we find that the observed upregulation of perforin was not simply a result of residual, unreleased perforin within granules. Within the first hour of activation, $CD8^+$ T cells degranulate and consequently appear perforin-negative. However, coincident with the upregulation of perforin mRNA, perforin protein begins to be detectable within CD107a-positive cells after two hours.

We made use of monensin to further characterize the two different anti-perforin clones. Given that this molecule is an ionophore that intercalates into membranes and mediates the passive exchange of monovalent cations, its effects on vacuolar pH are almost immediate. Indeed, directly after the addition of monensin, we observed a decline in the ability of the δG9 clone to detect perforin in resting $CD8^+$ T cells. However, the perforin signal was not lost because of degradation as the D48 clone was able to detect equivalent levels of perforin in the presence or absence of monensin. This result is interesting because prior work has shown that the long-term stability of perforin most likely requires an acidic environment (Kataoka et al., 1994; Kataoka et al., 1997). Most of the proteolytic enzymes in the cytotoxic granules, which are secretory lysosomes, require an acidic environment to be fully active. The rise in granule pH caused by the action of monensin probably occurs quickly enough to protect perforin from proteolytic degradation. Additionally, it is known that perforin binds to the proteoglycan serglycin in cytotoxic granules (Masson et al., 1990; Metkar et al., 2002) and increases in pH disrupt this association (Masson et al., 1990; Persechini et al., 1989). It is these interactions with

granule proteoglycans that might prevent perforin degradation by proteolytic enzymes found with cytotoxic granules under normal conditions.

Overall, these data suggest that the δG9 clone is limited to detecting a form of perforin either found only in the acidic milieu of cytotoxic granules or a conformation that is dependent on an interaction with serglycin. The D48 clone, on the other hand, is able to recognize both mature perforin as well as its newly synthesized form. Other groups have previously been unable to detect perforin upregulation because the δG9 clone cannot recognize perforin trapped in an ER/Golgi compartment by secretion inhibitors in ICS assays.

The fact that we do not detect perforin upregulation in every antigen-specific $CD8^+$ T cell response is noteworthy. For example, in donor ND171 we mapped two HCMV-specific epitopes that induced a measurable cytokine response. Compared to the TM10 peptide, SL12 was able to stimulate a higher degree of *de novo* perforin production in conjunction with cytokine synthesis and degranulation. The discrepancies observed in perforin upregulation between various responses indicates that some populations of $CD8^+$ T cells are better equipped to lyse targets. It has already been established that $CD8^+$ T cells can simultaneously polarize lytic granules toward multiple target cells (Wiedemann et al., 2006). The now appreciated ability of $CD8^+$ T cells to upregulate perforin, which can traffic directly to immunological synapses (Makedonas et al., 2009), provides another mechanism for the elimination of multiple infected targets before the re-loading of cytotoxic granules can occur. In conclusion, the ability to detect multiple forms of perforin suggests that many aspects of its protein expression, trafficking, structure, and mechanism of action remain to be elucidated.

CHAPTER 3

PERFORIN EXPRESSION DIRECTLY *EX VIVO* BY HIV-SPECIFIC CD8$^+$ T CELLS IS A CORRELATE OF LONG-TERM VIROLOGIC CONTROL OF HIV

Summary

Many immune correlates of CD8$^+$ T cell-mediated control of HIV replication, including polyfunctionality, proliferative ability, and inhibitory receptor expression, have been discovered. However, no functional correlates using *ex vivo* cells have been identified with the known ability to cause the direct elimination of HIV-infected cells. We have recently discovered the ability of human CD8$^+$ T cells to rapidly upregulate perforin - an essential molecule for cell-mediated cytotoxicity - following antigen-specific stimulation. Here, we examined perforin expression capability in a large cross-sectional cohort of chronically HIV-infected individuals with varying levels of viral load: elite controllers (n=35), viremic controllers (n=29), chronic progressors (n=27), and viremic nonprogressors (n=6). Using polychromatic flow cytometry and standard intracellular cytokine staining assays, we measured perforin upregulation, cytokine production, and degranulation following stimulation with overlapping peptide pools encompassing all proteins of HIV. We observed that HIV-specific CD8$^+$ T cells from elite controllers consistently display an enhanced ability to express perforin directly *ex vivo* compared to all other groups. This ability is not restricted to protective HLA-B haplotypes, does not require proliferation or the addition of exogenous factors, is not restored by HAART, and primarily originates from effector CD8$^+$ T cells with otherwise

limited functional capability. Notably, we found an inverse relationship between HIV-specific perforin expression and viral load. Thus, the capability of HIV-specific $CD8^+$ T cells to rapidly express perforin defines a novel correlate of control in HIV infection.

Introduction

Approximately 35-40 million people are currently infected with HIV worldwide. Most of these individuals fail to control HIV replication, and ultimately progress to acquired immune deficiency syndrome (AIDS) if left untreated. However, a subset (< 1%) of the HIV-infected population, termed elite controllers (EC), can spontaneously control viral replication to undetectable levels (Deeks and Walker, 2007; Lambotte et al., 2005; Lefrere et al., 1999). Understanding the mechanisms of immunologic control of HIV replication in EC may identify candidate markers of immune control useful for assessing HIV vaccine strategies.

The host immune response, in particular HIV-specific $CD8^+$ T cells, is at least partially responsible for the control of viral replication in many EC. For example, EC are enriched for certain HLA alleles, such as HLA-B13, B15, B51, B27, B57, and B58 (Emu et al., 2008; Frahm et al., 2006; Honeyborne et al., 2007; Migueles et al., 2000). EC contain a greater fraction of HIV-specific $CD8^+$ T cells that can degranulate, produce multiple functional cytokines and chemokines and display markedly better proliferative potential upon HIV peptide stimulation than individuals with progressive disease (Almeida et al., 2007; Betts et al., 2006; Boaz et al., 2002; Emu et al., 2008; Horton et al., 2006; Migueles et al., 2002; Zimmerli et al., 2005). Additionally, recent evidence has demonstrated that HIV-specific $CD8^+$ T cells from EC have enhanced cytotoxic

capabilities compared to progressors: Several groups have shown that HIV-specific $CD8^+$ T cells from EC display a superior ability to suppress the replication of HIV during extended culture (Chen et al., 2009; O'Connell et al., 2009b; Saez-Cirion et al., 2007). Using $CD8^+$ T cells expanded *in vitro* for six days, Migueles and colleagues observed a higher cytotoxic capacity on a per-cell basis of HIV-specific $CD8^+$ T cells from EC (Migueles et al., 2008). Collectively, these findings suggest that $CD8^+$ T cells may be critical to the control of HIV replication *in vivo*.

$CD8^+$ T cells are thought to kill virally-infected cells predominantly through the release of lytic proteins - mainly perforin and granzymes - that are secreted via exocytosis of pre-formed granules following recognition of infected targets (Russell and Ley, 2002; Shiver and Henkart, 1991; Shiver et al., 1992). Granule-mediated killing by $CD8^+$ T cells occurs within minutes to hours of target cell recognition; however, the reconstitution of intracellular perforin following degranulation has been reported to first require cellular proliferation (Meng et al., 2006; Migueles et al., 2002; Sandberg et al., 2001). We have recently identified another mechanism by which perforin-mediated $CD8^+$ T cell killing can take place: the rapid upregulation and targeted release of newly produced perforin, which traffics to the immunological synapse via a route that largely bypasses cytotoxic granules (Makedonas et al., 2009). *De novo* synthesis of perforin by human $CD8^+$ T cells can be detected by flow cytometry in conjunction with standard intracellular cytokine-staining (ICS) (Hersperger et al., 2008), thus permitting simultaneous assessment of $CD8^+$ T cell cytotoxic potential and cytokine production.

Here, we measured the ability of HIV-specific $CD8^+$ T cells to express perforin in a cross-sectional cohort of chronically-infected individuals that differentially control viral

replication. Several previously published studies have examined perforin expression in HIV-specific $CD8^+$ T cells in both progressive and nonprogressive infection (Andersson et al., 1999; Appay et al., 2000; Migueles et al., 2002; Zhang et al., 2003). However, due to the nature of the anti-perforin antibody employed (Makedonas et al., 2009), these studies have uniformly assessed only pre-formed, granule-associated perforin present within resting or long-term activated HIV-specific $CD8^+$ T cells. In this work we demonstrate that HIV-specific $CD8^+$ T cells from EC, compared to progressors, have a superior ability to express perforin immediately upon activation, without the need for prior proliferation or the addition of exogenous cytokines. Overall, this work identifies the rapid expression of perforin as a novel correlate of control of HIV replication and urges a closer examination of $CD8^+$ T cell polyfunctionality in HIV infection.

Materials and Methods

<u>*Human subjects*</u>: We examined *ex vivo* HIV-specific $CD8^+$ T cell responses from 35 elite controllers (EC), 29 viremic controllers (VC), 27 chronic progressors (CP), and 6 viremic nonprogressors (VNP). Most EC and VC were recruited from outpatient clinics at local Boston hospitals as well as from providers throughout the United States (Pereyra et al., 2008). Several EC were also recruited from clinics associated with the University of Toronto. PBMC samples from CP were from clinics associated with the University of Pennsylvania Center for AIDS Research, the University of Toronto, Case Western Reserve University, and the University of Alabama at Birmingham. VNP samples were obtained from the University of Toronto and Case Western Reserve University. PBMC samples from 15 HAART-suppressed patients were obtained from Harvard University

and the University of Toronto.

EC were defined by consistent plasma HIV RNA levels below the limit of detection (e.g. < 75 copies/mL by bDNA or < 50 copies/mL by ultrasensitive PCR) in a minimum of three determinations of plasma HIV RNA spanning at least a 12-month period. VC consistently maintained viral load between 50 and 2,000 copies/mL, while the majority of viral load measurements of CP were above 10,000 copies/mL. $CD4^+$ T cell counts were not considered for inclusion criteria in the EC, VC, or CP groups. VNP were identified as subjects with consistently high viremia (above 10,000 copies/mL on average) but with relatively stable $CD4^+$ T cell counts after long-term infection. It is the relative preservation of $CD4^+$ T cell numbers in spite of sustained high level HIV replication that was used to distinguish the VNP group clinically from CP. All subjects from the EC, VC, CP, and VNP groups were off antiretroviral therapy for at least 6 months prior to the sampling date; yet most subjects were treatment-naive. Refer to **Table 1** for more detailed information on the study cohort.

Antibodies: The following antibodies were used in this study: anti-CD4 PE Cy5.5, anti-CD14 APC Alexa 750, anti-CD19 APC Alexa 750, anti-CD8 Texas Red-PE, anti-IFN-γ Alexa 700 (Invitrogen, Carlsbad, CA), anti-CD107a FITC, anti-IL-2 APC, anti-TNFα PE Cy7 (BD Pharmingen, San Diego, CA), anti-MIP1α PE (R&D Systems, Minneapolis, MN), anti-CD27 PE Cy5 (Beckman Coulter, Fullerton, CA), anti-CD57 Qdot 565, anti-CD3 Qdot 585, and anti-CD45RO Qdot 605 or 705 (custom). Custom conjugations to Quantum (Q) dot nanocrystals were performed in our laboratory with reagents purchased

from Invitrogen. The anti-perforin antibody (B-D48 clone) was purchased from Diaclone (Besancon, France) and conjugated to Pacific Blue (Invitrogen) in our laboratory.

PBMC stimulation assays: Cryopreserved PBMC were thawed and subsequently rested overnight at 37°C, 5% CO_2 in complete medium (RPMI supplemented with 10% FBS and 1% L-glutamine). The following morning, the cells were washed with complete medium and resuspended at a concentration of $2x10^6$ cells/mL if sufficient cell numbers were available. Costimulatory antibodies (anti-CD28 and anti-CD48d; each at 1 μg/ml final concentration; BD Biosciences; San Jose, California), monensin (1 μg/ml final concentration; BD Biosciences; San Jose, California) and Brefeldin A (1 μg/ml final concentration; Sigma-Aldrich; St. Louis, Missouri) were also added to each condition. Anti-CD107a was added at the start of all stimulation periods, as described previously (Betts et al., 2003). PBMC were incubated at 37°C, 5% CO_2 for six hours with overlapping 15-mer peptide pools encompassing HIV-1 (clade B) Gag, Pol, Env, Nef, and the viral accessory proteins (TRVVV) [as 5 separate conditions]. PBMC from many of the subjects were also stimulated with a CEF peptide pool, which contains peptides derived from CMV, EBV, and Influenza virus. Each individual peptide in the pools was at a final concentration of 2 μg/mL for all stimulations.

At the end of six hours, cells were stained with Aqua amine-reactive dye (Invitrogen; Carlsbad, California) for 15 minutes in the dark at room temperature in order to later identify viable cells. A cocktail of antibodies was then added to the cells to stain for surface markers for an additional 20 minutes. Following staining for cell surface molecules, cells were permeabilized using the Cytofix/Cytoperm kit (BD Biosciences;

San Jose, California) according to the manufacturer's instructions. A cocktail of antibodies against intracellular markers was then added to the cells and allowed to incubate for one hour in the dark at room temperature. Finally, cells were fixed in 1x PBS containing 1% paraformaldehyde (Sigma-Aldrich; St. Louis, Missouri) before being stored in the dark at 4°C until the time of collection on the flow cytometer.

Flow cytometric analysis: For each stimulation condition, at least 500,000 total events were acquired using a modified LSRII (BD Immunocytometry Systems, San Jose, California). Data analysis was performed using FlowJo (version 8.8.4; TreeStar, Ashland, Oregon) and Spice (version 4.2.3, Dr. Mario Roederer, NIH, Bethesda, Maryland). Reported data have been corrected for background, and only responses with a total frequency above 0.25% of memory $CD8^+$ T cells (after background subtraction) were considered for analysis. Boolean gating analysis was carried out once positive gates were established for each functional parameter. This analysis resulted in 64 possible combinations of the 6 measured functions. Importantly, two combinations were ignored in all analyses: (1) events negative for all measured functional parameters and (2) perforin single-positive cells. By analyzing the data in such a manner, we only examined perforin expression resulting from HIV-specific stimulation. For this reason, perforin expression was only considered within activated, HIV-specific $CD8^+$ T cells expressing at least one other functional parameter. Naïve cells ($CD27^+CD45RO^-$) were excluded during all analyses, except where indicated.

Statistical analysis: All graphing and statistical analysis was performed using R (version 2.8.1), JMP (version 7), or GraphPad Prism software (version 5.0a). Functionality was compared between study groups using nonparametric tests (Mann-Whitney test for two groups; Kruskal-Wallis test followed by a Dunns test for multiple comparisons when comparing three or more groups). Correlations between viral load and perforin expression were based on Spearman correlation coefficients. Comparisons between groups of specific functional permutations were based on a Lachenbruch's Two-part Wilcoxon test. This analysis simultaneously tests for a difference in the proportion of subjects who have an above zero response and a difference in the magnitude of the response (Lachenbruch, 2001; Nason, 2006). Only those functional combinations for which the average response was greater than zero were considered to be relevant for consideration. Functional permutations were considered significantly different if the p value was below 0.01. In all figures, * denotes a p value < 0.05, ** denotes a p value < 0.01, and *** denotes a p value < 0.001. Unless otherwise noted, error bars represent the standard deviation.

<u>Table 1</u>: Clinical parameters of the HIV-infected study cohort to examine perforin expression.

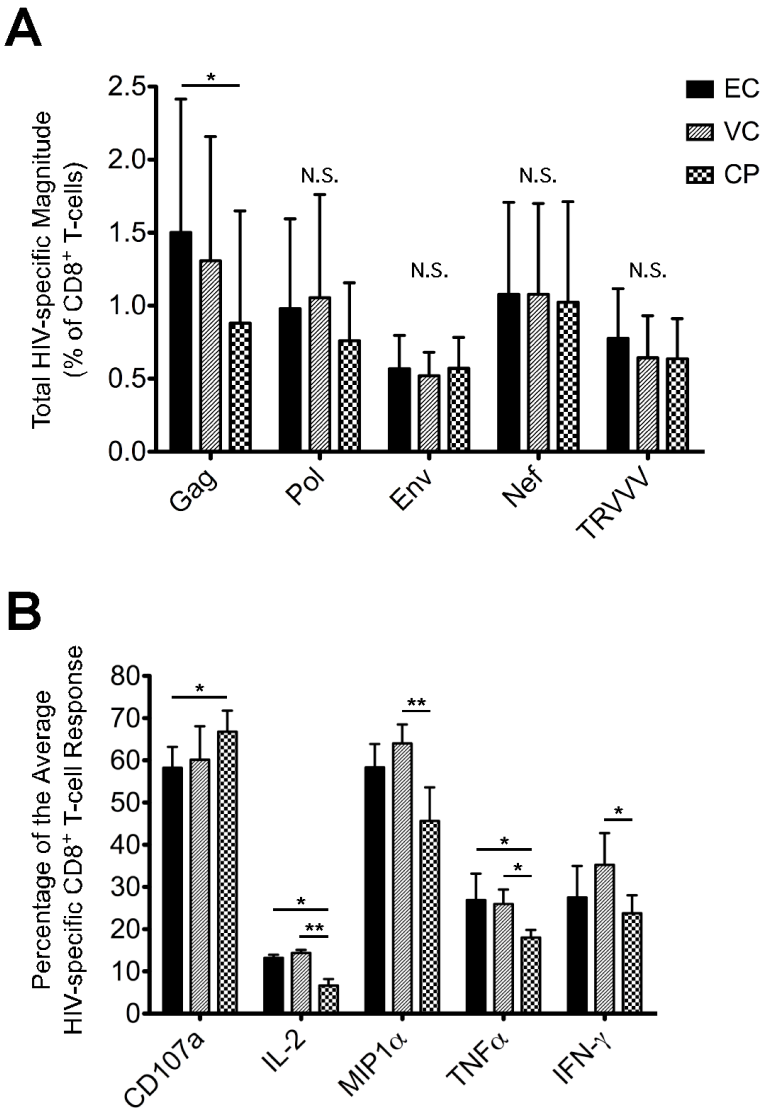
	Elite Controller	Viremic Controller	Chronic Progressor	Viremic Nonprogressor	HAART-treated
Number of subjects	35	29	27	6	15
Plasma HIV RNA, **median** (*IQR*), copies/mL	undetectable	**396** (*82-874*)	**24,121** (*18,000-41,579*)	**35,000** (*29,672-101,500*)	undetectable
$CD4^+$ T cell count, **median** (*IQR*), cells/mm^3	**811** (*702-1,068*)	**576** (*449-785*)	**508** (*401-599*)	**557** (*439-625*)	**440** (*301-610*)
Decline in $CD4^+$ T cell count per year, **median** (*IQR*), cells/mm^3	Not determined	Not determined	**170** (*103-319*)	**36** (*26-47*)	Not determined
Infection duration, **median** (*IQR*), years	**17** (*13-21*)	**12** (*8-19*)	**7** (*4-13*)	**20** (*16-22*)	**16** (*12-20*)
Duration of HAART treatment prior to PBMC sample, **median** (*IQR*), years	N/A	N/A	N/A	N/A	**2** (*1-6*)

Results

HIV-specific CD8$^+$ T cell response between EC and CP did not vary greatly in total magnitude, degranulation, or cytokine production

We assessed the magnitude and functional characteristics of HIV-specific CD8$^+$ T cells by stimulating PBMC from 35 elite controllers (EC), 29 viremic controllers (VC), and 27 chronic progressors (CP) (**Table 1**) with overlapping peptide pools encompassing all HIV-1 (clade B) proteins. We developed a flow cytometric staining panel that simultaneously measured memory phenotype (CD27, CD45RO, and CD57), degranulation [surface expression of CD107a (Betts et al., 2003)], cytokine expression (IFN-γ, TNFα, and IL-2), and chemokine production (MIP1α). As a sixth functional parameter, we included an anti-perforin antibody (clone B-D48) to measure perforin upregulation as described in Chapter 2 (Hersperger et al., 2008; Makedonas et al., 2009).

As shown in **Figure 7A**, the total HIV-specific CD8$^+$ T cell response magnitude to Pol, Env, Nef, or TRVVV stimulation did not differ substantively across the groups, but EC displayed a somewhat higher Gag-specific response. The lack of large differences in response magnitude is in agreement with previous studies that measured the total magnitude of CD8$^+$ T cell responses in EC and CP using flow cytometry (Betts et al., 2006; Emu et al., 2008; Migueles et al., 2002). We next determined the relative contribution of CD107a, IFN-γ, TNFα, IL-2, and MIP1α to the HIV-specific CD8$^+$ T cell response (**Fig. 7B**). In general, no clear trends emerged in overall functionality between the groups. For example, compared to EC, CP demonstrated a slightly enhanced ability to degranulate, lower levels of TNFα, but no statistically significant difference in the proportion of the average HIV-specific CD8$^+$ T cell response comprised of either IFN-γ

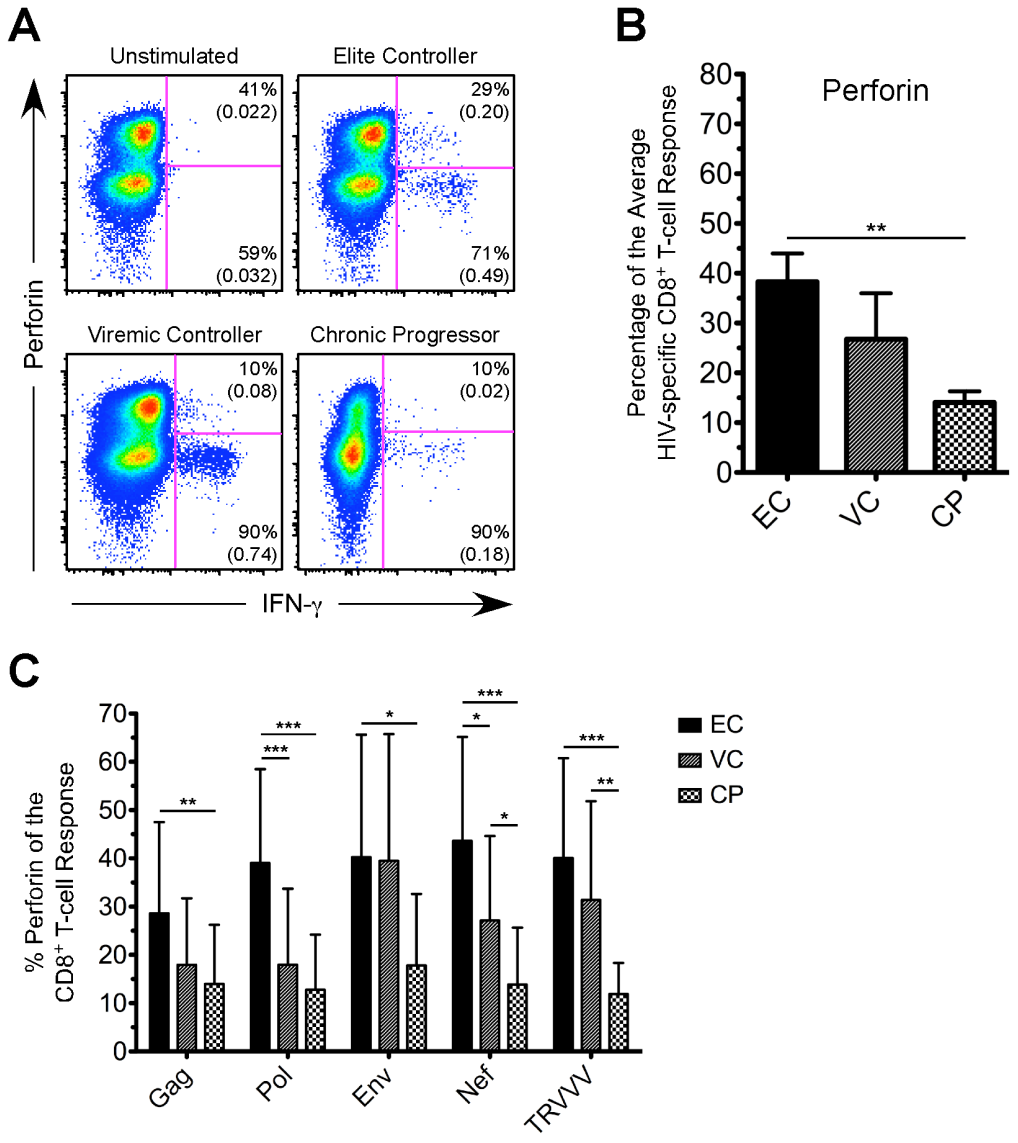


<u>Figure 7</u>: Average HIV-specific CD8$^+$ T cell response magnitude, degranulation capability, and cytokine production do not vary widely between the cohort groups. **(A)** The CD8$^+$ T cell response magnitude to all HIV peptide pools was calculated for EC, VC, and CP and plotted as percent of CD8$^+$ T cells (excluding naïve cells). The total magnitude was calculated by summing across all functional combinations. **(B)** The proportion of the average HIV-specific CD8$^+$ T cell response comprised of each single functional parameter (except perforin) is shown for EC, VC, and CP. **(A and B)** Statistical analysis was carried out using one-way ANOVA tests (nonparametric; Kruskal-Wallis) followed by a Dunns test for multiple comparisons. * denotes a p value < 0.05 and ** denotes a p value < 0.01. All bars represent the mean and error bars indicate the standard deviation.

or MIP1α. The largest difference in functionality was IL-2 expression, which was higher among EC and VC compared to CP. Previous studies have also shown enhanced production of IL-2 after HIV-specific stimulation in subjects with low or undetectable viremia (Betts et al., 2006; Emu et al., 2008; Zimmerli et al., 2005). Similar overall observations were found for the individual HIV antigens as well (data not shown).

HIV-specific CD8$^+$ T cells in EC demonstrated a greater ability to express perforin than VC and CP

We next assessed perforin expression by HIV-specific CD8$^+$ T cells in each cohort group. We consistently observed higher co-expression of perforin within responding cells from EC compared to VC or CP for all HIV antigens (**Fig. 8A** shows representative Nef-specific responses producing IFN-γ; other HIV antigens are not shown but yielded similar results). In fact, perforin expression comprised a significantly greater proportion of the average HIV-specific CD8$^+$ T cell response in EC than in CP (**Fig. 8B**). The relative contribution of perforin to the CD8$^+$ T cell response was significantly higher (~3 fold) in EC compared to CP for all of the individual HIV antigens (**Fig. 8C**). In addition to the proportion of the HIV-specific CD8$^+$ T cell response comprised of perforin, EC also displayed a greater magnitude of perforin expression upon stimulation by all HIV antigen pools compared to both VC and CP (data not shown). However, we found no correlation among EC between the total magnitude of an HIV-specific response and the corresponding amount of perforin expression (data not shown).



<u>Figure 8</u>: EC demonstrate an enhanced ability to express perforin directly *ex vivo*. **(A)** Representative flow cytometric plots of perforin versus IFN-γ are shown from one representative EC, VC, and CP. Percentages represent the proportion of functional cells that stain either positive or negative for perforin. Values in parentheses are the magnitude of each population and denote percent of $CD8^+$ T cells (excluding naïve cells). **(B)** The proportion of the average HIV-specific $CD8^+$ T cell response comprised of perforin is shown for EC, VC, and CP. **(C)** The relative contribution of perforin to the Gag-, Pol-, Env-, Nef-, and TRVVV-specific $CD8^+$ T cell responses is shown for EC, VC, and CP. **(B and C)** Statistical analysis was carried out using one-way ANOVA tests (nonparametric; Kruskal-Wallis) followed by a Dunns test for multiple comparisons. * denotes a p value < 0.05, ** denotes a p value < 0.01, and *** denotes a p value < 0.001. All bars represent the mean and error bars indicate the standard deviation.

There was some variability among EC subjects in the contribution of perforin to the HIV-specific $CD8^+$ T cell response. Within some EC there was low perforin expression induced by one HIV antigen (e.g. Gag) but higher perforin production to another peptide pool (e.g. Pol). Some EC demonstrated high HIV-specific perforin in response to every antigen. Although several EC did express low levels of HIV-specific perforin, only 20% of all EC in the cohort failed to achieve 30% perforin of the $CD8^+$ T cell response to at least one of the antigen pools (data not shown). In contrast, only 15% of CP demonstrated even one HIV antigen-specific $CD8^+$ T cell response comprised of 30% perforin (data not shown). Thus, our data suggests that EC are not simply a homogenous group of HIV-infected individuals and do demonstrate some variability, in agreement with previous findings (Emu et al., 2008; Saez-Cirion et al., 2009).

Next, we examined the functional profile of the average Nef-specific $CD8^+$ T cell response among the EC, VC, and CP groups (**Fig. 9A and 9B**; the other HIV antigens are not shown but yielded similar results). Only the functional combinations that were significantly different between at least two of the groups are shown in **Figure 9A**. We rarely observed simultaneous expression of all six functions because perforin and IL-2 are generally not co-expressed by the same cell (Cellerai et al., 2010; Makedonas et al., 2010). The average Nef-specific functional profile in EC and VC was composed of more $CD8^+$ T cells than in CP that simultaneously expressed five functions (**Fig. 9B**). Additionally, the percentage of the Nef-specific response that was perforin-positive (black arcs in **Fig. 9B**) was significantly higher among EC (44%) compared to VC (27%; $p<0.05$) or CP (14%; $p<0.001$). Similar findings were observed for Gag-, Pol-, Env-, and TRVVV-specific responses (data not shown).

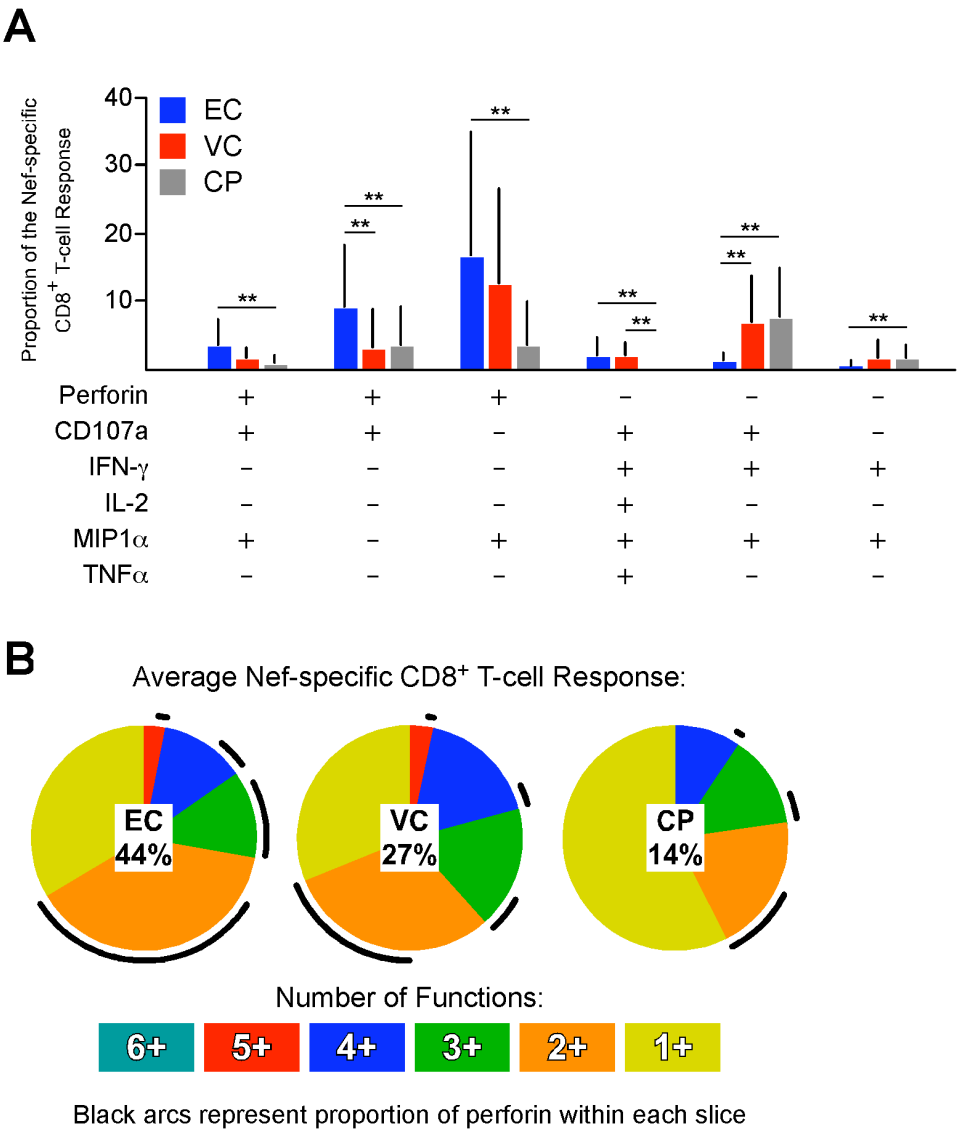


Figure 9: The majority of perforin expression comes from cells with otherwise limited functional capability. (A) The functionality of the average Nef-specific $CD8^+$ T cell response is shown; only the functional permutations that varied significantly between at least two of the groups are shown. ** denotes a p value < 0.01 based on a Lachenbruch's Two-part Wilcoxon test as described in the Methods. All bars represent the mean and error bars indicate the standard deviation. **(B)** The average Nef-specific $CD8^+$ T cell functional profile is shown for EC, VC, and CP. Responses are grouped according to the number of positive functions. The relative amount of perforin positivity within each functional group (i.e. each pie slice) is depicted as black arcs. The relative contribution of perforin (mean value) to the entire response is represented by the percentage in the center of each pie.

Interestingly, the majority of perforin was produced by cells expressing only a single other function: CD107a or MIP1α (**Fig. 9B**). The $CD8^+$ T cells that co-expressed CD107a and perforin likely upregulated perforin *de novo* since a cell that was $CD107a^+$ presumably lost all (or nearly all) of its preformed perforin through the process of degranulation. Notably, the proportion of the HIV-specific response in EC that was both $CD107a^+$ and $perforin^+$ was significantly higher than CP for all HIV antigens (data now shown). The second major population of $perforin^+$ cells co-expressed only MIP1α. The relevance of this population is unclear. However, we have previously shown that activated $CD8^+$ T cells can transport newly synthesized perforin directly to the immunological synapse without trafficking first through cytolytic granules (Makedonas et al., 2009). Thus, despite the absence of apparent degranulation, $MIP1\alpha^+$ $perforin^+$ cells may potentially be involved in ongoing cytolytic activity.

Perforin expression is not restricted to the presence of protective HLA-B alleles

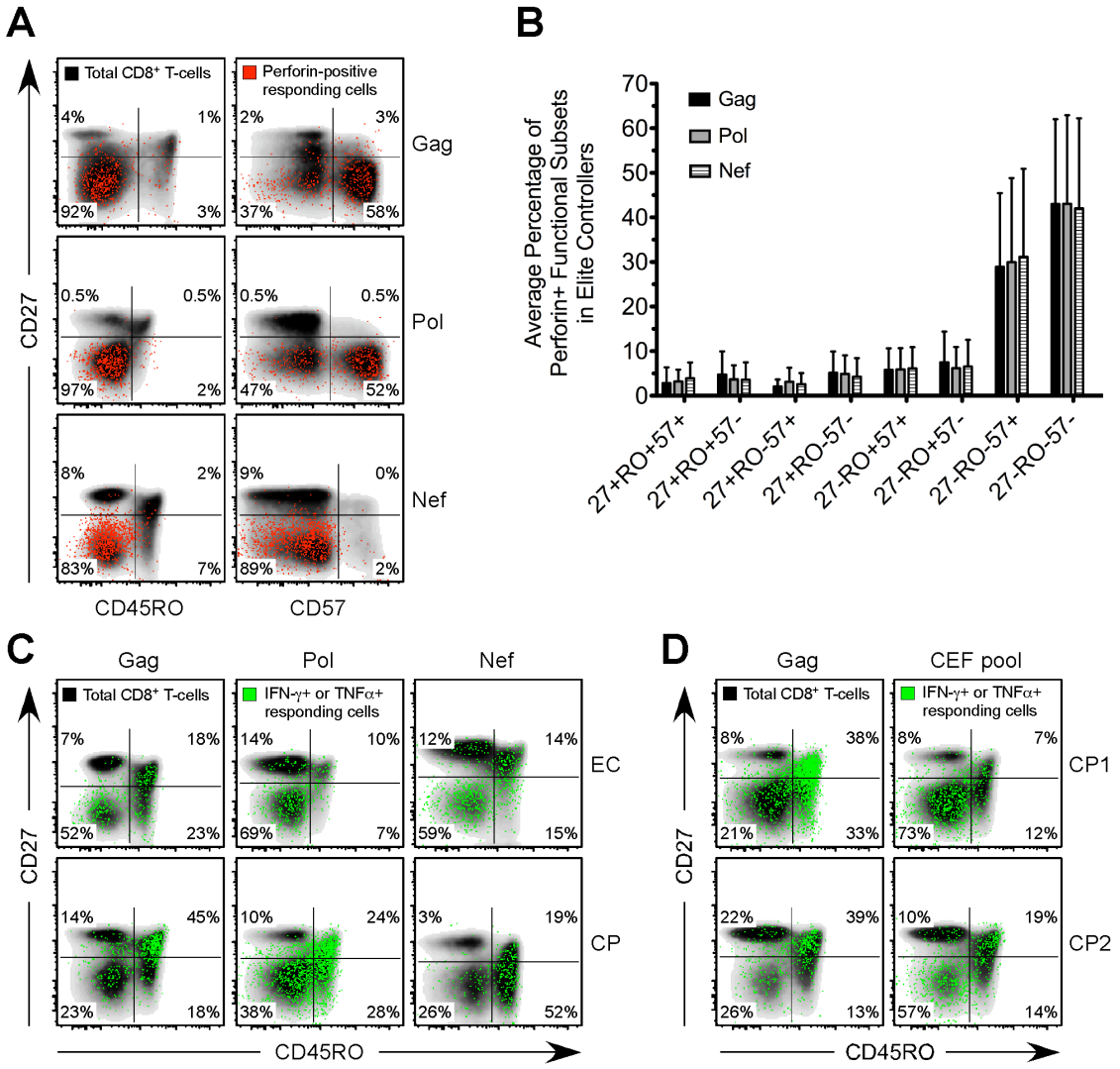
One consistent host factor associated with durable control of HIV is the presence of certain HLA class I alleles, particularly HLA-B27 and B57 (Goulder et al., 1997; Kiepiela et al., 2004; Kiepiela et al., 2007; Lambotte et al., 2005; Migueles et al., 2000). Other HLA-B alleles have also been associated with delayed disease progression or lower viral loads, including HLA-B13, B15, B51, and B58 (Frahm et al., 2006; Honeyborne et al., 2007). Among EC in our cohort, 54% of the subjects expressed HLA-B27 or B57, while 32% of VC carried these alleles. Additionally, 43% of EC in the study cohort expressed either HLA-B13, B15, B51, or B58, while 32% of VC carried these alleles. Overall, we found no association between protective HLA-B status and perforin expression to any individual HIV peptide pool in either EC or VC, or when perforin

expression to all HIV peptide pools was averaged within each subject (data not shown). Thus, there was no apparent relationship between protective HLA-B alleles and the capacity of HIV-specific $CD8^+$ T cells to express perforin after stimulation.

Distinct expansion of HIV-specific effector $CD8^+$ T cells in EC

Much work has been done to characterize the memory phenotype of HIV-specific $CD8^+$ T cells and to compare them to the memory phenotype of $CD8^+$ T cells specific for other persistent viruses, such as CMV or EBV (Appay et al., 2002; Champagne et al., 2001). In this study, we included reagents in the polychromatic flow cytometric staining panel to detect surface expression of CD27, CD45RO, and CD57. Some studies have used CD45RA to determine the memory phenotype of human $CD8^+$ T cells (Appay et al., 2002; Hamann et al., 1997). However, CD45RO and CD45RA are isoforms of the same molecule and share a reciprocal relationship to memory status; memory cells, which contain enhanced frequencies of antigen-reactive cells, typically appear as $CD45RO^+$ (Merkenschlager and Beverley, 1989). Effector $CD8^+$ T cells are almost universally $CD27^-$ and can be either positive or negative for CD45RO expression. $CD27^-$ effector cells usually display more cytotoxic potential and contain a higher proportion of perforin and granzyme containing cells than $CD27^+$ subsets (Hamann et al., 1997; Takata and Takiguchi, 2006). CD57 is a marker of terminal differentiation; $CD57^+$ $CD8^+$ T cells usually lack a strong proliferative potential but maintain a strong cytolytic capacity due to high expression of both perforin and granzymes (Brenchley et al., 2003; Chattopadhyay et al., 2009). $CD27^-CD45RO^-CD8^+$ T cells are considered differentiated effector cells and often display high surface expression of CD57.

Next, we examined the memory phenotype, based on surface expression of CD27, CD45RO, and CD57, of HIV-specific $CD8^+$ T cells in each group. The majority of HIV-specific $CD8^+$ T cells that expressed perforin in EC, VC, and CP were $CD27^-CD45RO^-CD57^{+/-}$ (**Fig. 10A and 10B** and data not shown), frequently considered an effector-type profile, which is in agreement with previous reports that examined the presence of perforin in various human $CD8^+$ T cell memory subsets (Chattopadhyay et al., 2009; Takata and Takiguchi, 2006). This phenotype was common to virtually all $perforin^+$ HIV-specific $CD8^+$ T cells regardless of their specificity for Gag, Pol, Nef, Env or TRVVV (**Fig. 10A** and data not shown). HIV-specific $CD8^+$ T cells among many CP subjects were skewed toward a $CD27^+CD45RO^{+/-}$ memory phenotype (**Fig. 10C**), as previously shown (Appay et al., 2002; Betts et al., 2006; Precopio et al., 2007). However, a higher proportion of HIV-specific $CD8^+$ T cells in EC than in CP displayed a memory phenotype consistent with differentiated effector cells (**Fig. 10C**). Overall, the presence of $CD27^-CD45RO^-$ HIV-specific $CD8^+$ T cells was less common among CP than EC, in agreement with a previous study (Saez-Cirion et al., 2007). The absence of effector-like HIV-specific $CD8^+$ T cells in CP is not, however, reflective of the total $CD8^+$ T cell pool in these individuals. Indeed, a substantial fraction of $CD8^+$ T cells in CP that responded to CEF (pool of CMV, EBV, and Flu peptides) stimulation were $CD27^-CD45RO^-$ (**Fig. 10D**). However, responding Gag-specific $CD8^+$ T cells within the same subjects were primarily $CD27^+CD45RO^+$ (**Fig. 10D**).

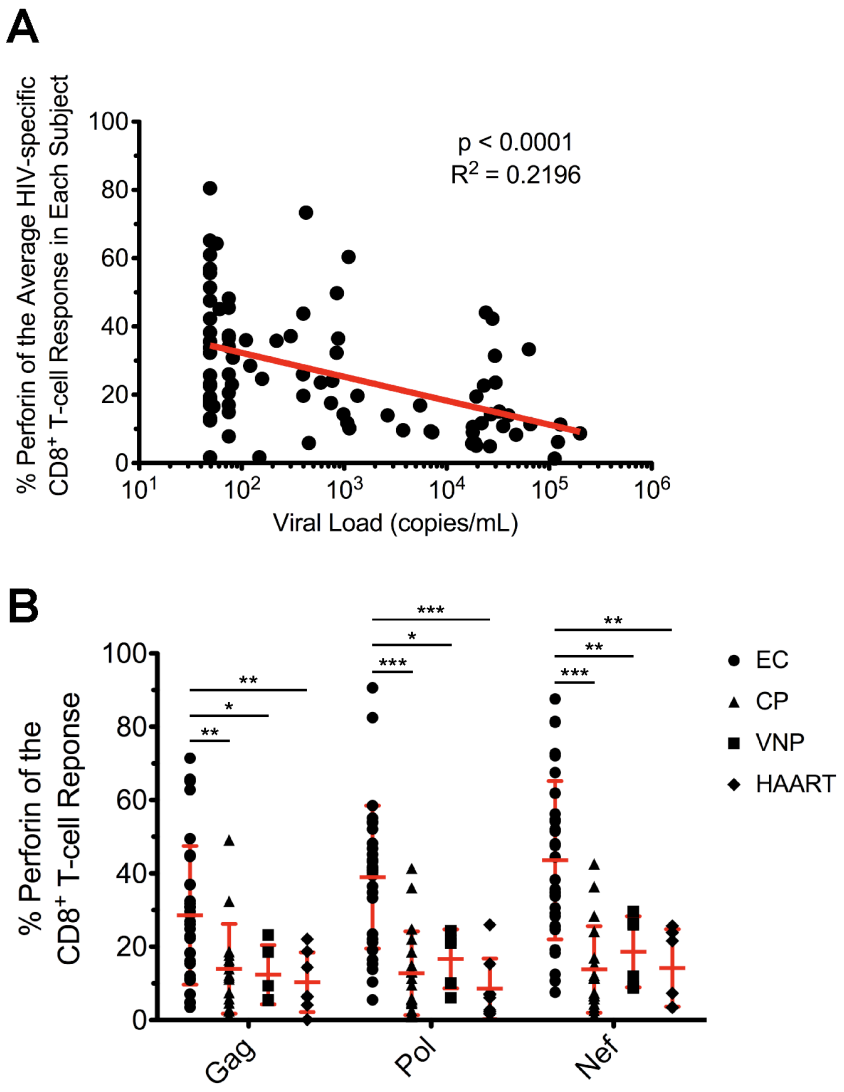


<u>Figure 10</u>: Distinct expansion of HIV-specific effector CD8$^+$ T cells in EC. (A) Gag-, Pol-, and Nef-specific perforin$^+$ functional subsets (red events) were overlaid onto a density plot (black shading) of the memory phenotype of the total CD8$^+$ T cell population in three representative EC subjects. **(B)** The memory phenotype of Gag-, Pol-, and Nef-specific perforin$^+$ functional subsets was determined for all EC. Bars represent the mean and error bars indicate the standard deviation. **(C)** Gag-, Pol-, and Nef-specific CD8$^+$ T cells, as defined by the production of IFN-γ or TNFα (green events), were overlaid onto a density plot (black shading) of the memory phenotype of the total CD8$^+$ T cell population in three separate EC and CP subjects. **(D)** Gag- and CEF-specific CD8$^+$ T cells, as defined by the production of IFN-γ or TNFα (green events), were overlaid onto a density plot (black shading) of the memory phenotype of the total CD8$^+$ T cell population in two separate CP subjects. **(A, C, D)** Percentages represent the fraction of overlaid cells that fall within each quadrant.

Inverse relationship between HIV-specific perforin expression and viral load

Having observed higher perforin expression in HIV-specific $CD8^+$ T cells in EC, we next examined the relationship between perforin expression and viral load. We found a significant inverse correlation between the average HIV-specific perforin expression within each subject and HIV viral load (**Fig. 11A**). This inverse relationship was found for every individual HIV antigen specificity and when only considering subjects with detectable viremia (data not shown). We also found a statistically significant positive correlation between $CD4^+$ T cell counts in the blood and HIV-specific perforin expression by $CD8^+$ T cells across all subjects (data not shown), a finding most likely driven by the high $CD4^+$ T cell counts among the EC subjects (**Table 1**). Furthermore, when we examined the other functional parameters in a similar manner, we only found a statistically significant inverse relationship between IL-2 expression and viral load (data not shown), which is an expected result based upon previous studies.

To better understand the relationship between viral load and perforin expression, we next compared HIV-specific $CD8^+$ T cell responses among EC to viremic nonprogressors (VNP), who maintain stable $CD4^+$ T cell counts in the face of consistently high viral loads (**Table 1**) without progressing to AIDS. The infection duration in both groups was similar (17 vs. 20 years in the absence of therapy; **Table 1**). Therefore, by comparing EC and VNP, we can control for the rate of $CD4^+$ T cell decline, progression status, and duration of infection. As shown in **Figure 11B**, perforin expression by HIV-specific $CD8^+$ T cells in VNP is significantly lower than EC and actually closely resembles the perforin levels observed in CP.



<u>Figure 11</u>: Inverse relationship between viral load and HIV-specific perforin expression, which is not rescued by HAART. (A) The average proportion of HIV-specific perforin expression within each subject was plotted against the HIV viral load from each respective subject. The most proximal viral load measurement to the time point of the PBMC sample was used in the analysis. Spearman correlation tests were performed to determine statistical significance. **(B)** The relative contribution of perforin to the Gag-, Pol-, and Nef-specific $CD8^+$ T cell responses is shown for all EC, CP, VNP, and HAART-suppressed subjects. Each symbol represents an individual study subject. One-way ANOVA tests (nonparametric; Kruskal-Wallis test) were performed followed by a Dunns test for multiple comparisons. * denotes a p value < 0.05, ** denotes a p value < 0.01, and *** denotes a p value < 0.001. The error bars represent the mean and standard deviation.

HIV-specific perforin expression is not recovered by HAART

In order to determine whether the low perforin expression associated with progression was reversible, we examined HIV-specific perforin expression by $CD8^+$ T cells in HAART-treated individuals with undetectable HIV viremia (**Table 1**). Compared to EC, the total $CD8^+$ T cell response magnitude was lower in HAART-treated subjects to Gag, Pol, and Nef stimulation; however, only the difference in the total Gag-specific magnitude reached statistical significance (data not shown). Despite some differences in total magnitude, there were no substantive differences in the relative contribution of degranulation, IFN-γ, TNFα, or MIP1α production to the HIV-specific $CD8^+$ T cell response between EC and HAART-suppressed subjects (data not shown). However, HIV-specific perforin expression in treated subjects was considerably lower than the levels observed in EC, and was similar to perforin expression in CP (**Fig. 11B**). Thus, the ability to express and rapidly upregulate perforin by HIV-specific $CD8^+$ T cells in chronic HIV infection was not recovered following HAART.

Discussion

While many cell surface markers, activation profiles, and functional parameters of both *ex vivo* HIV-specific $CD8^+$ and $CD4^+$ T cells have been shown to correlate with control of viremia (Betts et al., 2006; Day et al., 2006; Day et al., 2007; Deeks et al., 2004; Hunt et al., 2008; Younes et al., 2003), few, if any, can potentially mediate direct control of HIV replication through the lysis of infected cells. Here we have shown that perforin expression by *ex vivo* HIV-specific $CD8^+$ T cells is significantly higher in EC compared to patients with uncontrolled viral replication. HIV-specific $CD8^+$ T cells that

express perforin bear predominantly an effector phenotype, indicating that effector populations, in addition to central memory populations (Betts and Harari, 2008; Pantaleo and Harari, 2006), may be critically important to the control of HIV infection. We also find an inverse correlation between perforin expression by HIV-specific $CD8^+$ T cells and viral load. Together, these results represent an unique assessment of HIV-specific immunity and provide a novel platform for measuring potential vaccine efficacy in clinical trials.

There is little question regarding the crucial importance of perforin in the control of infectious pathogens. Indeed, mutation or dysregulation of perforin in humans results in compromised cellular immunity and enhanced susceptibility to viral infections (Molleran Lee et al., 2004). Previous reports on *ex vivo* HIV-specific $CD8^+$ T cells have uniformly found low or absent perforin expression in both CP and EC and no detectable differences in perforin levels between the groups (Appay et al., 2002; Appay et al., 2000; Lieberman et al., 2001; Migueles et al., 2002; Zhang et al., 2003). However, these studies have in retrospect only defined the level of granule-associated perforin within resting HIV-specific $CD8^+$ T cells due to unforeseen limitations in the anti-perforin antibody employed in these studies (Hersperger et al., 2008; Makedonas et al., 2009). Due to chronic activation and continual presence of viral antigen - albeit extremely low levels in EC (Hatano et al., 2009; Pereyra et al., 2009) - HIV-specific $CD8^+$ T cells are unlikely to reach a true resting state; therefore, it is unlikely these cells accumulate cytolytic granules containing perforin *in vivo*. However, our results indicate that this does not preclude their ability to upregulate new perforin upon antigen-specific stimulation, a killing mechanism

that we have recently shown potentiates the cytotoxic ability of human $CD8^+$ T cells (Makedonas et al., 2009).

We have shown previously that both the commonly used anti-perforin antibody (δG9 clone) and the anti-perforin antibody used in this study (B-D48 clone) stain resting $CD8^+$ T cells equivalently (Hersperger et al., 2008). Thus, previous research that found no difference in the levels of perforin within resting HIV-specific $CD8^+$ T cells between EC and CP (Appay et al., 2002; Appay et al., 2000; Lieberman et al., 2001; Migueles et al., 2002; Zhang et al., 2003) were not necessarily incorrect. Here, we have shown using a perforin antibody that can detect both granule-associated and granule-independent forms of perforin that HIV-specific $CD8^+$ T cells from EC express this protein to a higher degree than patients with uncontrolled viremia. It is important to note, though, that the B-D48 clone cannot specifically distinguish pre-formed from newly upregulated perforin using flow cytometric-based assays. Nevertheless, to identify the potential contribution of perforin produced *de novo*, we examined the proportion of the HIV-specific $CD8^+$ T cell response that both degranulated ($CD107a^+$) yet remained $perforin^+$ after six hours of stimulation. These $CD8^+$ T cells that co-expressed CD107a and perforin likely upregulated new perforin; they have presumably lost most or all of their pre-formed perforin through the process of degranulation. By analyzing this specific population, we found that the proportion of the HIV-specific $CD8^+$ T cell response in EC that co-expressed $CD107a^+$ and $perforin^+$ was significantly higher than CP to all HIV antigens. However, we have also shown that newly synthesized perforin largely bypasses cytotoxic granules (Makedonas et al., 2009); therefore, we are almost certainly underestimating the levels of perforin upregulation by focusing only on cells that have degranulated.

The capacity of unstimulated $CD8^+$ T cells from EC to begin to eliminate HIV-infected autologous $CD4^+$ T cell targets within several hours of co-incubation has been previously reported (Saez-Cirion et al., 2007). The results from that study suggested that HIV-specific $CD8^+$ T cells were responsible for the elimination of infected $CD4^+$ T cells through a mechanism dependent on cell-to-cell contact and MHC-I restriction. Our findings on perforin upregulation by HIV-specific $CD8^+$ T cells shortly after stimulation are consistent with the results of Saez-Cirion and colleagues (Saez-Cirion et al., 2007) and may even be a mechanism to explain their findings. Moreover, another previously published report indicated that HIV-specific $CD8^+$ T cells kill targets through the use of cytotoxic granules and not by the Fas/FasL pathway (Shankar et al., 1999). Therefore, available evidence indicates that the perforin/granzyme pathway of cytotoxicity is likely the primary means by which HIV-specific $CD8^+$ T cells kill infected cells *in vivo*.

Our findings here suggest that HIV-specific $CD8^+$ T cells in EC have a superior cytotoxic potential by expressing higher levels of perforin. This supposition is supported by recent work from Migueles and colleagues (Migueles et al., 2008). These authors showed that HIV-specific $CD8^+$ T cells from EC accumulated more granule-associated perforin as a result of their superior ability to proliferate *in vitro* compared to $CD8^+$ T cells from progressors. They also found that higher amounts of perforin (and granzyme B) in HIV-specific cells translated into an enhanced ability to lyse infected targets. Thus, together with the previous work of Migueles et al., our results show that EC have an enhanced ability to upregulate perforin either directly *ex vivo* or after *in vitro* proliferation. Given what is known about the importance of perforin in orchestrating cytotoxicity, we can conclude that HIV-specific $CD8^+$ T cells from EC certainly have the

potential to elicit elimination of infected targets to a greater degree than progressors, which may directly impact viral load. Furthermore, we know that newly synthesized perforin traffics directly to the immunological synapse - the site of action of cytotoxicity (Makedonas et al., 2009).

Besides differences in cytotoxic capabilities, HIV-specific $CD8^+$ T cells from EC have also been shown to be more polyfunctional in nature; they can simultaneously degranulate and produce multiple functional molecules, such as IL-2, IFN-γ, and TNFα, to a greater extent than $CD8^+$ T cells from progressors (Betts et al., 2006; Ferre et al., 2009). Our results here confirm and extend these findings. Polyfunctional HIV-specific $CD8^+$ T cells were also found in this study to comprise a greater fraction of the response in EC than in CP. Interestingly, we rarely observed HIV-specific $CD8^+$ T cells capable of producing all six functions simultaneously. This results from a dichotomous relationship between perforin and IL-2 production from the same cell (Cellerai et al., 2010; Makedonas et al., 2010). The implications of this dichotomy are profound for our understanding of effective HIV-specific $CD8^+$ T cell responses: IL-2 producing $CD8^+$ T cells will presumably not have immediate cytolytic activity; conversely, perforin producing $CD8^+$ T cells may be inherently reliant upon production of IL-2 from cells in their surrounding environment for maintenance or modulation. Both cell types are most likely crucial to maintaining protective immunity. The IL-2 producing cells may be part of a population of $CD8^+$ T cells that can maintain itself through autocrine production of IL-2. This ability may be important in the setting of diminished $CD4^+$ T cell help in HIV infection (Zimmerli et al., 2005). Alternatively, these cells may represent a self-renewing memory population of $CD8^+$ T cells responsible for long-term maintenance of effector

cells. IL-2 producing cells likely do not display any direct anti-viral capability directly after activation but may be able to differentiate into perforin producing effector cells. The increased IL-2 production observed by both HIV-specific $CD8^+$ T cells and $CD4^+$ T cells (Younes et al., 2003) in EC may also directly increase cytotoxic potential, as has recently been reported (Kalia et al., 2010; Pipkin et al., 2010b).

Interestingly, we found that a substantial fraction of the total perforin production by HIV-specific $CD8^+$ T cells among EC comes not from polyfunctional populations but instead from cells that elicit only a single other measured functional parameter: specifically MIP1α or CD107a. In previous studies, where perforin upregulation was not measured, the potential importance and cytotoxic capabilities of these populations was not appreciated. On this note, the degree of functionality of a $CD8^+$ T cell response is only reflective of what functional parameters are actually being measured. For example, we find that most $CD8^+$ T cells that upregulate perforin also produce granzyme B upon stimulation (Makedonas et al., 2010). Therefore, many of the $CD8^+$ T cells found in this study to co-express perforin with MIP1α and/or CD107a, may actually be highly "polyfunctional" if we had also examined the expression of other parameters critical for cytotoxicity, such as granzyme B.

Our data show that perforin expressing cells bear effector-like phenotypic markers. Thus, while a central memory phenotype is often considered a protective phenotype in HIV infected individuals, our results suggest that effector cells are also of significance. It should be noted, however, that simply achieving effector status does not guarantee the expression of perforin. Indeed, some HIV-specific $CD8^+$ T cells in both EC and CP were $CD27^-CD45RO^-$ yet did not express perforin. Our results suggest that

effector status is necessary but not sufficient for perforin upregulation. The importance of effector cells in the control of HIV infection is further supported by recent observations by Picker and colleagues who found that a rhesus-CMV-based SIV vaccine vector could stimulate protective effector SIV-specific $CD8^+$ T cells (Hansen et al., 2009).

Finally, our results suggest that perforin expression by HIV-specific $CD8^+$ T cells is not readily recovered by inhibition of viral replication or reduction in chronic immune activation by HAART - a finding which is consistent with a previous report showing that HAART treatment does not restore other functional parameters, such as proliferative capacity, polyfunctionality, or cytotoxic activity (Migueles et al., 2009). We also found that perforin production does not appear to be directly influenced by beneficial HLA-B haplotypes or the relative maintenance of $CD4^+$ T cell levels over time. Whether perforin expression is lost early, late, or progressively during infection remains unclear. Further studies are necessary to identify the mechanism(s) underlying the relative absence of perforin upregulation in progressive HIV infection, and, if possible, to discover a means by which this critical function can be regained or elicited through therapeutic intervention.

CHAPTER 4

T-BET EXPRESSION IS STRONGLY LINKED TO THE CYTOTOXIC POTENTIAL OF ANTI-VIRAL CD8$^+$ T CELLS AND IS ASSOCIATED WITH LONG-TERM VIROLOGIC CONTROL OF HIV

Summary

Recent data suggest that CD8$^+$ T cell effector activity is an important component to the control of HIV replication in elite controllers (EC). One critical element of CD8$^+$ T cell effector function and differentiation is the T-box transcription factor T-bet. Here, we assessed T-bet expression, together with the effector proteins perforin, granzyme A, granzyme B, and granulysin, in HIV-specific CD8$^+$ T cells from EC (n=20), chronically-infected progressors (n=18), and HAART-suppressed individuals (n=19). When compared to the other cohort groups, HIV-specific CD8$^+$ T cells among EC demonstrated a superior ability to express perforin and granzyme B but with no detectable difference in the levels of granzyme A or granulysin. Notably, we observed higher levels of T-bet in HIV-specific CD8$^+$ T cells from EC, with an ensuing positive correlation between T-bet and levels of both perforin and granzyme B. Moreover, HIV-specific CD8$^+$ T cells in EC upregulated T-bet to a greater extent than progressors after *in vitro* expansion with concomitant upregulation of perforin and granzyme B. Collectively, these results suggest that T-bet may be playing an important role in driving effector function and its modulation may lead to enhanced effector activity against HIV.

Introduction

HIV infection is typically associated with high viral loads and steadily declining $CD4^+$ T cell counts until eventual immune system collapse with the onset of AIDS. However, a rare subset of HIV-infected individuals, termed "elite controllers" (EC), can spontaneously control viral load to extremely low levels without the intervention of anti-retroviral therapy. Understanding the mechanism(s) by which EC are able to control HIV replication is an area of intense interest that may provide necessary insights for the development of vaccines and therapeutics to combat HIV (Deeks and Walker, 2007; O'Connell et al., 2009a).

Recent data has shown that HIV-specific $CD8^+$ T cells from EC have enhanced cytotoxic function compared to progressors. $CD8^+$ T cells from EC displayed a superior ability to suppress the replication of HIV in autologous $CD4^+$ T cells during extended culture (O'Connell et al., 2009b; Saez-Cirion et al., 2007). $CD8^+$ T cells from EC that were expanded *in vitro* for 6 days following HIV-specific stimulation demonstrated enhanced proliferation and upregulation of perforin and granzyme B (Migueles et al., 2002; Migueles et al., 2008). The upregulation of these cytotoxic granule-resident proteins during culture translated into a greater capacity to induce target cell death on a per cell basis (Migueles et al., 2008). In addition, EC express higher levels of perforin immediately after antigen recognition, resulting in a greater *ex vivo* cytotoxic potential (Hersperger et al., 2010). Collectively, these findings suggest that $CD8^+$ T cells play a critical role in the control of HIV replication, particularly within EC.

$CD8^+$ T cells directly inhibit viral replication and subsequent dissemination within a host via the elimination of infected cells. The two major means of target cell cytolysis

are via cytotoxic granule exocytosis and the Fas/FasL pathway (Chavez-Galan et al., 2009). Cytotoxic granules are secretory lysosomes (Peters et al., 1991) that contain multiple proteins - including perforin, granzymes, and granulysin - that work in concert to induce apoptosis in infected cells. The cytotoxic granule pathway is likely the principal mechanism by which HIV-specific $CD8^+$ T cells eliminate HIV-infected cells (Migueles et al., 2008; Shankar et al., 1999). Perforin is a pore-forming protein essential for the entry of various pro-apoptotic proteases known as granzymes, including granzyme A and B, into infected target cells (Bolitho et al., 2007; Chowdhury and Lieberman, 2008; Pardo et al., 2009a). Granulysin, a member of the saposin-like protein family, may be important to the control of a wide variety of pathogenic bacteria, fungi, and parasites and has also been implicated in tumor surveillance (Clayberger and Krensky, 2003; Krensky and Clayberger, 2009; Stenger et al., 1998).

The transcriptional regulation of cytolytic effector cells has recently been an area of immense interest (Glimcher et al., 2004). One transcription factor, T-bet, has been shown to play a pivotal role in the development, differentiation, and function of effector cells. A member of the T-box family of transcription factors (Papaioannou, 1997; Smith, 1997; Zhang and Yang, 2000), T-bet was originally discovered as the major determinant of Th1 lineage commitment in $CD4^+$ T cells (Szabo et al., 2000). Subsequent work has demonstrated that it is also important for the effector differentiation of $CD8^+$ T cells (Intlekofer et al., 2007; Joshi et al., 2007; Sullivan et al., 2003). T-bet is essential for the development of many experimental autoimmune diseases, including type 1 diabetes and myocarditis in transgenic mice (Juedes et al., 2004; Taqueti et al., 2006); in both models, pathogenic $CD8^+$ T cells are thought to be causally associated with the onset of disease,

which is mitigated or prevented in the absence of T-bet. Additionally, T-bet can bind to the promoter regions of perforin and granzyme B in both human and mouse cells (Townsend et al., 2004) and influences the levels of expression of these cytolytic molecules (Intlekofer et al., 2005; Pearce et al., 2003). Although the biology of T-bet has been well studied in experimental murine models, the significance of this transcription factor in the context of human infection is less well defined.

Here, we examined the expression of T-bet in both resting and activated $CD8^+$ T cells from HIV-infected and uninfected individuals. We hypothesized that T-bet may play a causative role in the increased cytotoxic potential that has been described for HIV-specific $CD8^+$ T cells from EC (Hersperger et al., 2010; Migueles et al., 2002; Migueles et al., 2008). We report that HIV-specific $CD8^+$ T cells from EC demonstrated higher T-bet expression after both short-term stimulation and *in vitro* proliferation than those from individuals who were not controlling HIV replication ("progressors") or who were controlling virus only through the use of highly active anti-retroviral therapy ("HAART-suppressed"). We also observed that T-bet expression correlated strongly with perforin and granzyme B. Our results suggest that T-bet may be influencing the effector function and cytotoxic potential of HIV-specific $CD8^+$ T cells and could be an attractive target for modulation to improve HIV-specific immunity.

Materials and Methods

<u>*Human subjects*</u>: We examined HIV-specific $CD8^+$ T cell responses in a cross-sectional cohort of 20 elite controllers (EC), 18 chronically-infected progressors (CP), and 19 individuals on HAART. The majority of peripheral blood mononuclear cell (PBMC)

specimens were obtained from patients enrolled in the SCOPE study at the University of California, San Francisco (UCSF) (Emu et al., 2008). PBMC samples from a small number of EC and CP were also obtained through clinics associated with the University of Toronto and Harvard University. EC were defined as therapy-naïve individuals who displayed an initial positive HIV antibody test >2 years prior to study entry and had a minimum of three documented viral loads of <75 copies/mL over a period of 12 months. CP were defined as untreated individuals with plasma HIV RNA levels consistently above 10,000 copies/mL. HAART-suppressed individuals maintained undetectable plasma viral loads (<75 copies/mL) for at least a two year span prior to inclusion. All subjects had proximal $CD4^+$ T cell counts above 350 cells/mm^3 and lacked evidence of an AIDS-defining illness (refer to **Table 2** for relevant clinical information). Additionally, PBMC from HIV-negative healthy donors were obtained from the University of Pennsylvania Center for AIDS Research Human Immunology Core.

Antibody reagents: The following antibodies were used in this study: anti-CD8 Qdot 605, anti-CD3 Qdot 655, anti-Granzyme B PE-Cy5.5 (Invitrogen, Carlsbad, CA), anti-TNFα PE-Cy7, anti-IFN-γ APC, anti-CD4 APC-Cy7, anti-CD14 APC-Cy7, anti-CD19 APC-Cy7, (BD Pharmingen, San Jose, CA), anti-Granulysin PE, anti-CD107a PE-Cy5, anti-CD27 PE-Cy5, anti-T-bet PE (clone 4B10) (eBioscience, San Diego, CA), anti-T-bet FITC (clone 4B10; Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD45RO ECD (Beckman Coulter, Miami, FL), anti-Granzyme A Alexa-700 (BioLegend, San Diego, CA), anti-Perforin PE (clone B-D48; Diaclone, Besancon, France), anti-CD57 Qdot 565,

and anti-Perforin Pacific Blue (custom). The anti-Perforin monoclonal antibody (clone B-D48) purchased from Diaclone was conjugated to Pacific Blue in our laboratory.

PBMC stimulation and proliferation assays: PBMC stimulations and intracellular cytokine staining (ICS) assays were carried out as described in the previous chapter. Briefly, PBMC were resuspended at a concentration of $2x10^6$ cells/mL in a total volume of 1 mL for each condition. Costimulatory antibodies (anti-CD28 and anti-CD48d, 1 μg/ml each; BD Biosciences), monensin (1 μg/ml; BD Biosciences) and Brefeldin A (BFA, 1 μg/ml; Sigma-Aldrich; St. Louis, MO) were also added. Anti-CD107a PE-Cy5 was included at the start of all stimulations to measure levels of degranulation (Betts et al., 2003). PBMC were incubated at 37°C, 5% CO_2 for six hours with peptide pools encompassing HIV-1 Gag (PTE), Nef (clade B consensus) [NIH AIDS Research and Reference Reagent Program], or human cytomegalovirus (CMV) pp65 and IE1 proteins [New England Peptide, Gardner, MA]. Each individual peptide in the pools was at a final concentration of 2 μg/mL. At the conclusion of the six hour stimulation period, cells were labeled with Aqua amine-reactive dye (Invitrogen) prior to staining for surface and intracellular markers, as previously described in detail (Makedonas et al., 2009).

In some experiments that assessed proliferation, PBMC were labeled with CFSE (Invitrogen) prior to stimulation according to the manufacturer's instructions. Cells were stimulated either with individual peptides (determined from prior epitope mapping), the aforementioned HIV Gag or Nef pools, or with a pool of approximately 100 previously identified optimal HIV (clade B) $CD8^+$ T cell epitopes ranging in length from 9 to 11 amino acids covering all regions of the viral proteome. Individual peptides were at a final

concentration of 2 μg/mL in each condition. At various time points after initial peptide stimulation, antigen-specific cells were identified using peptide:MHC-I tetramer (conjugated to APC) or after peptide re-stimulation for six hours in the presence of BFA.

Flow cytometric analysis: For each stimulation condition, at least 500,000 total events were acquired using a modified LSRII (BD Immunocytometry Systems, San Jose, CA). Data analysis was performed using FlowJo (version 8.8.4; TreeStar, Ashland, OR) and Spice (version 4.2.3, Dr. Mario Roederer, NIH, Bethesda, MD). Reported data have been corrected for background based on the negative (no peptide) control, and only responses with a total frequency above 0.10% of total $CD8^+$ T cells (after background subtraction) were considered as positive responses. The contribution of perforin, grz A, grz B, or granulysin to all HIV- or CMV-specific $CD8^+$ T cell responses was calculated individually in combination with IFN-γ^+, TNFα^+, or $CD107^+$ cells. Therefore, in each instance, a total of 16 possible functional combinations were possible. Similar to the analysis carried out in Chapter 3, two combinations were ignored: (1) events negative for all parameters (i.e. IFN-γ^-TNFα^-CD107a$^-$cytolytic protein$^-$) and (2) cytolytic molecule single-positive cells (i.e. perforin single-positive cells, grz A single-positive cells, etc).

Statistical analysis: All statistical analysis was performed using GraphPad Prism software (version 5.0a). Functionality was compared between study groups using nonparametric tests (Mann-Whitney when comparing only two groups; Kruskal-Wallis followed by a Dunns test for multiple comparisons when comparing three or more groups). Correlations were assessed using the Spearman test.

Table 2: Clinical parameters of the HIV-infected study cohort to examine T-bet expression.

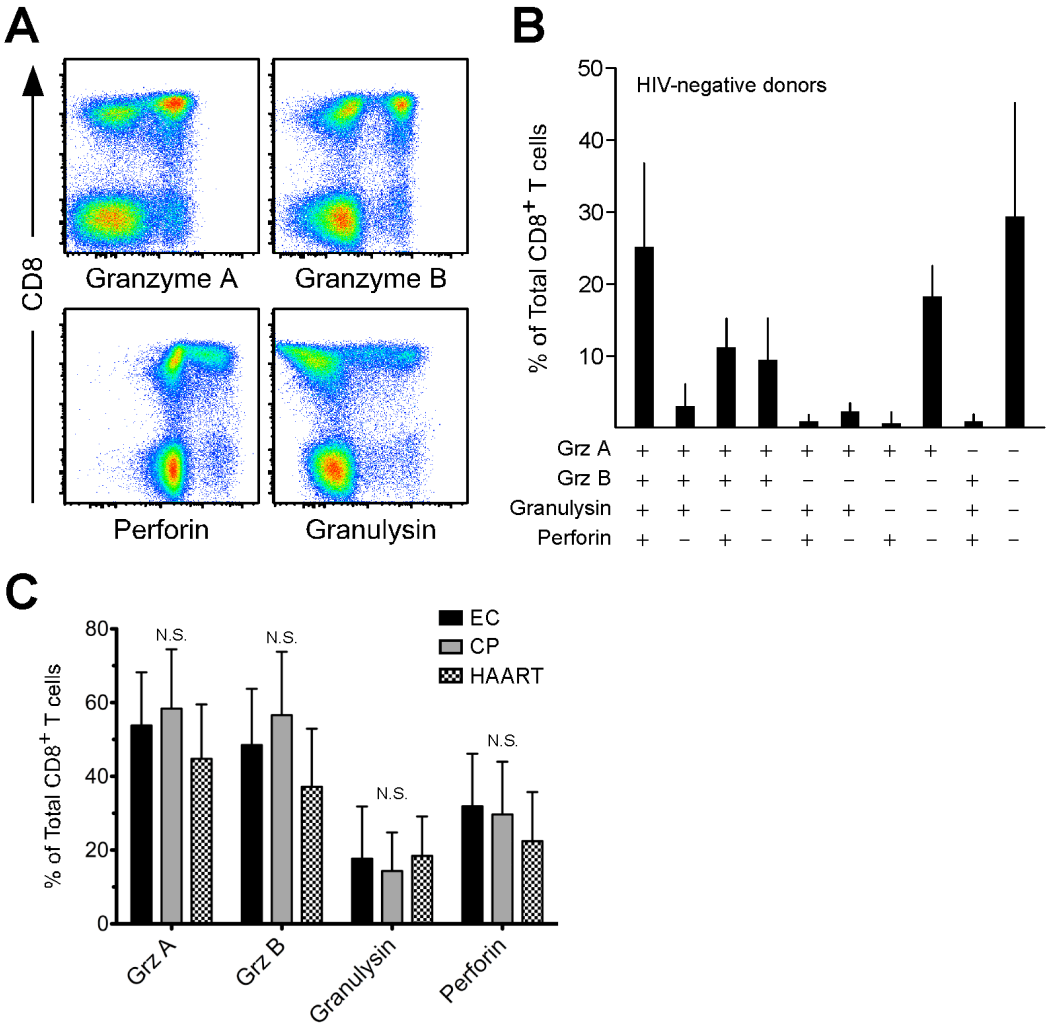
	Elite Controllers	Chronic Progressors	HAART-suppressed
Number of subjects	20	18	19
Plasma HIV RNA, **median** (*IQR*), copies/mL	undetectable	**28,908** (*15,904-49,686*)	undetectable
$CD4^+$ T cell count, **median** (*IQR*), cells/mm^3	**772** (*664-1,133*)	**622** (*461-734*)	**675** (*493-868*)
Infection duration, **median** (*IQR*), years	**17** (*11-20*)	**6** (*3-16*)	**14** (*10-20*)
Duration of HAART treatment prior to specimen sample, **median** (*IQR*), years	N/A	N/A	**5** (*4-7*)

Results

Expression pattern of cytotoxic granule-resident proteins in bulk CD8$^+$ T cells

We recently reported that HIV-specific CD8$^+$ T cells from elite controllers (EC) demonstrated an enhanced ability to express perforin directly *ex vivo* when compared to chronically-infected progressors (CP) (Hersperger et al., 2010). Here, we sought to gain a more complete understanding of CD8$^+$ T cell cytotoxic potential among HIV-positive subjects that demonstrate differential control of virus replication. We studied CD8$^+$ T cell responses using a flow cytometric staining panel that was designed to simultaneously measure the expression of the granule-associated proteins granzyme A (grz A), granzyme B (grz B), granulysin, and perforin (representative staining of each shown in **Fig. 12A**). Importantly, we used an anti-perforin antibody that can detect both pre-formed and newly upregulated perforin after activation (Hersperger et al., 2008; Hersperger et al., 2010; Makedonas et al., 2009).

Initially, we characterized the co-expression of these four molecules in resting bulk CD8$^+$ T cells among HIV-negative donors (n=4; **Fig. 12B**). In general, grz A was the most ubiquitously expressed molecule; it was present in virtually every combination of cytolytic molecules, in agreement with previous findings (Harari et al., 2009; Takata and Takiguchi, 2006). Approximately 25% of the total CD8$^+$ T cell compartment co-expressed all four molecules while on average 50% of all CD8$^+$ T cells expressed either none of the cytolytic proteins or grz A alone. The majority of granulysin was co-expressed with grz A, grz B, and perforin; however, a sizeable fraction of granulysin was observed with either grz A or grz B but not perforin. Upon examination of the HIV-positive cohort, the overall expression patterns of grz A, grz B, granulysin, and perforin



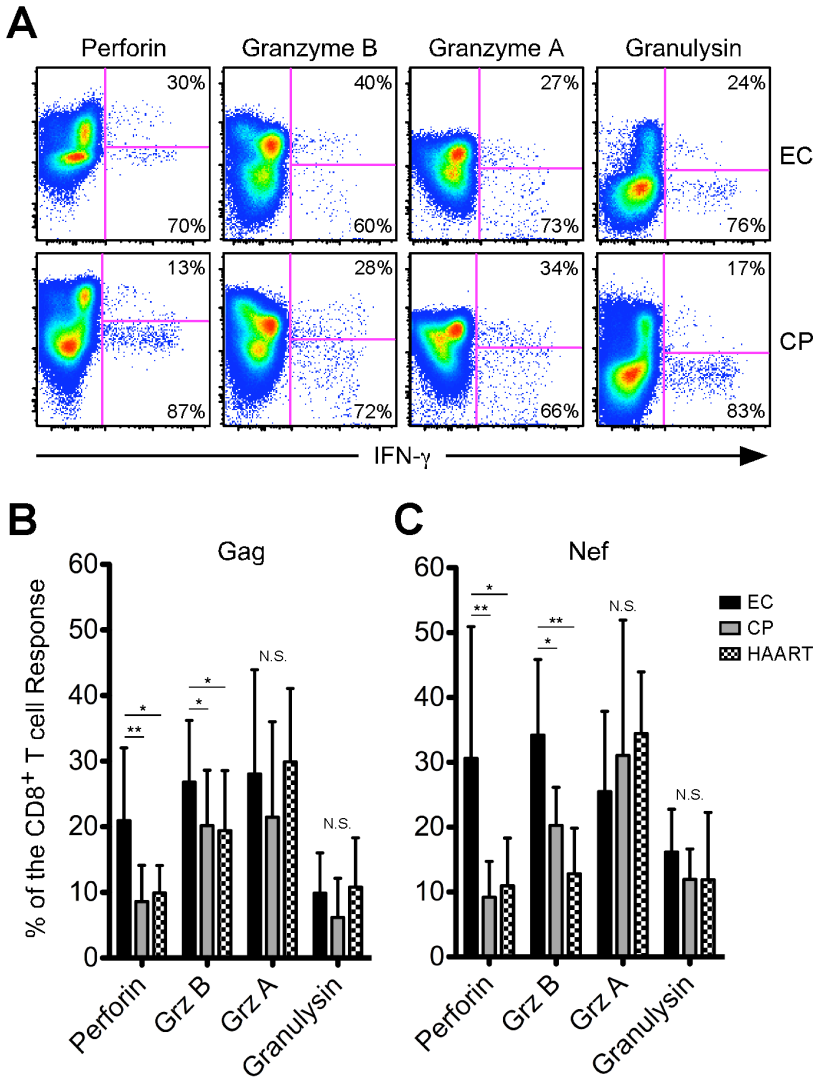
<u>Figure 12</u>: Expression profile of cytotoxic granule-resident proteins among human CD8$^+$ T cells. (A) Representative staining of grz A, grz B, perforin, and granulysin in human lymphocytes. Events shown have been gated on CD3$^+$ T cells. **(B)** The average expression profile of grz A, grz B, perforin, and granulysin in the total CD8$^+$ T cell compartment is shown from four HIV-negative subjects. Permutations that comprised less than 0.5% of CD8$^+$ T cells were omitted. **(C)** The proportion of the total CD8$^+$ T cell pool that expressed grz A, grz B, granulysin, or perforin as single parameters was determined among EC, CP, and HAART-suppressed patients. For each cytolytic molecule, statistical analysis was carried out using one-way ANOVA tests (nonparametric; Kruskal-Wallis) followed by a Dunns test for multiple comparisons. **(B and C)** All bars represent the mean and error bars indicate the standard deviation.

in the total $CD8^+$ T cell compartment was found to be similar (data not shown). Additionally, we found no statistically significant differences between EC, CP, or HAART-suppressed patients in the proportion of the total $CD8^+$ T cell pool that expressed grz A, grz B, granulysin, or perforin as single parameters (**Fig. 12C**).

HIV-specific $CD8^+$ T cells in EC demonstrated a greater ability to express perforin and grz B compared to CP and HAART-suppressed subjects

We next identified HIV- and CMV-specific $CD8^+$ T cell responses among subjects in the HIV-infected cohort by measuring the production of IFN-γ, TNFα, and degranulation following stimulation with overlapping peptide pools. The total $CD8^+$ T cell response magnitude to HIV- or CMV-specific stimulation was similar between the groups (data not shown), consistent with previous studies (Emu et al., 2008; Gea-Banacloche et al., 2000; Hersperger et al., 2010; Migueles et al., 2002). However, the Gag-specific response magnitude was significantly higher in EC than HAART-suppressed individuals. As expected (Sylwester et al., 2005), the total CMV-specific response was greater than the HIV-specific response across all groups.

We next determined the contribution of grz A, grz B, granulysin, and perforin to the HIV-specific $CD8^+$ T cell response in each subject. We consistently observed higher expression of perforin and grz B within responding cells from EC compared to CP or HAART-suppressed individuals (**Fig. 13A** shows example Nef-specific responses producing IFN-γ in a EC and CP subject; HAART-suppressed example not shown). However, co-expression of grz A and granulysin within responding cells appeared similar across the cohort (**Fig. 13A**). Upon examination of the entire HIV-infected cohort in a quantitative manner, perforin and grz B were found to comprise a significantly greater



<u>Figure 13</u>: HIV-specific CD8$^+$ T cells from EC express higher amounts of perforin and granzyme B than progressors after short-term stimulation. (A) Representative flow cytometric plots showing perforin, grz B, grz A, and granulysin versus IFN-γ from one EC (top row) and CP (bottom row). Percentages represent the proportion of IFN-γ expressing cells that were either positive or negative for each cytolytic protein. **(B and C)** The relative contribution of perforin, grz B, grz A, and granulysin to **(B)** Gag- and **(C)** Nef-specific CD8$^+$ T cell responses is shown for EC, CP, and HAART subjects. For each cytolytic molecule, statistical analysis was carried out using one-way ANOVA tests (nonparametric; Kruskal-Wallis) followed by a Dunns test for multiple comparisons. * denotes a p value < 0.05 and ** denotes a p value < 0.01. All bars represent the mean and error bars indicate the standard deviation.

proportion of the average Gag- (**Fig. 13B**) and Nef-specific (**Fig. 13C**) responses in EC compared to both CP and HAART-suppressed subjects; whereas the proportion of the HIV-specific $CD8^+$ T cell response comprised of grz A or granulysin did not differ between the groups (**Fig. 13B and 13C**). We also examined the levels of grz A, grz B, granulysin, and perforin in CMV-specific responses in each group. We found no difference between EC and progressors in the proportion of CMV-specific responses comprised of any measured cytolytic molecule (data not shown).

In summary, HIV-specific $CD8^+$ T cells from EC produced higher levels of perforin and grz B directly *ex vivo* compared to either CP or HAART subjects, but we found no difference in the levels of grz A or granulysin between any cohort group after either HIV- or CMV-specific stimulation. Due to the lack of difference in the levels of grz A or granulysin between the cohort groups, we largely focused on perforin and grz B for the remainder of the this study.

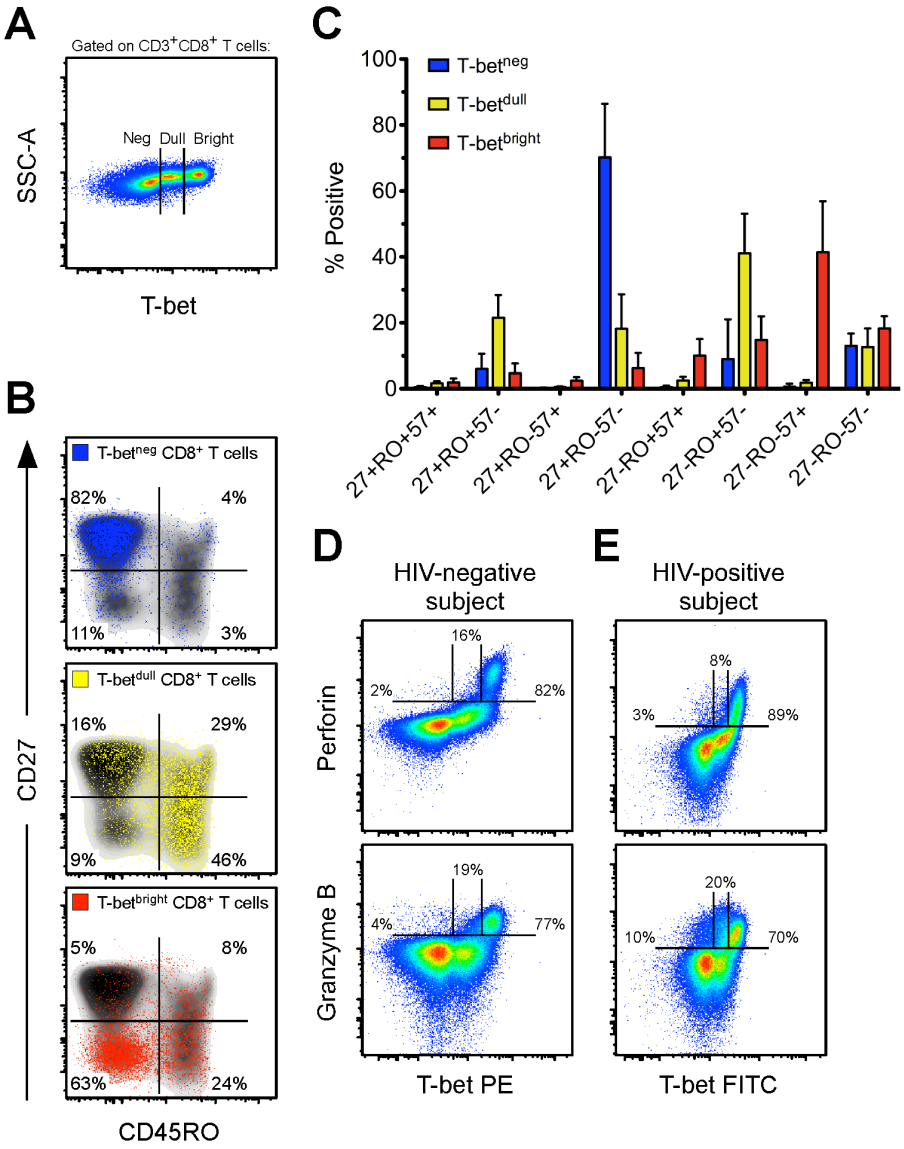
T-bet expression was highly associated with effector $CD8^+$ T cell phenotype and cytotoxic potential

Given that the T-box transcription factor T-bet has been shown to influence effector $CD8^+$ T cell differentiation in mice (Intlekofer et al., 2007; Joshi et al., 2007; Sullivan et al., 2003) and previous work from our laboratory has associated T-bet with effector $CD8^+$ T cells in humans (Makedonas et al., 2010), we hypothesized that T-bet may be influencing the effector differentiation and cytotoxic potential of HIV-specific $CD8^+$ T cells within EC. Initially, we characterized in detail the memory phenotype and cytotoxic potential of human $CD8^+$ T cells that express T-bet. We determined the expression patterns of T-bet among bulk $CD8^+$ T cells from a group of HIV-negative

donors (n=8). As shown in **Figure 14A**, we consistently observed three populations of T-bet in total $CD8^+$ T cells: (1) T-$bet^{negative}$, (2) T-bet^{dull}, and (3) T-bet^{bright}. The T-bet^{neg} cells were almost exclusively $CD27^+CD45RO^-$, the majority of T-bet^{dull} cells were $CD45RO^+$ with variable expression of CD27, and the T-bet^{bright} cells were primarily $CD27^-CD45RO^-$ (**Fig. 14B**). Therefore, the majority of T-bet^{bright} cells displayed the typical phenotypic characteristics of differentiated effector cells (Hamann et al., 1997).

CD57 is a memory marker that is commonly associated with an effector phenotype (Brenchley et al., 2003; Chattopadhyay et al., 2009). $CD8^+$ T cells that lacked or expressed intermediate levels of T-bet were virtually all $CD57^-$ (**Fig. 14C**). The majority of T-bet^{bright} cells also expressed CD57; however, a sizeable fraction were $CD27^-CD45RO^{+/-}CD57^-$ (**Fig. 14C**). Thus, CD57 was associated with the highest levels of T-bet expression, but T-bet^{bright} cells were not exclusively $CD57^+$.

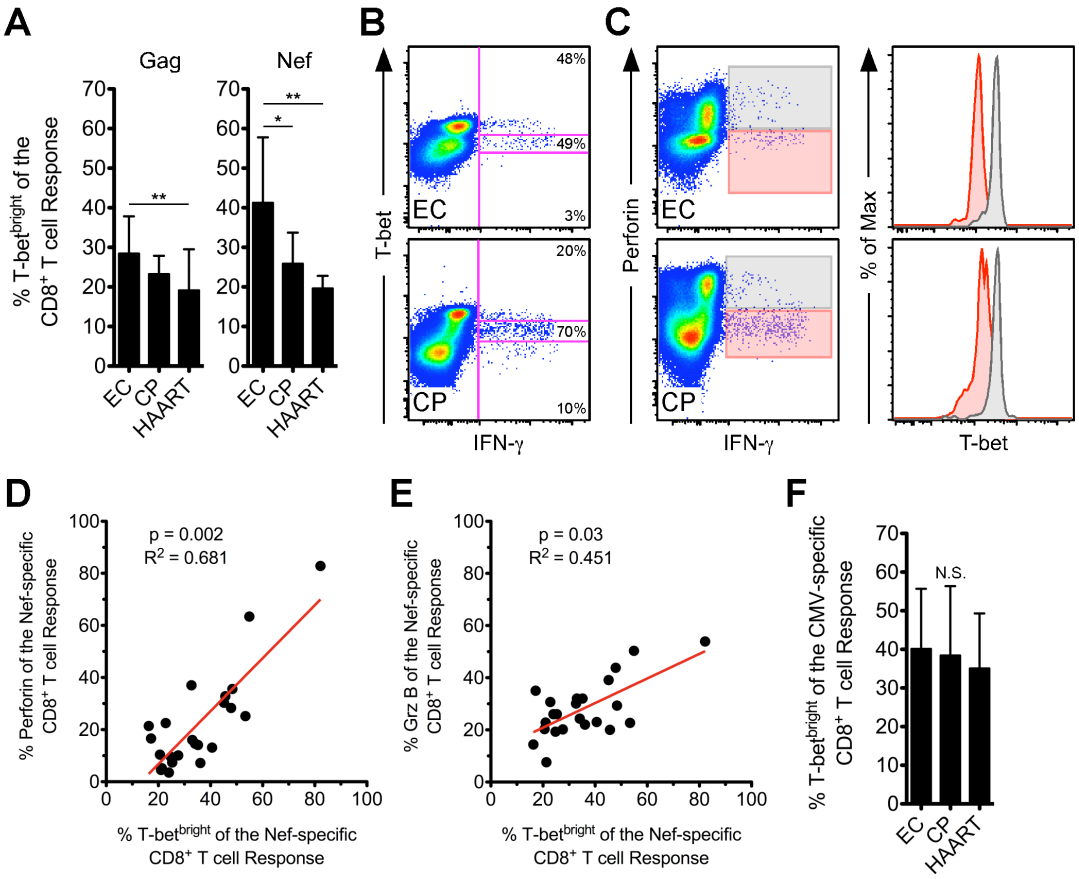
Collectively, these data suggest that most T-bet^{bright} $CD8^+$ T cells are differentiated effector cells. Cells of this memory phenotype have been shown previously to express high levels of perforin and grz B (Chattopadhyay et al., 2009; Takata and Takiguchi, 2006). Consequently, T-bet^{bright} $CD8^+$ T cells expressed perforin and grz B most abundantly compared to both the T-bet^{neg} or T-bet^{dull} $CD8^+$ T cell populations (representative example shown in **Fig. 14D**). These associations between T-bet and cytolytic protein expression were maintained in the HIV-infected cohort as well (representative example shown in **Fig. 14E**). Furthermore, we found a highly significant positive correlation between T-bet^{bright} levels and the expression of both perforin and grz B in the total $CD8^+$ T cell pool among the entire HIV-positive cohort (data not shown).



<u>Figure 14</u>: Effector CD8$^+$ T cells contain the highest levels of T-bet, which is associated with perforin and granzyme B expression. (A) A representative flow cytometric plot of the T-bet staining profile within CD3$^+$CD8$^+$ T cells. **(B)** Representative plots of the memory distribution of the three T-bet populations, which were overlaid onto density plots (black shading) of total CD8$^+$ T cells. Percentages represent the fraction of overlaid cells that fell within each quadrant. **(C)** The memory distributions of the three T-bet populations were established in a quantitative manner for HIV-negative subjects (n=8). All bars represent the mean and error bars indicate the standard deviation. **(D and E)** Representative plots showing T-bet against both perforin and grz B among total CD8$^+$ T cells from a **(D)** HIV-negative and **(E)** HIV-positive subject. Percentages represent the fraction of perforin or grz B that was either negative, dull, or bright for T-bet expression.

HIV-specific CD8$^+$ T cells from EC expressed higher levels of T-bet than CP or HAART-suppressed subjects

We next examined T-bet expression within the HIV-positive cohorts. A significantly higher fraction of Gag- and Nef-specific CD8$^+$ T cell responses were T-bet^{bright} in EC than in CP and/or HAART-suppressed individuals (**Fig. 15A**). However, there was no difference between the groups in T-bet expression among bulk CD8$^+$ T cells (data not shown). We observed that a larger percentage of responding HIV-specific CD8$^+$ T cells among EC fell within the T-bet^{bright} gate than progressors (example Nef responses shown in **Fig. 15B**) or HAART-suppressed subjects (data not shown). Consistent with the observations from total resting CD8$^+$ T cells (**Fig. 14**), perforin$^+$ HIV-specific CD8$^+$ T cells from both EC and CP demonstrated higher levels of T-bet than perforin$^-$ cells (representative examples shown in **Fig. 15C**). Additionally, we found a positive correlation between the levels of Nef-specific perforin (**Fig. 15D**) and grz B (**Fig. 15E**) and the fraction of the Nef response that was T-bet^{bright}; however, we found no correlation between levels of Nef-specific grz A or granulysin and the fraction of the response that was T-bet^{bright} (data not shown). Finally, despite the difference in T-bet expression among HIV-specific responses between the cohort groups, no such difference in the levels of T-bet was observed for CMV-specific CD8$^+$ T cell responses between EC, CP, or HAART subjects (**Fig. 15F**), which suggests that progressors do not suffer from a global immune defect.

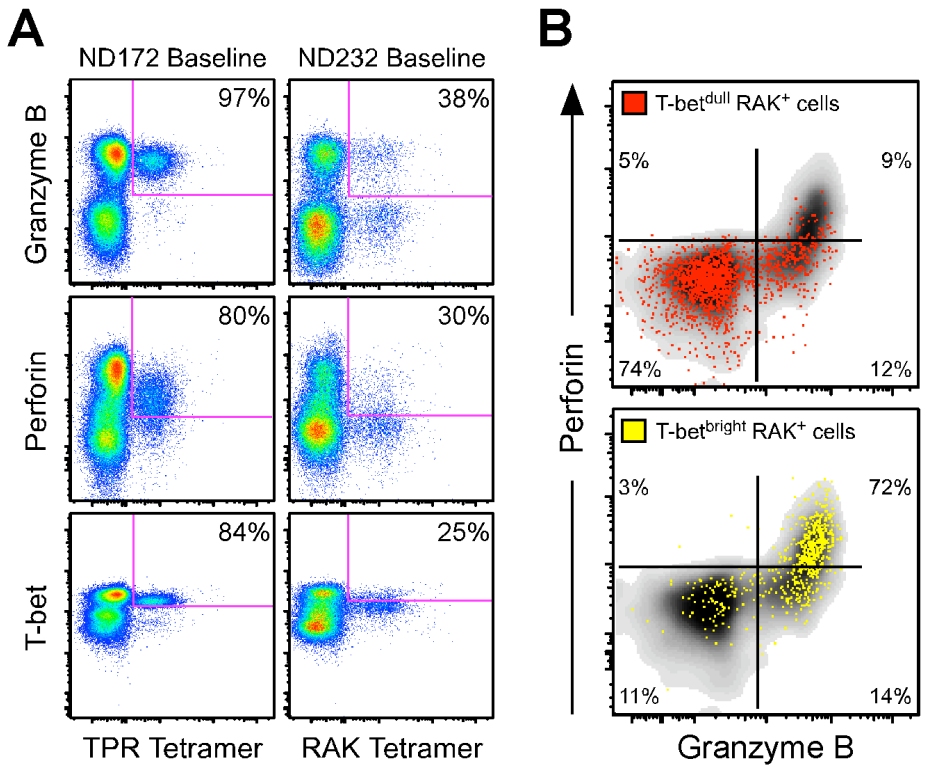


<u>Figure 15</u>: HIV-specific CD8$^+$ T cells from EC express higher amounts of T-bet than progressors after short-term stimulation. (A) The fraction of Gag- or Nef-specific CD8$^+$ T cells that fell within the T-bet^{bright} gate was determined for EC, CP, and HAART subjects. **(B)** Representative flow cytometric plots from an individual EC and CP showing the fraction of the Nef-specific response, as determined by the production of IFN-γ, that fell within the three T-bet gates. Events shown have been gated on CD8$^+$ T cells. **(C)** Relative levels of T-bet expression are shown for perforin$^+$IFN-γ$^+$ (gray) and perforin$^-$IFN-γ$^+$ (red) CD8$^+$ T cells from a Nef-specific response in the same EC (top) and CP (bottom) as shown in (B). **(D and E)** The proportion of Nef-specific **(D)** perforin and **(E)** grz B expression was plotted against the fraction of the response that was T-bet^{bright} within each EC and CP subject. HAART-suppressed individuals were not included. Spearman correlation tests (nonparametric; two-tailed) were performed to determine statistical significance. **(F)** The fraction of CMV-specific CD8$^+$ T cells that fell within the T-bet^{bright} gate was determined for EC, CP, and HAART subjects. **(A and F)** Statistical analysis was carried out using one-way ANOVA tests (nonparametric; Kruskal-Wallis) followed by a Dunns test for multiple comparisons.* denotes a p value < 0.05 and ** denotes a p value < 0.01. All bars represent the mean and error bars indicate the standard deviation.

T-bet is rapidly upregulated along with perforin and grz B during virus-specific recall responses

Next, we determined how the expression of T-bet was modulated over the course of an antigen-specific recall response. We began by examining two HIV-negative donors in whom we had previously identified high magnitude peptide-specific $CD8^+$ T cell responses. ND172 had a $CD8^+$ T cell response directed against the HLA-B7-restricted epitope TPRVTGGGAM from the HCMV pp65 tegument protein. Approximately 10% of all $CD8^+$ T cells were TPR-specific by tetramer staining directly *ex vivo* in this donor (data not shown). The second subject, ND232, had a $CD8^+$ T cell response to the HLA-B8-restricted epitope RAKFKQLL derived from the EBV BZLF1 protein. Approximately 5% of total $CD8^+$ T cells were RAK-specific by tetramer staining at baseline (data not shown). Prior to *in vitro* stimulation, a much higher fraction of the TPR-specific cells compared to the RAK-specific cells were positive for perforin and grz B (**Fig. 16A**). Additionally, 84% of TPR-specific cells were T-bet^{bright} at baseline, whereas only 25% of RAK-specific cells achieved a similar level of T-bet expression (**Fig. 16A**). Of the T-bet^{dull} RAK-specific cells, only 9% were perforin$^+$grz B$^+$, whereas 72% of T-bet^{bright} RAK-specific cells were positive for both cytolytic proteins (**Fig. 16B**).

We subsequently performed *in vitro* proliferation assays using CFSE-labeled PBMC and examined levels of T-bet, perforin, and grz B within antigen-specific cells. After 24 hours, there was complete loss of the ability to identify TPR- or RAK-specific $CD8^+$ T cells using tetramer (data not shown). Thus, in order to identify antigen-specific cells, we performed overnight IFN-γ ICS assays using TPR or RAK peptides. There were no substantial changes in overall T-bet, perforin, or grz B levels at this time point



<u>Figure 16</u>: Baseline staining for T-bet, perforin, and granzyme B in antigen-specific CD8$^+$ T cells prior to stimulation. (A) The degree of grz B, perforin, and T-bet^{bright} positivity was determined at baseline for TPR- and RAK-specific CD8$^+$ T cells (labeled by peptide:MHC-I tetramer) from two HIV-negative donors, ND172 and ND232, respectively. Percentages represent the fraction of tetramer$^+$ cells that fell within each gate. **(B)** Perforin and granzyme B expression are limited to RAK-specific cells that express the highest amounts of T-bet. RAK-specific CD8$^+$ T cells were identified at baseline in ND232 by tetramer staining as in (A). RAK-specific cells that were T-bet^{dull} (red) or T-bet^{bright} (yellow) were overlaid onto a density plot (black shading) of total CD8$^+$ T cells showing perforin vs. grz B expression. Percentages represent the fraction of overlaid cells that fell within each quadrant.

compared to the expression profiles at baseline (data not shown). This result was confirmed in five additional HIV-negative subjects by comparing T-bet expression levels at six and 24 hours after SEB stimulation in responding $CD8^+$ T cells (data not shown).

After two days, there was still no detectable tetramer staining (data not shown); consequently, we carried out peptide re-stimulation ICS assays to identify RAK- and TPR-specific cells. As shown in **Figure 17A**, we observed a dramatic change in the expression of T-bet, perforin, and grz B within RAK-specific cells. By 48 hours after initial activation, all detectable RAK-specific cells were now positive for T-bet, perforin, and grz B; a similar phenomenon was also observed for TPR-specific cells (data not shown). Interestingly, the median fluorescence intensity (MFI) of T-bet within RAK-specific cells at day two was higher than the MFI of the T-bet^{bright} population at baseline in ND232 (compare **Fig. 17A to 16A**). In fact, an apparent fourth population of T-bet-expressing cells (T-bet^{+++}) appeared by 48 hours (**Fig. 17A**); this phenomenon was not observed in the negative (DMSO/no peptide) control (data not shown). The ability to stain RAK-specific cells with tetramer returned by day three (**Fig. 17B**). Consistent with the 48 hour time point, all RAK tetramer$^+$ cells were grz B$^+$, perforin$^+$, and T-bet^{+++}; peptide re-stimulation at day three revealed similar results (**Fig. 17C**). Notably, the appearance of a T-bet^{+++} population also occurred in ND172 after TPR peptide stimulation (data not shown).

We next examined the relationship between cell division and the modulation of T-bet, perforin, and grz B. At day five, we noticed that even the small number of RAK-specific cells that did not divide had upregulated T-bet, perforin, and grz B to a similar level as cells that had diluted CFSE (**Fig. 18A**), which is consistent with data from

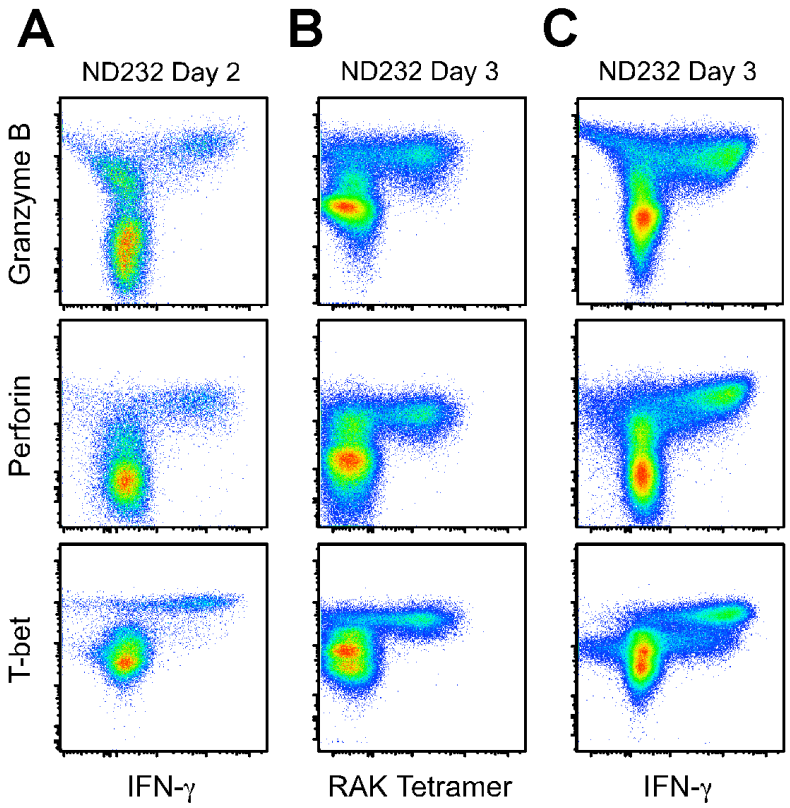
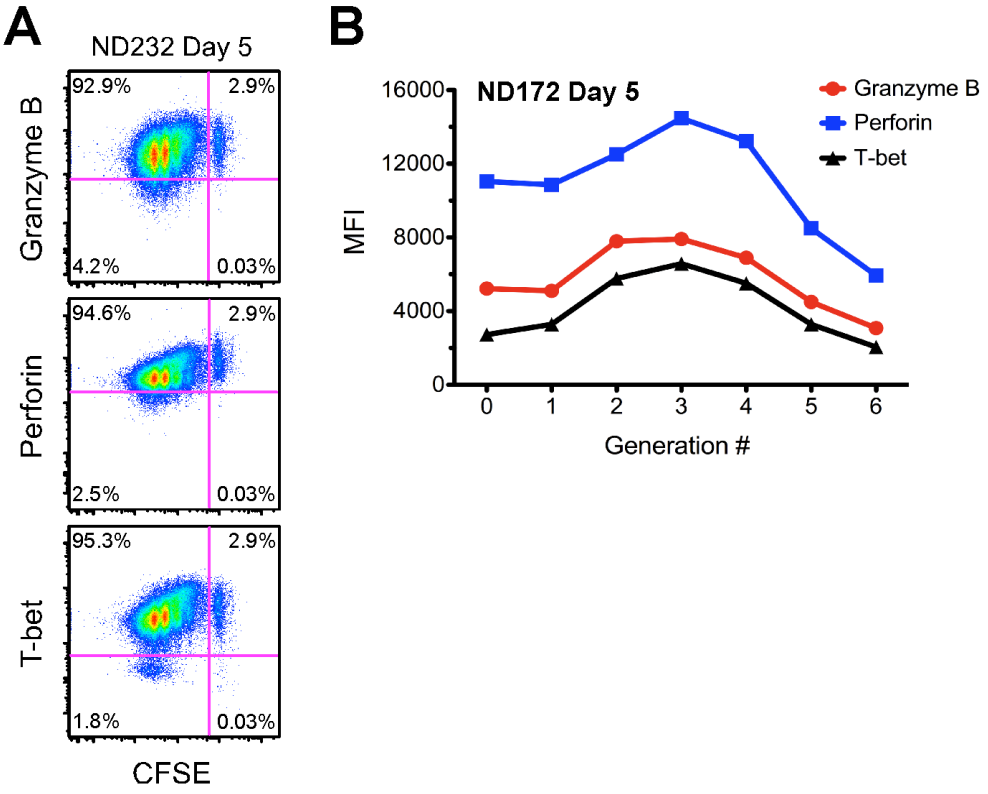


Figure 17: Dramatic concurrent increase in T-bet, perforin, and granzyme B expression occurs shortly after antigen-specific stimulation. (A-C) PBMC from ND232 were incubated for either **(A)** two or **(B and C)** three days in the presence of RAK peptide. The levels of grz B, perforin, and T-bet are shown within RAK-specific cells, which were identified either by **(A and C)** peptide re-stimulation for six hours followed by staining for IFN-γ or by **(B)** tetramer staining. All events shown have been gated on $CD8^+$ T cells.



<u>Figure 18</u>: The kinetics of T-bet, perforin, and granzyme B upregulation are tightly linked during an antigen-specific recall response. (A) CFSE-labeled PBMC from ND232 were incubated for 5 days in the presence of RAK peptide and the expression of grz B, perforin, and T-bet was subsequently determined within RAK-specific cells. All flow cytometric plots show only RAK-specific cells, as determined by tetramer staining. Percentages represent the fraction of tetramer^{+} cells that fell within each quadrant. **(B)** CFSE-labeled PBMC from ND172 were incubated for 5 days in the presence of TPR peptide. The median fluorescence intensity (MFI) of grz B, perforin, and T-bet within TPR-specific cells was plotted against each generation number, which was established partly based on the dilution of CFSE dye by the FlowJo analysis software.

mouse models showing T-bet upregulation after TCR stimulation in the absence of proliferation (Lighvani et al., 2001). We gated on each successive generation (zero through six) of tetramer$^+$ cells at day five and plotted the MFI of T-bet, perforin, and grz B against the respective division number. The kinetics of T-bet, perforin, and grz B upregulation were tightly linked for both TPR-specific cells in ND172 (**Fig. 18B**) and RAK-specific cells in ND232 (data not shown).

HIV-specific CD8$^+$ T cells from EC display higher T-bet expression after proliferation

We next studied the levels of T-bet, perforin, grz B, and grz A after proliferation of HIV-specific CD8$^+$ T cells. We compared the expression of these proteins in a subset of EC and CP following five days of *in vitro* stimulation with various HIV peptide pools. As expected (Horton et al., 2006; Migueles et al., 2002), HIV-specific CD8$^+$ T cells from EC demonstrated a greater proliferative ability than CP (representative examples shown in **Fig. 19A**). A median of 1.01% of CD8$^+$ T cells diluted CFSE after HIV-specific stimulation in EC whereas only a median of 0.41% of CD8$^+$ T cells did so in CP (p=0.03; data not shown). A significantly higher fraction of IFN-γ^+CFSE^{low} cells from EC expressed perforin and grz B compared to CP at day five (**Fig. 19B**). However, there was no difference in the upregulation of grz A between the two groups (**Fig. 19B**), in agreement with a prior report (Migueles et al., 2008). Notably, there was significantly higher levels of T-bet within IFN-γ^+CFSE^{low} cells from EC compared to CP **(Fig. 19C and 19D)**. Additionally, we found a positive correlation between the levels of T-bet within IFN-γ^+CFSE^{low} cells and the corresponding amount of perforin and grz B expression for each respective subject (data not shown).

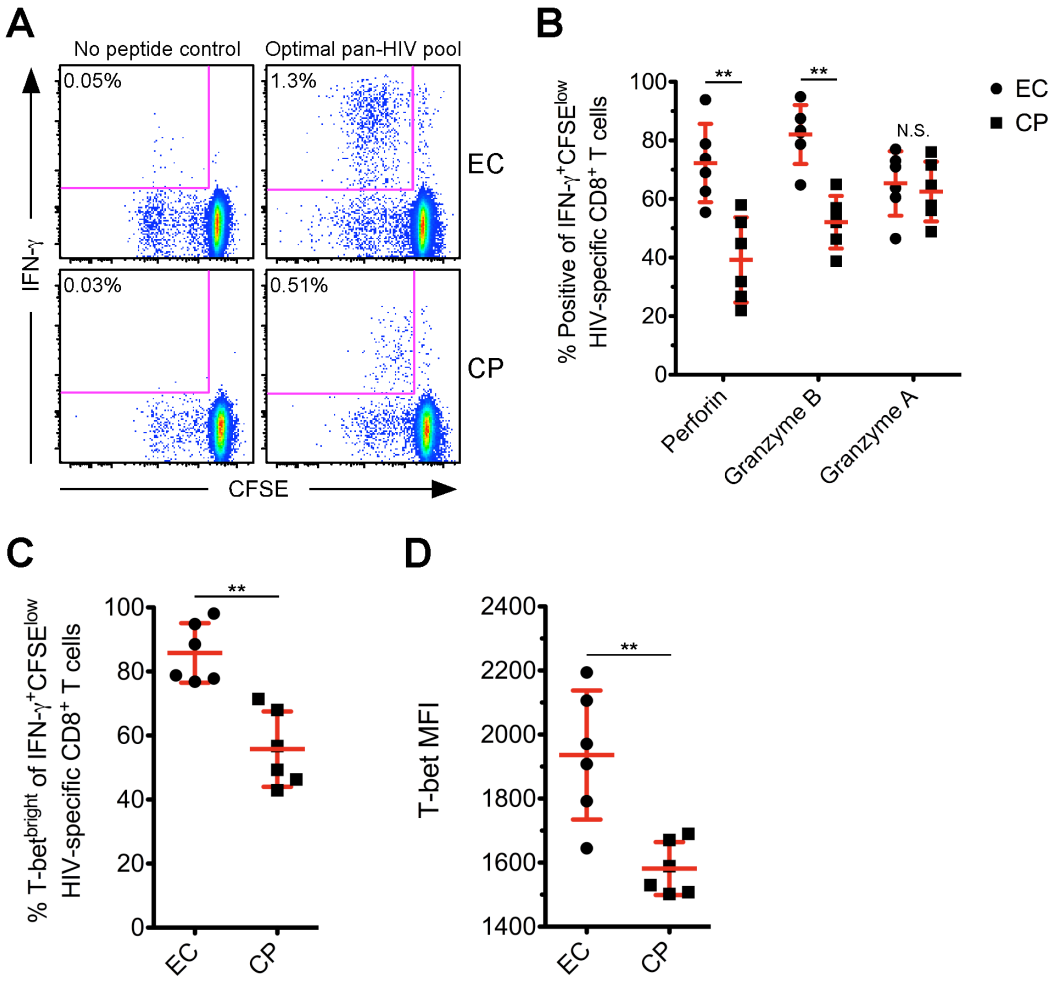


Figure 19: HIV-specific CD8$^+$ T cells from EC express higher amounts of T-bet than progressors after proliferation. (A) CFSE-labeled PMBC from a subset of EC and CP were stimulated for 5 days in the presence of a pool of Nef, Gag, or optimal 9-11mer HIV peptides. Representative flow cytometric plots show HIV-specific CD8$^+$ T-cells, as determined by the production of IFN-γ following re-stimulation, that have proliferated. Percentages represent the fraction of total CD8$^+$ T-cells within each gate. **(B)** The proportion of IFN-γ$^+$CFSE^{low} HIV-specific CD8$^+$ T-cells positive for perforin, grz B, and grz A was determined on day five for each EC and CP subject. **(C and D)** T-bet expression was also determined on day five following HIV-specific stimulation with levels reported as the **(C)** percentage or **(D)** MFI among IFN-γ$^+$CFSE^{low} HIV-specific CD8$^+$ T-cells. **(D)** The reported values indicate the difference between the T-bet^{neg} population and the T-bet MFI of the IFN-γ$^+$CFSE^{low} cells for each subject. **(B-D)** Mann-Whitney tests (nonparametric; two-tailed) were performed to compare EC to CP for each functional parameter. ** denotes a p value < 0.01. Error bars indicate the mean and standard deviation.

Discussion

Several lines of evidence have suggested that the presence and maintenance of effector function by HIV-specific $CD8^+$ T cells is critical for control of viral replication. In this study, we comprehensively examined cytolytic protein expression by HIV-specific $CD8^+$ T cells. We found that HIV-specific $CD8^+$ T cells from EC demonstrated higher perforin and grz B expression than HIV-infected progressors or HAART-suppressed individuals; however, we found no difference in the levels of grz A or granulysin between the groups. We observed that T-bet, a critical transcriptional regulator of effector $CD8^+$ T cell differentiation and function (Intlekofer et al., 2007; Joshi et al., 2007; Sullivan et al., 2003), was expressed to a higher degree in HIV-specific $CD8^+$ T cells from EC compared to progressors or HAART suppressed subjects. Moreover, the link between T-bet, perforin, and grz B was observed in both short-term ICS assays and during proliferation of antigen-specific $CD8^+$ T cells.

As mentioned above, we observed higher expression of perforin and grz B from HIV-specific $CD8^+$ T cells in EC compared to progressors using *ex vivo* ICS assays. It is known that there is a tendency for perforin-expressing $CD8^+$ T cells to co-express grz B both at rest (Takata and Takiguchi, 2006) and after activation (Makedonas et al., 2010). In agreement with this, we found a positive correlation between the expression of perforin and grz B within activated $CD8^+$ T cells (data not shown). These data suggest that HIV-specific $CD8^+$ T cells in EC have a superior cytotoxic potential, which can be attributed to their higher expression of perforin and grz B. Indeed, recent work from Migueles and colleagues (Migueles et al., 2008) demonstrated that higher amounts of perforin and grz B within *in vitro* expanded HIV-specific $CD8^+$ T cells translated into an

enhanced ability to lyse infected targets. Therefore, our findings suggest that HIV-specific $CD8^+$ T cells in EC have a higher capacity to eliminate HIV-infected targets than progressors due to their higher expression of cytolytic molecules. Yet the fact that there was no difference in the production of perforin or grz B by CMV-specific $CD8^+$ T cells between EC and CP implies an HIV-specific phenomenon rather than a global deficiency in effector function within progressors. Additionally, our results indicate that low HIV viral load by itself (through the use of HAART) is unlikely to be an explanation for the higher levels of perforin and grz B seen in EC, a finding that is consistent with previous reports (Hersperger et al., 2010; Migueles et al., 2009).

HIV-specific $CD8^+$ T cells from CP contain more grz A on average than perforin or grz B. While HIV-specific $CD8^+$ T cells from EC certainly do express grz A, they generally contain higher amounts of perforin and grz B relative to progressors. Notably, prior research has implicated grz B as the major effector granzyme that induces target cell death, whereas grz A has been shown to be less cytotoxic or not at all (Pardo et al., 2009a). Accordingly, Harari and colleagues reported a positive correlation between target cell lysis and the levels of perforin and/or grz B in virus-specific $CD8^+$ T cells (Harari et al., 2009). However, no relationship was found between levels of grz A and target cell destruction in the same report. Therefore, HIV-specific $CD8^+$ T cells from EC in general contain higher amounts of the cytotoxic molecules previously shown to be crucial to target cell cytolysis, which most likely explains the increased cytotoxic capabilities of HIV-specific $CD8^+$ T cells from EC (Chen et al., 2009; Migueles et al., 2008; O'Connell et al., 2009b; Saez-Cirion et al., 2007).

We recently reported that there is an enrichment of HIV-specific effector $CD8^+$ T cells in EC that express perforin (Hersperger et al., 2010). The memory phenotype of these perforin-expressing cells was primarily $CD27^-CD45RO^-CD57^{+/-}$. The fact that T-bet^{bright} cells displayed a strikingly similar memory phenotype - combined with the positive correlation with perforin - led us to hypothesize that there may be higher levels of T-bet within HIV-specific $CD8^+$ T cells from EC compared to CP. While originally discovered as the major determinant of Th1 lineage commitment in $CD4^+$ T cells (Szabo et al., 2000), T-bet has subsequently been shown to be important for the differentiation and function of effector $CD8^+$ T cells (Intlekofer et al., 2007; Joshi et al., 2007; Sullivan et al., 2003). In this study, we identified three distinct populations of T-bet within human $CD8^+$ T cells: a bright, dull, and negative population. Interestingly, we found that the T-bet^{bright} population expressed the highest levels of perforin and granzyme B. Therefore, high T-bet expression is indicative of elevated cytotoxic potential within human antigen-specific $CD8^+$ T cells. The association we found between T-bet, perforin, and grz B in resting or short-term activated $CD8^+$ T cells was accentuated following *in vitro* proliferation. For example, EBV-specific $CD8^+$ T cells from one of our HIV-negative donors demonstrated a dramatic capacity to simultaneously upregulate T-bet, perforin, and grz B within two days of *in vitro* activation despite initially expressing low levels of these proteins. Additionally, we observed that dividing HIV-specific $CD8^+$ T cells from EC displayed a greater ability than CP to upregulate T-bet, perforin, and grz B after *in vitro* stimulation, an extension of the prior work performed by Migueles, Connors, and colleagues (Migueles et al., 2002; Migueles et al., 2008). Collectively, these data suggest that the underlying defect(s) in effector function by HIV-specific $CD8^+$ T cells from

progressors lie not in the cytolytic proteins themselves, but rather in the elements controlling their expression, including T-bet.

In summary, T-bet appears to be an excellent marker for the presence of effector function and cytotoxic capacity in the monitoring of HIV vaccine and therapeutic efforts. Our work reinforces the importance of studying the regulatory elements that influence effector function and the expression of cytolytic molecules, which is fortunately an area of increasing attention (Araki et al., 2008; Cruz-Guilloty et al., 2009; Glimcher et al., 2004; Intlekofer et al., 2005; Lu et al., 2003; Pearce et al., 2003; Pipkin et al., 2010a; Taylor et al., 2010). Additionally, modulation of T-bet may lead to enhanced effector activity against HIV with the goal of improved clinical outcomes and a delay in disease progression among infected individuals.

CHAPTER 5

EPILOGUE

Implications and considerations

At the beginning of this work in Chapter 2, we described the rapid upregulation of perforin following activation as a novel functional capability of human antigen-specific $CD8^+$ T cells. We characterized the flow cytometric detection of *de novo* perforin synthesis by using an anti-perforin monoclonal antibody, generated against a recombinant form of the protein, that is able to bind to a conformationally-independent epitope and, therefore, amenable to intracellular cytokine staining (ICS) formats. The ability to detect multiple forms of perforin suggests that many aspects of its protein expression, trafficking, structure, and mechanism of action remain to be elucidated. Indeed, we have recently published that newly produced perforin can traffic directly to the immunological synapse without first being loaded into cytotoxic granules (Makedonas et al., 2009).

Since we are primarily interested in studying the immune response in humans to viral pathogens, we next made use of this ability to measure perforin upregulation in a large cohort of HIV-infected patients that differentially controlled viral replication. This was a unique collection of infected individuals whereby some controlled HIV naturally to undetectable levels (elite controllers; EC), maintained viral replication within a low but detectable range (viremic controllers; VC), demonstrated high viral loads with declining $CD4^+$ T cell levels (chronic progressors; CP), displayed high levels of viremia but were nonprogressors in clinical terms (viremic nonprogressors; VNP), or controlled HIV to undetectable levels only because of drug therapy (HAART-suppressed). Due to the

central role played by perforin in cell-mediated cytotoxicity and target cell destruction, we hypothesized that HIV-specific $CD8^+$ T cells among EC may have an enhanced capacity to upregulate this protein compared to the other cohort groups that are unable to control HIV. As reported in Chapter 3, this is indeed what we observed.

While the majority HIV-infected individuals progress to AIDS with the first decade of infection, EC rarely develop opportunistic infections and also typically display sustained control over viral replication for many years. However, the mechanism(s) to explain their ability to naturally control HIV has remained largely unknown. Recent evidence has shown that HIV-specific $CD8^+$ T cells from EC exhibit enhanced functionality compared to individuals with progressive disease and, therefore, may play an important role in the favorable clinical outcomes witnessed among EC. We showed in this work that the ability to control HIV replication in EC is associated with the *ex vivo* upregulation of perforin, a critical molecule that enables $CD8^+$ T cells to directly kill infected cells. Thus, we identified a functional capability of $CD8^+$ T cells, readily measured by standard flow cytometric assays, that potentially has a direct impact on HIV replication *in vivo*. Collectively, our findings point to the importance of measuring the effector capability of immune responses and may even provide an important qualifier for future HIV vaccine research.

The study of mouse immunology has greatly advanced our understanding of the generation and maintenance of $CD8^+$ T cell cytolytic functionality. Through the ability to knock-out certain genes and manipulate various aspects of the mouse immune system, some of the critical factors that influence effector differentiation and cytolytic capacity have been elucidated (Glimcher et al., 2004). Two T-box transcription factors, T-bet and

Eomesodermin (Eomes), have been shown in the murine system to be particularly critical for the expression of perforin, granzyme B, and other features of effector $CD8^+$ T cells. As reported in Chapter 4, we examined the expression of T-bet within human $CD8^+$ T cells in both a global and virus-specific manner.

Similar to the previous study, we assembled a cohort of HIV controllers (EC), non-controllers (CP), and HAART-suppressed subjects. We studied the expression of T-bet within $CD8^+$ T cells together with a collection of granule-resident proteins: perforin, granzyme A, granzyme B, and granulysin. We found that when compared to the other cohort groups, HIV-specific $CD8^+$ T cells from EC demonstrated a superior ability to express perforin [thereby corroborating the results of our previous study] and granzyme B but with no detectable difference in the levels of granzyme A or granulysin. We also observed higher levels of T-bet in HIV-specific $CD8^+$ T cells from EC, with an ensuing positive correlation between T-bet and levels of both perforin and granzyme B. Moreover, HIV-specific $CD8^+$ T cells in EC upregulated T-bet to a greater extent than progressors after *in vitro* proliferation with concomitant upregulation of perforin and granzyme B. Therefore, these results suggest that T-bet may be playing an important role in driving effector function in human $CD8^+$ T cells and its modulation may lead to enhanced effector activity against HIV.

Although the biology of T-bet has been well studied in experimental murine models, the significance of this transcription factor in the context of human infection is much less well defined. Here, we have demonstrated an association between T-bet expression within HIV-specific $CD8^+$ T cells and the ability to control HIV replication.

Notably, a recently published study from van Lier and colleagues that examined CMV-specific $CD8^+$ T cells corroborates our findings from the setting of HIV infection. These authors performed a comprehensive longitudinal analysis of CMV-specific $CD8^+$ T cell populations from transplant patients (seropositive donor organ into a seronegative recipient) after defined primary infection with CMV (Hertoghs et al., 2010). At all stages CMV infection, it was found that CMV-specific $CD8^+$ T cells expressed T-bet in parallel with the continuous expression of perforin and granzyme B. Therefore, it appears that T-bet is tightly linked to the effector function of human $CD8^+$ T cells in the setting of multiple viral infections. These data reinforce the importance of studying the regulatory elements that influence effector function and the expression of cytolytic molecules.

Despite high rates of seroprevalence in humans, CMV infection is largely benign except in immunocompromised individuals (Britt, 2008). CMV-specific $CD8^+$ T cell responses are among the highest magnitude immune responses witnessed in humans (Sylwester et al., 2005) and are thought to play a major role in keeping the replication of CMV in check. $CD8^+$ T cells specific for CMV antigens have been well studied and are considered as prototypical effectors in many studies. They maintain high levels of perforin and granzymes (Chattopadhyay et al., 2009; Harari et al., 2009) and display phenotypic characteristics of differentiated effector $CD8^+$ T cells [i.e. downmodulation of CD27 and CD28 and surface expression of CD57 and CD45RA] (Appay et al., 2002; Kern et al., 1999). Due to the effector status of CMV-specific $CD8^+$ T cells, they generally lack lymphoid homing capability and instead traffic to peripheral tissues and other extralymphoid sites (Pipeling et al., 2008; Pitcher et al., 2002).

The effector-like nature of CMV-specific $CD8^+$ T cells, coupled with their ability to seed peripheral sites, has led some researchers to propose using replication-competent CMV vectors as a vaccination strategy to induce anti-HIV T cell responses (Hansen et al., 2009). Broadly speaking, the major goal of a T cell-based vaccine is to elicit HIV-specific $CD8^+$ T cells that can limit acute-phase HIV replication, thereby decreasing subsequent chronic-phase viremia. Importantly, a lower set point viral load is associated with improved clinical outcomes, longer duration of AIDS-free survival, and a reduced possibility of transmission to an uninfected person. Studies of vaccinated monkeys challenged with SIV have supported the concept that vaccine-induced T cells can restrict primary infection or partially control chronic viral replication (Horton et al., 2002; Letvin et al., 2006; Liu et al., 2009; Wilson et al., 2006). Unfortunately, a recent attempt to induce HIV-specific T cells in humans using replication-incompetent Adenovirus vectors failed to achieve similar results; indeed, the vaccine did not show any protection or efficacy (Buchbinder et al., 2008).

The vast majority of current T cell vaccine platforms use nonpersistent vectors that only produce antigens for a limited time. Consequently, once the vaccine-associated antigens disappear, the elicited T cells circulate primarily within lymphoid tissues and achieve many characteristics of central memory cells (Robinson and Amara, 2005). While central memory T cells generally demonstrate a high proliferative ability upon antigen re-exposure (Wherry et al., 2003), they do not possess immediate effector function and require differentiation before trafficking to peripheral tissues (Sallusto et al., 2004). Additionally, central memory $CD8^+$ T cells express extremely low levels of perforin and granzyme B and do not demonstrate immediate cytotoxic capacity (Takata

and Takiguchi, 2006; Wolint et al., 2004). In contrast, vaccine strategies that provide a persistent level of antigen, such as live-attenuated viral vectors, maintain differentiated effector and effector memory T cells in extralymphoid sites (Gauduin et al., 2006; Robinson and Amara, 2005). Since attenuated strains of HIV carry many risks, some groups have turned to persistent vectors like CMV to produce effector T cells that persist long-term in peripheral sites (Pipeling et al., 2008; Pitcher et al., 2002).

Given that HIV is predominantly transmitted via mucosal routes, the presence of preexisting HIV-specific effector $CD8^+$ T cells at the portal of entry could be profound in terms of limiting the initial burst of viremia during primary infection and subsequent peripheral dissemination. It has already been established that effector memory T cells are the principal T cell population in mucosal sites (Grossman and Picker, 2008; Picker et al., 2006), and recent work has shown that in the majority of new HIV infections a single founder virus is transmitted to previously uninfected hosts (Keele et al., 2008). Therefore, an effector HIV-specific T cell response at the outset of mucosal infection may improve vaccine efficacy when the virus is possibly most susceptible to immune-mediated control. As a proof-of-concept for this idea, Picker and colleagues exploited rhesus CMV to induce durable SIV-specific $CD8^+$ and $CD4^+$ effector memory T cells in rhesus macaques (Hansen et al., 2009). Compared to control monkeys, vaccinated macaques showed increased resistance to acquisition upon repeated low-dose intrarectal challenge with SIVmac239, including four animals who completely controlled initial infection without systemic dissemination.

Collectively, the results reported in this thesis - together with a growing amount of data from other groups (Hansen et al., 2009; Hertoghs et al., 2010; Migueles et al.,

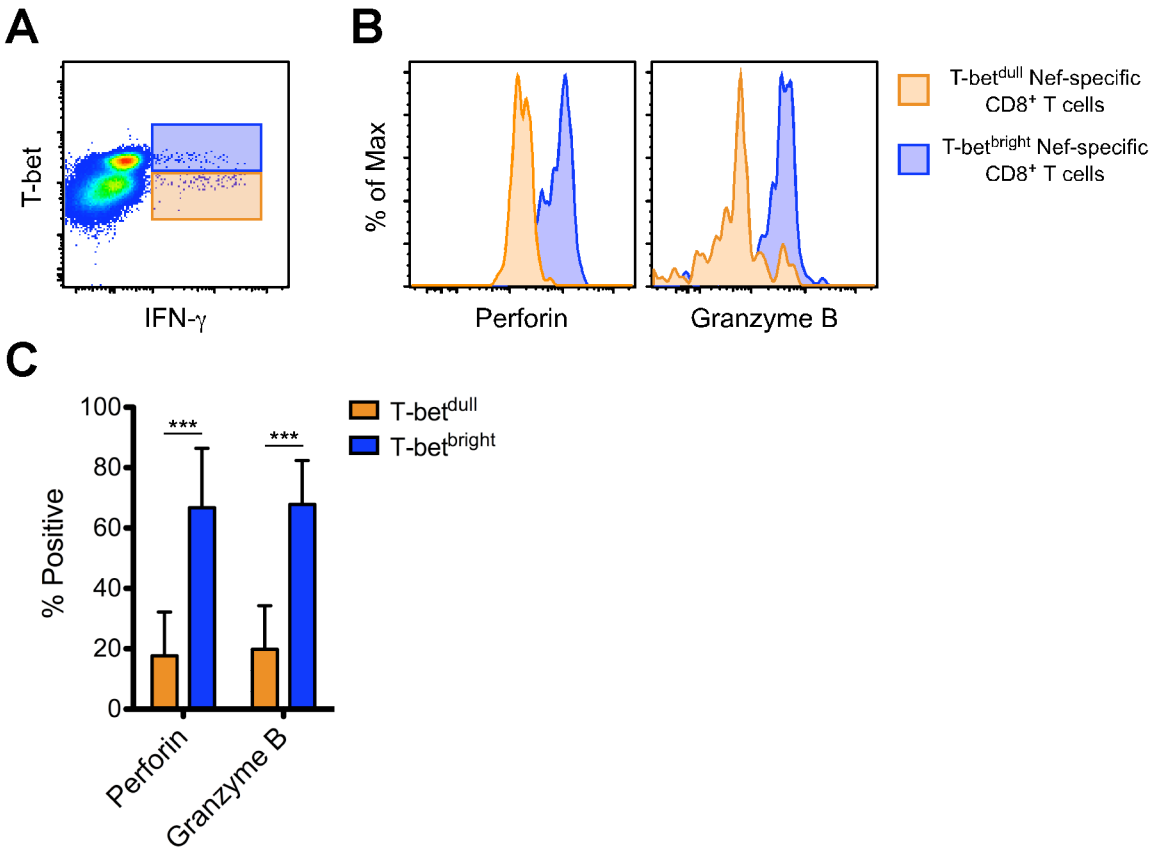
2008; Vine et al., 2004) - indicate that effector function by anti-viral $CD8^+$ T cells is crucial for the containment of viral replication in the context of several human pathogens. We found that T-bet is associated with increased expression of perforin and granzyme B within antigen-specific $CD8^+$ T cells. Notably, higher levels of all three of these proteins are found within HIV-specific $CD8^+$ T cells from patients who demonstrate durable long-term control over HIV compared to patients with progressive disease and detectable viral loads. The importance of effector cells in the control of HIV infection has been experimentally supported by recent observations from Picker and colleagues who found that a rhesus CMV-based SIV vaccine vector could stimulate protective effector SIV-specific T cells (Hansen et al., 2009). To date, this study by Hansen and colleagues provides some of the best evidence that effector T cells can actually prevent systemic dissemination following acute SIV infection, and our results support the notion that effector T cells can be vital to the containments of viral replication during the chronic-phase of infection.

Summary and future directions

Throughout this thesis, we attempted to stress the fact that we observed a strong correlation between high levels of T-bet and the presence of perforin and granzyme B within both resting and activated human $CD8^+$ T cells. For example, we showed in **Figure 15C** that antigen-specific $CD8^+$ T cells expressing perforin consistently contain more T-bet than perforin-negative responding cells. The same antigen-specific $CD8^+$ T cell responses can also be analyzed in a reciprocal fashion, as presented in **Figure 20** below. We examined expression of perforin and granzyme B within T-bet^{bright} versus

T-bet$^{dull/neg}$ responding Nef-specific CD8^{+} T cells among EC subjects (**Fig. 20A**; similar trends were also observed for Nef-specific responses in progressors but that data is not shown). Notably, we consistently observed a higher degree of perforin and granzyme B production from T-bet^{bright} Nef-specific CD8^{+} T cells (**Fig. 20B**). Upon quantification of all Nef responses among EC, a significantly higher fraction of T-bet^{bright} CD8^{+} T cells were positive for both perforin and granzyme B compared to T-bet^{dull} Nef-specific CD8^{+} T cells (**Fig. 20C**). We also found similar results when examining Gag-specific responses in the HIV-infected cohorts (data not shown).

Further studies need to be done that manipulate T-bet expression (knockdown or overexpression) within human CD8^{+} T-cells to formally show a causative role for T-bet in promoting the expression of perforin and/or granzyme B. However, our data does show a very tight association between T-bet and the presence of these cytolytic molecules (**Fig. 20**). Therefore, at the very least, T-bet could be used as a marker for cytotoxic potential and effector status in the monitoring of human immune responses. While we focused solely on T-bet expression within this study, future studies should address the potential contribution of other regulatory factors to the expression of cytolytic molecules in the context of HIV infection or other human pathogens. For example, several groups have reported that Eomesodermin, a T-box family member with T-bet, can be important to effector differentiation in the murine system, as we previously mentioned. Given the reduced T-bet expression among HIV-specific CD8^{+} T cells from CP, it is possible that therapeutic manipulation of T-bet expression in progressors may lead to improved *in vivo* control over HIV replication and improved clinical outcomes.



<u>Figure 20</u>: Higher levels of T-bet within Nef-specific CD8$^+$ T cells are associated with greater perforin and granzyme B production. (A) Representative flow cytometric plot from an EC subject showing a Nef-specific response, as determined by the production of IFN-γ. The T-bet^{bright} and T-bet$^{dull/neg}$ responding cells have been highlighted by blue and orange boxes, respectively. Events shown have been gated on CD8$^+$ T cells. **(B)** The relative levels of perforin and granzyme B are shown for the T-bet^{bright} and T-bet$^{dull/neg}$ cells as shown in (A). **(C)** The fraction of T-bet^{bright} and T-bet$^{dull/neg}$ Nef-specific CD8$^+$ T cells positive for perforin or granzyme B expression was determined among all EC subjects. Mann-Whitney statistical tests (nonparametric; two-tailed) were performed to determine statistical significance. *** denotes a p value < 0.001. Error bars indicate the mean and standard deviation.

Future work needs to uncover why EC demonstrate a greater propensity for T-bet expression - and concomitant upregulation of perforin and granzyme B - among HIV-specific $CD8^+$ T cell responses compared to progressors. We consistently observe three populations of T-bet within human $CD8^+$ T cell populations, but only the highest levels of T-bet expression are associated with the presence of perforin or granzyme B. Therefore, it may be the relative concentration of T-bet in relation to other transcription factors that determines effector function and/or differentiation. Also, perhaps there is a threshold level of T-bet necessary to turn on the production of various effector proteins, including perforin and granzyme B.

This work raises many questions with regard to the reason behind the higher T-bet expression within HIV-specific $CD8^+$ T cells from EC compared to CP. The perforin and granzyme B loci with EC may be in a more "open" configuration of chromatin, which allows for a higher number of T-box binding sites in the upstream regulatory regions of these cytolytic genes to be available for T-bet binding. Additionally, the increased levels of antigen load and immune activation found in progressive HIV infection may negatively impact the ability for T-bet production within HIV-specific $CD8^+$ T cells from CP. In summary, this thesis confirms and extends the findings of previous groups showing that HIV-specific $CD8^+$ T cells are important to the containment of HIV replication within EC, but further research is needed to understand the mechanism(s) behind the increased expression of T-bet, perforin, and granzyme B exhibited by EC. Because there is currently no effective vaccine against HIV, these issues become all the more important to the treatment and possible prevention of HIV worldwide.

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