HIV specific CD8+ T cells from Elite Controllers are Primed for Survival

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Running Title: Elite Controllers’ CD8 T cells resist apoptosis

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Abstract

HIV specific cytotoxic T lymphocytes (CTL) are preferentially primed for apoptosis and this may represent a viral escape mechanism. We hypothesized that HIV individuals that control virus to undetectable levels without antiretroviral therapy (ART), elite controllers (EC), have the capacity to up-regulate survival factors allowing them to resist apoptosis. To address this, we performed cross-sectional and longitudinal analysis of pro-apoptotic (cleaved caspase-3) and anti-apoptotic (Bcl-2) markers of CMV and HIV specific CD8 T cells in a cohort of HIV infected subjects with varying degrees of viral control on and off ART. We demonstrated that HIV specific CTL from EC are more resistant to apoptosis than those with pharmacologic control, successfully treated (ST), despite similar in vivo conditions. Longitudinal analysis of chronically infected persons starting ART revealed that the frequency of HIV-specific T cells prone to death decreased suggesting that this phenotype is partially reversible even though it never achieves levels present in EC. Elucidating the apoptotic factors contributing to the survival of CTL in EC is paramount to our development of effective HIV-1 vaccines. Furthermore, a better understanding of cellular markers that can be utilized to predict response durability in disease or vaccine elicited responses will advance the field.
Introduction

Without anti-retroviral therapy (ART), the majority of HIV-infected individuals progress to AIDS. However, a small portion of those infected demonstrate evidence of immune control of the virus, namely long term non-progressors (LTNP) and elite controllers (EC). These individuals can maintain low or undetectable viral loads without ART. Elucidating the mechanism of viral control in these unique patients remains an area of intense investigation. Despite scientific efforts over the past two decades, the design of an effective preventive vaccine for HIV still eludes us. Given the important role of CD8 T cell responses in viral control seen in non-human primate and human data(35, 39), and the fact that HLA class I alleles are associated with differences in disease progression(16, 31, 37, 43, 44), many current efforts are focused on defining an optimal CD8 T cell immune response to guide effective vaccine design. Unfortunately, studies have failed to consistently demonstrate clear associations of breadth or magnitude of the cytotoxic T lymphocyte (CTL) response with plasma viral load (pVL) (1, 5, 23, 49). However, polyfunctional T cell responses, including the capacity to secrete cytokines, degranulate, and proliferate in response to antigen, correlate with clinical markers of disease progression (2, 10, 18, 32, 45). Maintenance of these types of responses also appears to be important as patients identified and treated early generate and maintain these responses while chronically infected patients with uncontrolled viremia and progressive disease lose these responses over time (3, 8, 9, 21, 34).

Although HIV specific CTL appear to control HIV replication in most patients in acute infection and in EC or LTNP, we still do not understand why these responses are lost in the majority of patients in chronic infection (3, 28). CD8 T cells in chronic HIV infection succumb to exhaustion and cell death in an environment of uncontrolled viremia and nonspecific immune activation (38, 53, 55). Surface markers including PD-1, CD160, 2B4 have provided insights
into predicting exhaustion and correlate with clinical parameters of disease progression (71).

Similarly, vaccine design must incorporate the capacity to generate effective responses and maintain cell-mediated immunity over time or with subsequent boosting. The results of the RV 144 “Thai trial” demonstrated modest protection overall that tended to be greatest in the first year but waned over time (57). This ‘waning’ of vaccine efficacy highlights the importance of gaining a better understanding of the mechanisms dictating immune memory and persistence of both antibodies and T cells. Furthermore, a better understanding of cellular markers that can be utilized to predict response durability in disease or vaccine-elicited responses would advance the field.

Apoptosis occurs through two main pathways. The extrinsic pathway is mediated by surface death receptors such as Fas/FasL. The intrinsic pathway is an intracellular process that can be initiated by a variety of mechanisms including lack of growth factors or cytokines resulting in mitochondrial damage(56). Pro-and anti-apoptotic members of the Bcl-2 family of proteins regulate the subsequent mitochondrial release of cytochrome C to induce apoptosis(19, 50, 61). Anti-apoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-XL negatively regulate the induction of the intrinsic apoptotic pathway(50). The intrinsic and extrinsic pathways converge on caspase-3, the effector caspase. Cleavage of caspase-3 results in a cascade of events leading to programmed cell death(60). Thus measurement of cleaved caspase-3 reflects cell death occurring by either the intrinsic or extrinsic pathways.

While most research on apoptosis and HIV has focused on death of HIV infected CD4 T cells, there is a growing body of literature investigating death of CD8 T cells and how this may represent an escape mechanism for the virus. This statement is especially pertinent in light of the evidence that apoptosis of the total CD8 T cell population correlates with disease progression (27, 38, 73). Furthermore, analysis of total CD8 T cell apoptosis in LTNP has demonstrated that LTNP have fewer apoptotic CD8 T cells (42, 46). The importance of this
phenomenon is more accurately reflected by studies of HIV specific CTL. In a cross sectional analysis of chronically infected patients, HIV specific CD8 T cells were more susceptible to Fas mediated cell death when compared to CMV specific CD8 T cells from the same patient (47), and had reduced levels of Bcl-2 and Bcl-X\textsubscript{L} (54). Furthermore, despite similar levels of Fas on CMV and HIV specific cells from the same patient, HIV specific CTL were 3-fold more prone to apoptosis (47). In \textit{vitro} studies also demonstrate that HIV infected, activated macrophages (M\textsubscript{Φ}) can induce apoptosis in HIV specific CTL suggesting that while engaging with HIV infected targets, CTL are at risk of being killed by the targets they are attempting to destroy (47). While the study of apoptosis of cytotoxic T cells suggests a putative mechanism that explains loss of viral control, studies to date have been limited, as cohorts have not been divided by immune status, pVL, or receipt of ART. In addition, previous studies have not analyzed EC in whom survival of optimal CTL may explain why these patients demonstrate durable viral control. We hypothesize that the capacity for prolonged survival rather than exhaustion and death, may be a unique and desirable quality of HIV specific T cells. Thus we sought to define the role of apoptosis resistance, via up-regulation of survival molecules such as Bcl-2, in the maintenance of effective CTL responses in EC and to elucidate the signaling mechanisms involved in generating those responses. This capacity to resist apoptosis may be an important defining advantage of EC in their control of viremia. Furthermore, CTL with increased survival capacity or apoptosis resistance may be protected from the deleterious effects of immune activation, exhaustion and death.

\textbf{Materials and Methods}

\textbf{Ethics Statement:} Certification has been issued by the UAB IRB indicating their review and approval of this activity in accordance with the Common Rule and any other governing regulations and is on file with the Department of Health and Human Services. Written, informed
consent, approved by the international review board, was obtained from each of the study subjects.

**Study Subjects and HLA Typing:** Peripheral blood samples were collected from healthy donors and HIV-infected volunteers at University of Alabama at Birmingham (UAB) and Alabama Vaccine Research Clinics. HLA typing was performed using the Micro SSP HLA Typing System. Table 1 lists the clinical parameters of the patient cohort which was divided into the following groups: Elite Controllers (EC), subjects maintaining plasma HIV-1 viral RNA loads (pVL) below the limit of detection by commercial assays (< 50 copies/mL) without antiretroviral therapy (ART) for at least 4 years; Successfully Treated (ST), subjects with undetectable pVL on ART for a mean of 22 months (range 10-47 months); Viremic controllers (VC), subjects with detectable pVL which always remains below 2000 copies/ml without ART; and Progressors (P), subjects with higher pVL (> 2000 copies/mL) off ART. Viral blips of < 200 copies/mL were acceptable in subjects with an undetectable viral load if subsequent viral load measurements were < 50 copies/mL. Healthy controls were HIV seronegative subjects (N=9). HLA specific analysis of CD8 T cells was restricted to HLA-A*2 and HLA-B*57. Table 2 shows detailed information on each patient including viral load, receipt of antiretrovirals, CD4 T cell count, age, gender, HLA type, IFN-γ ELISpot data and frequency of HIV and CMV specific T cells as measured by tetramer.

**Quantitation of HIV-1 RNA in plasma and absolute CD4+ T cell count:** Plasma HIV-1 RNA levels were measured using Amplicor Ultra Sensitive HIV-1 Monitor assay at UAB hospitals (version 1.5; Roche Diagnostics Systems). Peripheral blood CD4+ T cells were determined as described elsewhere (4). Patients are routinely seen at three-month intervals and CD4 counts and HIV viral load measurements are obtained at each clinic visit.
**Antigens:** HLA-restricted HIV-specific responses were determined using the interferon (IFN)-
γ enzyme-linked immunospot (ELISPOT) and optimized 9-11mer peptides (Los Alamos National
Laboratory HIV Immunology Database). PBMC were stimulated using optimized HLA-
restricted CD8 T cell epitopes, HLA B*57 restricted Gag-p24 KF11 (KAFSPEVIPMF) and HLA-
A*2 restricted Gag-p17 SL9 (SLYNTVATL), (10 μmol/L), cytomegalovirus (CMV) pp65 NV9
(NLVPMVATV) (10 μmol/L), or staphylococcal enterotoxin B (SEB (1 μg/mL) (positive control).

Peptides previously shown to elicit interferon-γ ELISPOT responses in the studied patients were
chosen for further studies.

**Ex vivo or In-vitro Culture** Cryopreserved PBMC were thawed, washed in complete RPMI
media (10% AB serum)(R-10) and rested overnight at 37° C in a 5% CO₂ incubator. Cells were
counted using Trypan Blue staining and 1x10⁶ PBMC were cultured in 500ul R-10 in short (6
hour) and long (4 day) term cultures at 37° C and 5% CO₂. The peptides HIV Gag-p24 KF11
and p17 SL9, or CMV pp-65 NV9 were used at concentration of 10μM for 4 day peptide
stimulation where indicated.

**Surface and Intracellular Cytokine Staining (ICCS)** PBMC from ex vivo and in vitro studies
were stained with tetramer (APC HLA-B*57-KF11, APC HLA-A*2-SL9, or PE HLA-A*2-CMV-
NV9) (Beckman Coulter), and surface markers (Pacific Blue anti-CD3, APC-Alexa Flour 750
anti-CD4, PerCpCy5.5 anti-CD8, and PeCy7 anti-CD45RO) (BD Biosciences). Intracellular
cytokine staining was performed as described elsewhere (6, 24, 59) and was utilized for the
detection of cleaved caspase-3 (CC-3) and Bcl-2. Briefly cells were washed with PBS, and
permeabilized using the Cytofix/Cytoperm (BD Biosciences). Intracellular staining was
performed using FITC anti-cleaved caspase-3 (Cell Signaling) and PE anti-Bcl-2 (BD Biosciences) according to the manufactures protocol.
Flow Cytometric Analysis: CompBeads (BD Biosciences) were used to establish fluorescence compensation setting for multicolor flow cytometric analysis. A minimum of $5 \times 10^5$ CD3+ events were collected using an LSRII flow cytometer (Becton Dickinson) and analyzed using FlowJo software version 8.5.2, as previously described (59). Since we were interested in studying apoptosis, we did not include a viability stain as this could reduce the events of interest. Lymphocytes were gated on forward scatter area (FSC-A) vs. side scatter area (SSC-A) and any cells that had low forward scatter with higher side scatter were excluded. Next, CD3+CD8+ T cells were selected. From this population, total CD8 T cells or tetramer specific T cells were analyzed for cleaved caspase 3 expression (see gating strategy Figure 1). Bcl-2, which is expressed in naïve cells, decreases at the peak of the expansion phase and remains low in cells that undergo apoptosis (30). Memory cells typically up-regulate Bcl-2 expression, a quality that aids in their survival (30). Thus, all Bcl-2 expression was analyzed from the CD45RO+ memory CD3+CD8+ gate to exclude activation induced Bcl-2 down regulation and allow comparison of the memory population (see gating strategy Figure 3A and Figure 4A) (30).

Statistics: Using Prism software (V.5 Graphpad), statistical analysis included nonparametric Mann-Whitney U test to compare differences between categorical variables. Spearman rank was used as the nonparametric test to compare the relationship between continuous variables. Statistically significant differences were defined as $p<0.05$. 
Results

**Ex vivo Expression of Cleaved Caspase 3 in HIV specific CTL**

To address the potential for apoptosis resistance as a mechanism of maintaining effective CD8 T cells in rare HIV infected subjects capable of immune control of HIV, we analyzed subjects who demonstrated varying levels of HIV control with and without anti-retroviral treatment (Table 1 and Table 2). We performed an *ex vivo* flow cytometric analysis on PBMC analyzing cleaved caspase 3 (CC-3), a marker of apoptosis. Caspase-3 is the terminal effector caspase for both intrinsic (mitochondrial dependent) and extrinsic (death receptor ligand dependent) apoptosis pathways. Thus measurement of CC-3 accurately reflects the induction of apoptosis resulting from cytokine deficiencies, and/or surface signaling via FAS and TRAIL. We performed tetramer staining using HLA-B*57 Gag KF-11, HLA-A*2 Gag SL-9, and HLA-A*2 CMV NV-9 tetramers to allow analysis of HIV and CMV specific responses. For HIV-specific T cells, we limited our analysis to HLA-B*57 and HLA-A*2 Gag restricted responses. Doing so allowed us to compare responses restricted by a favorable HLA (i.e. B*57) and a neutral HLA (i.e. A*2).

The HLA of each patient from each patient group can be found in the detailed Table 2. A representative gating strategy to measure CC-3 on total and HIV specific CD8 T cells is shown (Figure 1A) as well as a summary of the entire cohort (Figure 1B). Similar to prior studies, we found that total CD8 T cells from HIV infected persons are more prone to apoptosis when compared to healthy controls (*p*=0.0001) and that HIV specific CD8 T cells (tetramer +) are 2-3 times more prone to cell death when compared to CMV specific cells from the same persons (*p*=0.02) (Figure 1B). When examining apoptosis of total CD8 T cells, aviremic HIV-infected persons (EC & ST) were similar to healthy controls (Figure 1C). In contrast, the total CD8 T cells from viremic patients (VC & P) were more prone to apoptosis compared to healthy controls (*p*=0.005 and *p*=0.0001 respectively) (Figure 1C). When comparing HIV-specific responses in these cohorts, we found no differences in cell death between viremic individuals (VC & P) despite viremic controllers maintaining pVL < 2,000 copies/mL without ART (Figure 1D). This
suggests that the mere presence of circulating virus resulted in an increased frequency of cells prone to death rather than the level of viremia. In contrast, we found significant differences in the frequency of HIV specific T cells prone to death as measured by CC-3 in the aviremic group, with EC having the lowest frequency of cells prone to apoptosis (p=0.048) (Figure 1D). We also examined HIV specific CD8 T cells targeting either HLA A*2 Gag SL-9 or HLA B*57 Gag KF-11 to determine if responses restricted by favorable HLA were more likely to survive under unfavorable conditions. The levels of CC-3 on HIV specific CD8 T cells were indistinguishable between these two HLA phenotypes within each of the groups (data not shown).

**Cleaved caspase 3 levels on HIV specific CTL after stimulation**

We found significant differences in the ex vivo levels of CC-3 in HIV specific (tetramer positive) CD8 T cells between groups. In vivo, CD8 T cells from EC and ST have minimal virus exposure. We next sought to extend these studies to determine the effect of HIV peptide stimulation over time on cell death and survival. For these studies, we cultured PBMC from HIV positive subjects for 4 days in the presence (stimulated) or absence (media alone) of HIV peptides (KF-11 or SL-9). In addition, PBMC from healthy HIV seronegative controls were cultured under similar conditions in the presence or absence of CMV peptide, NV-9. In our flow cytometric analysis of CC-3 under ex vivo conditions, we consistently found that the subset of HIV specific CD8 T cells that had down regulated CD8, “CD8dim”, were the most prone to cell death (as determined by increased levels of CC-3) (Figure 2A & 2D). In fact, 80-90% of the CC-3hi HIV specific CD8 T cells originated from the CD8dim subset of CTL (Figure 2B and D). This was observed across all HIV subgroups. We therefore analyzed both populations (CD8hi and CD8dim T cells) for CC-3 levels within our viremic and aviremic cohorts in an ex vivo and 4 day culture (media alone or with simultaneous peptide stimulation) assay. Across all HIV subgroups we observed lower levels of CC-3 in the CD8hi T cell group (Figure 2A & C). Interestingly, peptide stimulation of PBMC over 4 days resulted in statistically higher levels of
CC-3 in the CD8{hi} population in both viremic populations (Figure 2B). Alternatively, we found that CD8{dim} cells were much more prone to death across the HIV cohort and under all conditions as evidenced by higher expression of CC-3 (Figure 2A & D). These cells demonstrated sensitivity to cell death in long-term culture even in the absence of peptide stimulation (Figure 2D- hatched vs. white bars), which was present regardless of patient group. However, the magnitude of the cell death was remarkably different across the cohort. Despite overall higher expression of CC-3 amongst the CD8{dim} cells in comparison to CD8{hi} cells, EC consistently maintained the lowest levels of CC-3 within the CD8{dim} population across the cohort. This observation was noted in both of the 4-day culture conditions where it was found to be 4 fold lower than viremic (VC and P) subjects and 2 fold lower than ST subjects (Figure 2D).

**Ex vivo Expression of Bcl-2 in Total and HIV specific CD8 T cells**

Since expression of anti-apoptotic molecules can affect the fate of the cell, we sought to determine ex vivo levels of anti-apoptotic Bcl-2 in cells derived from the same patient cohort. When examining Bcl-2 levels in total CD8 T cell populations, VC were different when compared to healthy individuals and exhibited significantly higher levels when compared to P (Figure 3B). Levels of Bcl-2 on total CD8 T cells observed in healthy, EC, and ST patients were comparable. However, upon analysis of HIV specific CD8 T cells (gag tetramer+) we found significant up regulation of Bcl-2 in EC and ST when compared to viremic subjects (Figure 3C). Amongst HIV specific CD8 T cells, EC exhibited approximately 2 fold higher levels of Bcl-2 compared to ST and greater than 3 fold higher levels compared to both viremic groups.

**HIV specific CTL from Elite controllers have a survival advantage**

Analysis of CD8 T cells in EC indicates increased levels of anti-apoptotic Bcl-2 and reduced levels of pro-apoptotic CC-3 when compared to cells obtained from other HIV-infected groups.
percentage of cells in each population that were more effectively primed for survival (CC-3lo/Bcl-2hi) (Figure 4A). We found significant differences between each group demonstrating that EC and ST have fewer cells at risk of apoptosis when compared to viremic subjects. Interestingly, when comparing those subjects with immune control of virus (EC) with pharmacologic control of virus (ST) in an ex vivo analysis, HIV specific CTL exhibited a survival advantage among the EC demonstrating preservation of cells primed for survival (CC-3lo/Bcl-2hi) (EC vs. ST p=0.038) (Figure 4B). This survival phenotype was not preserved in viremic controllers over progressors despite their having some level of immune viral control. In contrast, EC maintained the lowest frequency of cells prone to death, CC-3hi/Bcl-2lo (data not shown).

Treatment partially reverses the susceptibility of HIV specific CTL to apoptosis

Since we found that the pattern of pro and anti-apoptotic molecule expression in ST patients more closely mimicked the EC group but never achieved the same levels, we extended these studies by performing a longitudinal analysis of four chronically infected persons at least one year prior to initiating ART and at one year post ART, once viremia was suppressed to undetectable levels (Figure 4C & D). We demonstrated that control of viremia with ART resulted in up-regulation of Bcl-2 and down-regulation of CC-3 in HIV specific CTL, a pattern consistent with improved cell survival (figure 4C). In addition, frequency of HIV specific CD8 T cells (closed symbols) prone to death, CC-3hi/Bcl-2lo, dramatically declined with suppression of HIV approaching the frequency of CMV specific CD8 T cells (open symbols) with the same phenotype (figure 4D). Thus, ART, while unable to reduce apoptotic cells to levels seen in EC, was able to partially reverse the survival defect demonstrated in HIV specific CTL.

Longitudinal data pre and post ART suggests that circulating virus, in part, contributes to enhanced cell death of HIV specific CD8 T cells. However, the finding that VC with pVL < 2,000 copies/mL have similar frequencies of cells prone to apoptosis as patients with poorly controlled
virus (Figure 1D) challenges the notion that circulating virus is the primary driver of cell death. To better understand the extent of viremia driving cell death, we compared the frequency of 
CC3hi total (Figure 5A) and HIV specific (Figure 5B) CD8 T cells with plasma viral load and 
found that viral load had a positive correlation, albeit weak, with CD8 T cell CC-3 expression ($R^2$ 
=0.20, $p=0.03$ for Total CD8 T cells, $R^2=0.29$, $p=0.001$ for HIV specific CD8 T cells). For a more 
stringent analysis, we excluded all subjects with an undetectable plasma viral load (pVL< 50) 
including elite controllers and successfully treated subjects. This analysis suggests that viral 
load was a poor correlate of apoptosis ($R^2=0.0007$, $p=0.738$) (Figure 5C).

**CMV specific Apoptosis in HIV seronegative and seropositive subjects**

While the focus of this work was on HIV specific CD8 T cells, we also examined 
susceptibility of CMV specific CD8 T cells from both HIV seronegative subjects and HIV infected 
subjects. CMV-specific CD8 T cells from HIV+ subjects are more prone to apoptosis than CMV 
specific CD8 T cells from HIV seronegative subjects (Figure 1B- closed squares vs open 
squares). Within HIV infected subjects, we observed a modest range of susceptibility of CMV 
specific T cells to apoptosis with EC and ST being the lowest and progressors being the highest 
(Figure 1D- gray symbols). Despite the range, all CMV specific CD8 T cells had relatively low 
CC-3 expression comparable to that of HIV-specific CD8 T cells from EC and ST (Figure 1D). 
This is perhaps a reflection that infection with CMV viremia is better controlled better than HIV. 
In long-term culture conditions, CMV specific T cells from healthy HIV seronegative controls had 
low levels of apoptosis in ex vivo and 4 day culture conditions (2B). Cell death increased with 
CMV peptide stimulation but the frequency of CC-3hi CD8+ cells were comparable between the 
CD8hi and CD8dim populations (2C & D). Finally, we examined coexpression of CC-3 and Bcl-2 
of CMV specific CD8 T cells from a progressor that was eventually treated with ART and was 
reanalyzed at a later time point when virus was controlled on ART (Figure 4C and D- open 
symbol/dotted line). In this longitudinal analysis, the CMV specific CD8 T cells had the highest


frequency of cells primed for survival (CC-3<sup>lo</sup>/Bcl-2<sup>hi</sup>) off ART compared to the HIV specific CD8 T cells (Figure 4C). Once HIV viremia was controlled, the frequency of CMV specific cells primed for survival or prone to death did not change significantly.
Discussion

The HIV specific CD8 T cell response is capable of controlling viral replication in acute HIV infection, and rarely in chronic infection (i.e. long term non progressors (LTNP) and elite controllers) for years. However, this initial response fails to control virus long-term in the majority of chronically infected patients. In the search for correlates of immunity to HIV, several qualities of HIV specific CD8 T cells have been identified that likely contribute to an effective anti-viral immune response. We reason that survival of CD8 T cells may also represent a key factor of an effective immune response. Antigen presenting cells provide costimulatory signals, which are necessary for the generation of polyfunctional CD8 T cell responses, and for the maintenance or long-term survival of these responses (67, 68). In the absence of these critical signals, cells are not only less functional with regards to cytokine secretion, proliferation, and lytic capacity, but are also more susceptible to apoptosis. Prior studies have demonstrated that HIV specific CD8 T cells are more prone to apoptosis when compared to CMV specific CD8 T cells within the same patient (47). Results of the present study demonstrate an important link in HIV immunopathogenesis that may explain in part the inability for HIV specific CD8 T cells to maintain long-term viral control.

The present study represents the first analysis of cell death and pro-survival factors in a cohort of viremic patients with varying levels of viral control but more importantly, in a cohort of aviremic patients with either immune control (EC) or pharmacologic control (ST) of HIV-1. Similar to previous groups, we demonstrate that HIV specific CD8 T cells are prone to apoptosis with viremic patients exhibiting the greatest susceptibility to apoptosis (47). Interestingly, in our cohort we determined this was the case regardless of the level of viral control as we did not observe significant differences in CC-3 levels between viremic progressors with higher viral loads and viremic controllers, those patients capable of maintaining pVL < 2,000 copies/mL. Despite this high degree of susceptibility to apoptosis, it is notable that chronically infected viremic patients have a high frequency of viral specific CD8 T cells (22). Some have significant
proliferation of viral specific effector populations as well as rapid turnover due to apoptosis.

Once treated, patients actually have a decline in the frequency of viral specific CD8 T cells, due
to decrease in antigenic stimulation and proliferation of effector T cells (17).

To our knowledge, this is the first study to also examine aviremic patients, VL < 50
copies/ml, who exhibited the lowest levels of CC-3 on HIV specific CD8 T cells. Interestingly
we found that elite controllers, those patients with immune control of HIV, exhibit lower levels of
CC-3 than successfully treated patients with virologic control on ART. These findings suggest
that in chronic infection, death of HIV specific CTL may contribute to the loss of viral control and
that resistance to apoptosis in CTL from EC may contribute to their ability to intrinsically control
viremia. In support of this, longitudinal studies of persons evolving from acute to chronic HIV
infection reveal HIV specific CD8 T cells with functional attributes associated with viral control
are often deleted in chronic infection, such as high avidity CTL (41). Similarly, preservation of
immunodominant responses into chronic infection is associated with lower viral load set point
and slower CD4 decline (63).

The susceptibility of CTL to apoptosis is likely related to environmental and intrinsic cellular
factors. HIV infection leads to a state of high immune activation, which is created by circulating
uncontrolled viremia and non-specific activation. The pathogenesis and demise of the immune
system is attributed in large part to a breakdown in the mucosal barrier and subsequent gut
translocation (14). This leads to further cellular activation, replication of virus, and loss of CD4 T
cells (13). To better understand how viral and non-viral mechanisms of cell death may
contribute to the loss of the CD8 T cell response, we examined the correlation of viral load and
expression of CC-3 on CD8 T cells. We attribute one-third of the HIV specific CD8 T cell death
observed to circulating virus (Figure 5B). The relatively poor correlation with viral load is
supported by the observation that progressors with higher viral loads do not exhibit significant
differences in expression of CC-3 when compared to viremic controllers, patients capable of
viral control to less than 2,000 copies/mL. Furthermore, viral load alone does not explain the observed differences between EC and ST patients, two groups with undetectable circulating plasma virus.

Non-specific immune activation, resulting from gut translocation likely contributes to immune cell death. Indeed, studies of large cohorts of HIV infected patients have demonstrated a strong correlation of plasma LPS with viral load and the activation state of CD8 T cells as measured by HLA-DR and CD38 (14). While gut translocation can account for some of the non-viral specific causes of cell death in viremic patients, it does not account for the observed differences between EC and ST. If differences in gut translocation and immune activation explain why ST patients have CTL more prone to apoptosis, we would expect ST to have higher LPS and immune activation when compared to EC. In fact, this is not the case. In large cohort studies, EC have higher levels of plasma LPS and higher levels of activated CD8 T cells when compared to ST patients (33). Thus if non-specific immune activation explained the observed differences in differential susceptibility we would expect the EC to have higher frequencies of HIV and CMV specific T cell apoptosis when compared to ST. Instead, within any given EC or ST subject, we observed comparable rates of HIV specific and CMV specific cell death (Figure 1 D). While beyond the scope of this work, the unique survival advantage of the HIV-specific CD8 T cells in EC may reflect differences in low-level immune activation from viral turnover in cellular reservoirs as it has been demonstrated that integrated viral DNA is significantly lower in EC when compared to ST (29).

We observed that decreased expression of CD8 on HIV specific T cells resulted in a greater frequency of cells prone to death as evidenced by increased expression of CC-3 (Figure 2). The down modulation of CD8 expression has been reported in other infection models including influenza and can result in T cell non-responsiveness (58, 64). CD8 down modulation has been postulated as a mechanism of immune regulation whereby activated T cells become
less or non-responsive. Such a response in a state of persistent viremia such as HIV infection likely contributes to disease pathogenesis.

The events proceeding cell deletion are an area of intense investigation in the field of chronic viral infections including HIV. While the generation of an effective immune response requires a combination of antigen presentation with costimulatory signals, the persistence of the response depends in part on negative or inhibitory signals. Persistent high antigen load results in progressive CD8 T cell exhaustion, which is characterized by loss of proliferation, ex vivo cytotoxicity, effector cytokine secretion, and eventually deletion(25, 66, 69, 72). Through genome-wide array analysis comparing exhausted CD8 T cells from chronic infection with those generated in acute LCMV infection, molecular signatures have provided insights into the signaling pathways resulting in exhaustion and deletion(70). Several inhibitory molecules are over-expressed in exhausted cells including PD-1, 2B4, TIM-3, Lag-3 and CD160 some of which correlate with clinical parameters of HIV disease progression (20, 36, 52, 70). Selective blockade of PD-1 and TIM-3 has been shown to restore effector functions(26, 65). More recent data demonstrates that the accumulation of multiple inhibitory factors, such as double positive CD160/PD-1, is associated with progressive exhaustion and cellular dysfunction(7, 11, 51). In contrast, CD8 T cells that express PD-1 without other inhibitory molecules can still recognize cognate antigen and generate a polyfunctional T cell response(51). Similarly, CD8 T cells from EC express mostly single positive CD160 CD8 T cells suggesting that this phenotype is more functional(51). Future studies incorporating pro and anti-apoptotic parameters with a comprehensive analysis of inhibitory factors would provide insight into the regulation of exhaustion and identify phenotypes which result in irreversible exhaustion and are destined for deletion.

Given the lack of evidence that external factors explain the observed differences in apoptosis between EC and ST, we looked to intrinsic factors to explain how cells might be
primed for survival. Costimulatory and cytokine signals (including IL-7 and IL-15) regulate T cell homeostasis and the expression of pro-survival molecules in the Bcl-2 family (12, 15, 40, 62, 67). In animal models, the timing of costimulatory signals via CD137 monoclonal antibodies (during the first few days of infection with either LCMV or influenza) significantly altered the persistence or deletion of viral specific CD8 T cells and the subsequent survival of mice (74). This compromised CD8 T cell response was accompanied by higher levels of IL-10 and TNF-α which were associated with loss of CD4 and CD8 T cells respectively (74). We therefore reason that rapid viral control in acute disease requires an optimal balance of costimulatory and cytokine signals. The observed differences in the survival advantage seen in EC over ST may be a result of the generation of cells primed for survival early in infection. In this scenario, ART treated patients, despite having undetectable pVL, may have cells prone to apoptosis, in part due to compromised APC and lack of optimal costimulation. To begin to investigate this, we examined expression of Bcl-2 in memory cells across the cohort. We focused our investigation on memory cells as effector populations down-regulate Bcl-2 expression (30). We found no differences when examining total CD8 T cells between EC and ST but marked differences between HIV-specific CD8 T cells (Figure 3). In fact, the highest expression was seen in elite controllers, with observed levels 2x that seen in the ST cohort. Finally, co-labeling analysis of CC3 and Bcl-2 demonstrates a phenotype of cells primed for survival (CC3<sub>lo</sub>/Bcl-2<sub>hi</sub>) (Figure 4B). In these analyses, EC possess a greater population of HIV specific CD8 T cells resistant to apoptosis by up-regulating anti-apoptotic molecules. In the longitudinal analysis of the viremic patients pre-and post ART, we demonstrated that although ART can result in up regulation of Bcl-2 on HIV specific CD8 T cells, the levels in “successfully treated patients” never achieve that seen in EC. This finding suggests that the HIV specific T cells were generated earlier, pre-viral control, rather than de novo after successful treatment with ART. Finally, to elucidate if priming for survival was restricted to “good alleles” such as B57, B27, we analyzed responses restricted by a favorable HLA B*57 and a neutral allele, A*2. We did not observe any selective advantage
based on HLA alone. This observation is promising as we translate this data into vaccine development where an optimal response would be desired in patients of any HLA type.

Our analysis of CMV specific CD8 T cells demonstrates that 1) CMV specific T cells from HIV seropositive subjects are more prone to apoptosis than CMV specific CD8 T cells from HIV seronegative subjects (figure 1B), 2) there is a range, albeit modest, of susceptibility to apoptosis of CMV specific CD8 T cells across the HIV cohort (figure 1D), and 3) ART does not significantly change either the frequency of cells prone to death (CC3\(^{hi}\)/Bcl-2\(^{lo}\)) or primed for survival (CC3\(^{lo}\)/Bcl-2\(^{hi}\)) (figure 4C and D open symbols). Collectively, these data demonstrate that the survival of CMV specific CD8 T cells is not compromised to the same degree that HIV specific T cells are within the same host. Interestingly, increasing data suggests that CMV specific T cells in aging and HIV infected populations are relatively resistant to apoptosis leading to higher frequencies of CMV specific T cells in these populations, a phenomenon which may contribute to immuosenescence and inflammation(48).

In summary, results of the present study demonstrate differences in survival of CTL in EC, a phenotype that is important for viral control. This work adds to a growing body of knowledge of generating and maintaining effective immune responses to HIV. Further work elucidating the pathways of achieving these responses will allow us to gain insight into the mechanisms of manipulating the immune response to induce long-lived responses in vaccine recipients.
Acknowledgements

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References


Figure Legends

Figure 1. Frequency of cleaved caspase-3+ CD8+ T cells in HIV-infected patients.

A) A representative polychromatic flow cytometry gating scheme for measurement of cleaved caspase-3 levels on total and tetramer specific CD8+ T cells is shown. B) Percentage of CC-3hi CD8+ T cells in healthy controls (open symbols) versus HIV subjects (closed symbols). In both groups, total CD8 T cells (circles) are compared to virus (HIV or CMV) tetramer specific CD8 T cells (squares). C) Frequency of CC-3hi Total CD8 T cells of healthy, aviremic (elite controllers and successfully treated) and viremic (viremic controllers and progressors). D) Frequency of CC-3hi Tetramer-specific (CMV or HIV) CD8 T cells from HIV aviremic and HIV viremic subjects. Note the symbols for panel D (diamonds=progressors; squares= viremic controllers; triangles= successfully treated; circle= elite controllers) apply to both HIV specific and CMV specific responses. (* p< 0.05)

Figure 2. Comparison of cell death between ex vivo, and long-term culture with and without peptide stimulation.

A) A representative polychromatic flow cytometry gating scheme showing the frequency of CC-3hi in HIV-specific CD8+ T cells (gated on tetramer + cells) in ex vivo (left panel) and 4-days in vitro stimulation with HIV-peptides (right panel) is shown. CD8 T cell population is divided to demonstrate CD8hi versus CD8dim, the subpopulation with CD8 down regulation. B) The frequency of CC-3hi antigen-specific CD8(hi + dim) cells (gated on tetramer + cells) across the cohort under different culture conditions (Black bar- ex vivo; hatched bar- 4 day culture in media alone; white bar-4 day culture with peptide stimulation). Antigen-specific responses for healthy individuals are to CMV (pp65-NV9 tetramer) and for HIV-infected individuals to HIV-1 (Gagp17-SL9 and Gagp24-KF11 tetramers). C) The frequency of CC-3hi antigen- specific CD8hi cells
(gated on tetramer + cells) across the cohort under different culture conditions. D) The frequency of CC-3\textsuperscript{hi} antigen-specific CD8\textsuperscript{dim} cells (gated on tetramer + cells) across the cohort under ex vivo, 4 day culture in media, and 4 day culture with peptide stimulation.

**Figure 3: Ex vivo Expression of Bcl-2 in Total and HIV specific CD8 T cells**

A. Representative flow cytometric gating scheme for measurement of Bcl-2 expression on total CD8\(^+\) T cells is shown. B. Total CD45R0\(^+\) CD8 T cells were examined for expression of the pro-survival molecule Bcl-2. The frequency of Bcl-2\textsuperscript{hi} total CD8\(^+\) T cells in healthy controls (open circles) versus HIV patients (closed symbols) is depicted including aviremic (elite controllers and successfully treated) and viremic (viremic controllers and progressors) subjects. C. HIV specific (Tetramer\(^+\)) CD45R0\(^+\) CD8 T cells were examined for expression of the pro-survival molecule Bcl-2. Frequency of Bcl-2\textsuperscript{hi} HIV-specific CD8 T cells are plotted across the cohort (* p<0.05).

**Figure 4: Coexpression of Bcl-2 and CC-3 on HIV specific CD8 T cells**

A) A representative polychromatic flow cytometry gating scheme for measurement of cleaved caspase-3 and Bcl-2 expression on CD45R0\(^+\) tetramer \(+\) CD8\(^+\) T cells is shown. Gating from lymphocytes to CD3\(^+\) CD8\(^+\) T cells is the same as depicted in Figure 1A. The frequency of CC-3\textsuperscript{lo}/Bcl-2\textsuperscript{hi} HIV specific CD8 T cells, “primed for survival”, or CC-3\textsuperscript{hi}/Bcl-2\textsuperscript{lo} HIV specific CD8 T cells, “prone to death”, was determined for each subject. B) The co-expression of CC-3 and Bcl-2 is plotted using the frequency of CC-3\textsuperscript{lo}/Bcl-2\textsuperscript{hi} HIV-specific CD8 T cells, “primed for survival for each subject across the cohort. C) Four patients were examined longitudinally pre ART and 1-year post ART. The frequency of CC-3\textsuperscript{lo}/Bcl-2\textsuperscript{hi} HIV specific CD8 T cells (gated on tetramer + cells), cells primed for survival, is plotted with connecting solid line to demonstrate ART effect. The dotted line connecting open symbols demonstrates the frequency CC-3\textsuperscript{lo}/Bcl-2\textsuperscript{hi} CMV specific CD8 T cells from one of the ART treated subjects pre and post ART treatment.
D) The frequency of CC-3\textsuperscript{hi}/Bcl-2\textsuperscript{lo} HIV specific CD8 T cells (gated on tetramer + cells), cells prone to death, is plotted with connecting solid line to demonstrate ART effect. The dotted line connecting open symbols demonstrates the frequency CC-3\textsuperscript{hi}/Bcl-2\textsuperscript{lo} CMV specific CD8 T cells from one of the ART treated subjects pre and post ART treatment.

Figure 5: Correlation of Frequency of CC-3\textsuperscript{hi} CD8 T cells with plasma viral load

A) For each patient the frequency of CC-3\textsuperscript{hi} Total CD8 T cells in ex vivo analysis is plotted versus pVL; B) CC-3\textsuperscript{hi} HIV specific CD8 T cells is plotted versus pVL; C) Aviremic subjects were excluded and the analysis of CC-3\textsuperscript{hi} HIV specific CD8 T cells is plotted versus pVL in viremic subjects only. Spearman rank correlation was performed.
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*Note these Patients have undetectable VL on ART.
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*Viral load is 178 copies/ml

**All prior viral load measurements ranged from 81,458 copies/ml

N/A: data not available; individuals did not have the HLA allele restricting the specific peptide tested.

: response not detected; <50 SFU/million cells.

ND: not done