Published in final edited form as:

J Acquir Immune Defic Syndr. 2011 October 1; 58(2): 132–140. doi:10.1097/QAI.0b013e318224d2e9.

Interleukin-2 Production by Polyfunctional HIV-1-Specific CD8 T-cells is Associated with Enhanced Viral Suppression

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Abstract

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

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

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

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

Keywords

HIV-1; CD8 T-cells; virus suppression; IL-2; polyfunctional; vaccines

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Meetings at which portions of this work were presented: Keystone Symposia, HIV Vaccines and Viral Immunity, Banff, Alberta, Canada, March 2010; 10th Annual Meeting of the Federation of Clinical Immunology Societies (FOCIS), Boston, MA, USA, June 2010.

INTRODUCTION

Immune correlates of protection for human immunodeficiency virus-1 (HIV-1) infection are poorly defined, presenting a significant challenge to the development of an effective vaccine.¹ The difficulty in defining these correlates relates to the fact that, without antiretroviral therapy (ART), more than 99% of HIV-1 infected individuals develop AIDS, as they lack an antiviral response that affords long-term protection from disease progression.^{2,3} Failure of the HIV-1-specific immune response is attributed to on-going depletion of CD4 T-cell populations,^{4,5} an established HIV-1 reservoir in latently infected cells,^{6,7} and virus escape from the host response.^{8–10} However, evidence of durable control exists among individuals identified as long-term nonprogressors (LTNP) or elite controllers (EC), who maintain low plasma viral loads (pVL) for many years without the use of ART.^{11–13} Hence, understanding the mechanisms that underlie delayed disease progression amongst controllers will aid in the identification of correlates of protection and design of a therapeutic HIV-1 vaccine.

The critical role CD8 T-cells play in controlling virus replication has been documented in several studies of HIV and SIV infection.^{14–19} Furthermore, the strong association between certain MHC class I molecules and delayed disease progression suggests CD8 T-cells contribute to viral control.²⁰ CD8 T-cells that are polyfunctional²¹ and target multiple Gag epitopes^{22–25} correlate with improved disease outcome, suggesting that the quality of response is an important determinant of their ability to restrict HIV-1 replication.

The Step Study, the first trial to evaluate a T-cell based HIV-1 vaccine, was closed when interim analysis revealed a possible enhanced risk of infection in vaccine recipients.²⁶ While the trivalent MRKAd5 construct used in this trial was highly immunogenic as analyzed by IFN- γ ELISPOT assays, this effector response did not equate with protection from infection or decreased viral load upon seroconversion.^{27,28} These results may be explained by a number of factors including poor induction of CD8 T-cells and a limited quality of response. The outcome of the Step Study underscores the need for improved methods to evaluate vaccine-induced T-cell responses.²⁹

Several groups have developed quantitative assays to measure inhibition of HIV-1 and SIV replication.^{30–36} Some studies have shown an association between enhanced antiviral efficacy and Gag specificity.^{32,37} Recently, analysis of HLA-B*2705-restricted responses indicated that inhibition of HIV-1 replication was related to the kinetics of infected target cell recognition by epitope-specific CD8 T-cells.³⁸ Despite these reports, the CD8 T-cell phenotype associated with enhanced control of virus replication remains unclear. Based on previous reports,^{21,39} we hypothesized that IL-2 production would identify a population of HIV-1-specific CD8 T cells with enhanced suppressive capacity and this population would be increased amongst controllers. To address this question, we sought to quantitatively assess CD8 T-cell-mediated suppression of HIV-1 replication using a modified *in vitro* suppression assay (iVSA).

METHODS

Study participants

Samples were collected from HIV-1 infected controllers ($n=4$) and progressors ($n=5$) enrolled at the University of Alabama at Birmingham (UAB) 1917 Clinic. Progressors had a pVL greater than 2,000 copies/mL and controllers were defined as having a pVL less than 2,000 copies/mL (Table, Supplemental Digital Content 1). CD4 T-cell counts were determined as previously described.²⁴ Plasma HIV-1 RNA levels were measured using Amplicor Ultra Sensitive HIV-1 Monitor assay (Version 1.5; Roche Diagnostic Systems,

Indianapolis, IN). All patients were off ART for at least six months at the evaluated time points. Genomic DNA extracted from PBMCs was used for PCR-based genotyping of HLA class I alleles.⁴⁰ Informed consent was obtained from all participants and the UAB Institutional Review Board approved the study.

IFN- γ ELISPOT Assay

Participants were screened for HIV-1-specific responses based on IFN- γ production. The ELISPOT assay was performed as previously described.⁴¹ Peptides were selected based on optimized CD8 epitopes predicted by each subject's HLA class I genotype and described in the LANL HIV Molecular Immunology Database (www.hiv.lanl.gov/content/immunology/index.html). A positive response was defined as values twice background (unstimulated cells) and greater than 55 SFC/10⁶ PBMCs.

Expansion of CD8 T-cell lines

To generate antigen-specific CD8 T-cell lines, immunodominant responses detected by ELISPOT were expanded *in vitro*. PBMCs were resuspended in serum-free media, and plated at 1.2 $\times$ 10⁶ cells/well. Non-adherent cells were removed after two-hour incubation at 37°C, 5% CO₂. Adherent cells, previously shown to be monocytes,^{42,43} were irradiated (33 gray), pulsed with HIV-1 peptide (10 μ g/mL) for two hours, and washed to remove excess peptide. Autologous CD8 T-cells were negatively isolated using the MACS CD8⁺ T cell isolation kit (Miltenyi Biotec, Gladbach, Germany), resuspended in complete media supplemented with IL-7 (25 ng/mL), and plated onto the peptide-pulsed monocytes at 0.5 $\times$ 10⁶ cells/well. Fresh IL-2 (50 U/mL) was added to cell cultures every 2–3 days. On day 7, CD8 T-cells were restimulated with peptide-pulsed monocytes. Expanded CD8 T-cell lines were tested for HIV-1-specific function on day 14 or 15.

Intracellular Cytokine Staining (ICS) Assay

ICS was performed as previously described.⁴⁴ Approximately 0.5–1 $\times$ 10⁶ cells were incubated with appropriate HIV-1 peptide (10 μ g/mL) for six hours in the presence of anti-CD107a. Cells were labeled with LIVE/DEAD fluorescent reactive dye (Invitrogen, Carlsbad, CA) and stained with antibodies against CD3, CD4, and CD8. Cells were then fixed, permeabilized (FIX & PERM, Invitrogen, Carlsbad, CA), and stained with antibodies for intracellular markers IFN- γ , TNF- α , IL-2, and perforin. A minimum of 100,000 events were acquired on an LSRII (BD Immunocytometry Systems, San Jose, CA) and data analyzed using FlowJo software (v7.6.1, TreeStar, Ashland, OR). Responses greater than 0.02% and twice the background response (negative control) were considered positive. Boolean gating was used to generate polyfunctional subsets. Analysis and presentation of distributions was performed using SPICE version 5.1, downloaded from <http://exon.niaid.nih.gov/spice>.⁴⁵

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

in vitro Suppression Assay (VSA)

HIV-1-specific CD8 T-cell lines were used as effector (E) cells in the VSA. To generate target cells (T), PBMCs were depleted of CD8⁺ cells using CD8 Dynabeads (Invitrogen, Carlsbad, CA). Enriched CD4⁺ cells were activated with IL-2 (50 U/mL) and PHA (2 μ g/mL) for 72 hours. Cell infection with HIV-1_{NL4-3} was optimized at a multiplicity of infection (MOI) of 0.001, minimizing virus-induced cytotoxicity. Effector cells were co-

cultured with autologous and non-autologous targets at multiple E:T ratios (range 0:1 to 5:1) in a 96-well plate for seven days. Supernatant was collected on days 0, 1, 3, 5, 7 and stored at -80°C until analysis. The TZM-bl reporter cell line was used for analysis of HIV-1 replication, in which luciferase expression is highly sensitive and linearly related to the quantity of infectious HIV-1.⁴⁶ Luciferase was quantified on a luminometer (Microplate Luminometer, Applied Biosystems, Foster City, CA). All cell lines tested in the *NSA* were run in duplicate. CD8 T-cell mediated suppression was quantified as percent suppression on the day of maximum HIV-1 replication.

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

Statistical Analysis

Clinical markers and T-cell functional responses between progressors and controllers were compared using the nonparametric Mann-Whitney test. Spearman rank correlation coefficient was calculated to analyze the relationship between suppression (E:T, 1:1) and functional/clinical parameters. Statistical analyses were performed with Prism software (GraphPad Software, La Jolla, CA). Comparison of distributions generated with SPICE was performed using a partial permutation test as described.⁴⁵

RESULTS

Identification and expansion of immunodominant HIV-1-specific responses

To determine if there is a signature phenotype for CD8 T-cells capable of virus suppression, we analyzed effector function in controllers with superior HIV-1 control, off ART, compared to individuals with progressive disease. Immunodominant CD8 T-cell responses are major determinants of CTL escape and such responses could be important mediators of virus suppression.^{47,48} We, therefore, identified immunodominant responses in each subject using the IFN- γ ELISPOT assay. IFN- γ production by virus-specific CD8 T-cells is often the last function detected before cells are fully exhausted, making it a reliable marker to identify HIV-1-specific responses during chronic infection.^{49,50} A positive HIV-1 response to at least one 9–11mer was detected in all individuals (range, 1–13 positive responses), with the magnitude ranging from 55 to 1438 SFC/ 10^6 PBMC in progressors and 55 to 4083 SFC/ 10^6 PBMCs in controllers (Supplemental Digital Content 2). Despite a significant difference in burden of disease as evidenced by pVL ($p=0.02$), there was no difference in the magnitude of response between progressors and controllers. Interestingly, the lowest ELISPOT responses were detected in an EC, C1655, who has maintained undetectable pVL for over twenty years, highlighting the limited utility of ELISPOT measurements as a correlate of an efficacious HIV-1-specific response.

Due to low frequencies of HIV-1-specific CD8 T-cells in controllers such as C1655,⁵¹ the potentially important information gleaned from *ex vivo* functional analysis of these cells is infrequently attempted. We therefore used a fourteen-day stimulation protocol to expand epitope-specific CD8 T-cells from cryopreserved PBMCs. Immunodominant responses detected by ELISPOT (Supplemental Digital Content 2, open symbols) were selected for expansion. Of fifteen CD8 T-cell lines, nine targeted epitopes restricted by HLA class I alleles associated with delayed disease progression (Table 1). Consistent with the dominance of Gag-targeting amongst controllers,^{24,52} all lines obtained from this group were specific to epitopes within p24.

Phenotypic analysis of HIV-1-specific CD8 T-cells

While expansion of epitope-specific CD8 T-cells is frequently performed, few studies have analyzed functional changes that occur after *in vitro* expansion. To establish the utility in

analyzing expanded HIV-1-specific CD8 T-cells, we used ICS to measure multiple effector functions and compared responses both *ex vivo* (Figure 1A) and *in vitro* (Figure 1B). We did not detect a significant difference between the median *ex vivo* or *in vitro* responses in progressors compared to that of controllers for any of the functions analyzed. As expected, *in vitro* expansion increased the frequency of antigen-specific CD8 T-cells (CD107a, IFN- γ , TNF- α , perforin, Figure 1C). In fact, in some ECs, this was the only method of detecting HIV-1-specific effector function. Interestingly, enhanced perforin mobilization was detected after expansion in progressors and controllers despite the fact that the former had few perforin-producing cells when analyzed *ex vivo*. Thus, while the frequency of epitope-specific responses increased after *in vitro* expansion, there was no preferential expansion of CD8 T-cells within either group.

Polyfunctional responses are a hallmark of non-progressive HIV-1 disease,^{21,53} we therefore analyzed these responses *ex vivo* and noted that controllers had an increased frequency of epitope-specific CD8 T-cells with three or more functions compared to progressors (Figure 2A). After *in vitro* expansion, however, these differences disappeared as cell lines derived from progressors were also polyfunctional (Figure 2B). This improved function was particularly prominent with perforin producing cells (orange arcs in Figure 2A, 2B), especially those that also expressed IFN- γ and CD107a (Figure 2D). There was no significant difference in the median frequency of IL-2⁺ cells between the two groups (Figure 1C). IL-2⁺ CD8 T-cells tended to be monofunctional in progressors (black arcs border gray slices, Figures 2A, 2B), although this finding was not significant ($p=0.145$). Amongst controllers, black arcs border the yellow, blue, and red pie slices, indicating IL-2 is being produced by cells capable of three or more functions. Figures 2C and 2D depict 31 unique combinations of CD8 T-cell responses detected *ex vivo* and after *in vitro* expansion. Analysis of these subsets revealed that polyfunctional CD8 T-cells which produce IL-2 tended to be enriched in controllers after expansion ($p=0.066$).

Epitope-specific CD8 T-cell lines derived from controllers demonstrate increased HIV-1 suppression

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

Total analysis of HIV-1-specific CD8 T-cell lines revealed that controllers effectively suppress virus replication compared to progressors (Figure 3E). The median percent suppression in progressors was 73.7% at an E:T ratio of 0.2:1, 76.0% at 0.5:1, compared to 92.9% and 98.8%, respectively in controllers. When the total analysis was adjusted by removing outlier data points (P6, P7, P9), we still detect significant differences in suppressive capacity, with controllers exhibiting enhanced virus suppression (0.2:1, $p=0.017$; 0.5:1, $p=0.004$, $p=0.0087$). At the highest effector cell concentration (E:T, 5:1), the

difference in suppression of autologous and non-autologous targets was no longer distinct, suggesting non-specific targeting of infected cells (Figure 3B, 3D).

Polyfunctional CD8 T-cells that maintain IL-2 production are associated with HIV-1 suppressive capacity

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

DISCUSSIONS

The qualitative features of virus-specific CD8 T-cells that contribute to protection from HIV-1 disease have not been clearly defined. In this study, we quantified the ability of epitope-specific CD8 T-cells to suppress HIV-1 replication, which may represent a more direct marker of antiviral effector function.^{29,54} When tested in the *IVSA*, CD8 T-cell lines derived from HIV-1 infected controllers showed significantly increased suppressive capacity compared to those from progressors. CD8 T-cells were tested against autologous CD4 T-cells, revealing a dose-dependent decrease in virus replication as effectors were added at increasing concentrations. As others have shown, our data provides further evidence that controllers maintain CD8 T-cell populations with potent anti-viral function. At high E:T ratios, virus suppression between the two groups was indistinguishable. As previously observed, this decreased sensitivity at high E:T ratios is likely due to non-specific targeting when CD8 T-cells are in excess.^{32,36,38} Furthermore, increased suppressive capacity may reflect the highly avid CD8 T-cell responses often detected in those with superior viral control.⁵⁵ Thus, progressors may require more CD8 T-cells to attain similar protection due to low avidity populations. Enhanced suppressive capacity is an identifiable marker of an effective HIV-1 specific CD8 T-cell response and is one plausible mechanism by which controllers delay disease progression.

For this study, expanded CD8 T-cell lines underwent two rounds of *in vitro* stimulation with peptide-pulsed, autologous monocytes. This protocol provides a method of expanding populations of low-frequency, antigen-specific cells, thereby permitting evaluation of CD8 T-cells that would otherwise not be analyzed *ex vivo*, but may play an important role in the host immune response. This is particularly important in view of the fact that many HIV-1 infected individuals with excellent viral control off ART have low to undetectable frequencies of HIV-specific CD8 T-cells.⁵¹ A potential caveat of this protocol is that we have enriched for epitope-specific CD8 T-cells with increased survival capacity.

While several groups have demonstrated that HIV-1-specific CD8 T-cells obtained from controllers are more efficient at viral suppression, a phenotypic profile of the CD8 T-cell capable of enhanced HIV-1 suppression has yet to be well defined.^{30–32,38} Results from a recent study suggested that antiviral function is dependent on antigen specificity, having observed increased HIV-1 inhibition by Gag-specific CD8 T-cells compared to Env-specific cells.³² Our study did not address Env-specific CD8 T-cells; however, Gag-specificity was not a universal predictor of HIV-1 suppressive capacity. In fact, Gag-specific CD8 T-cells derived from progressors lacked suppressive function. While suppressive capacity may

depend upon which epitopes are targeted, a larger sample size will be needed to address this question.

Production of soluble effector molecules may be an important predictor of antiviral efficacy as polyfunctional HIV-1-specific CD8 T-cells are more frequently observed among controllers.²¹ Comparison of *ex vivo* CD8 T-cell responses between progressors and controllers did not reveal a significant difference in production or mobilization of any individual function (CD107a, IFN- γ , TNF- α , IL-2, perforin). This was also true for responses detected after *in vitro* expansion in both groups. As previously reported,³⁸ we observed that expanded HIV-1-specific CD8 T-cells had an enhanced functional phenotype when compared to *ex vivo* responses, with increased production of CD107a, IFN- γ , TNF- α , and perforin. Polyfunctional, IL-2 producing CD8 T-cells were present in controllers after expansion, in contrast to progressors in whom IL-2 producing CD8 T-cells were predominantly monofunctional. CD8 T-cell lines able to produce IL-2 in combination with at least two other effector molecules exhibited increased virus suppression ($r=0.56$; $p=0.03$). The same function, measured *ex vivo*, trended towards an association with virus suppression, which may be related to the relatively small sample size and limited detection of IL-2 producing CD8 T-cells.

Earlier studies have associated IL-2 production by HIV-1-specific CD8 T-cells with viral control.^{39,56} More recently, polyfunctional CD8 T-cell responses were associated with increased suppressive capacity;³⁷ however it remains unclear if IL-2 production, polyfunctionality, or both are involved in viral control or if these phenotypes are the result of controlled virus replication. While our association of virus suppression with polyfunctional, IL-2 production likely represents a means to identify CD8 T-cells that have matured in the context of viral control, it may indicate that IL-2 is involved in this control. One potential mechanism is that IL-2 producing CD8 T-cells have a better proliferative capacity,^{57,58} and these may transition to an effector phenotype thus providing a stable population of cells able to maintain control of HIV-1 replication *in vivo*.

Other groups have been able to correlate degranulation and mobilization of perforin and granzymes with enhanced suppression *in vitro*.³⁴ In contrast, we found that expanded CD8 T-cells obtained from progressors have no difficulty increasing perforin production after cell expansion. These observed differences might be due, in part, to the fact that previous studies used a perforin antibody recognizing pre-formed perforin (clone $\delta G9$), but not newly synthesized perforin. We utilized a perforin antibody (clone D48), able to detect *de novo* synthesis of the protein.⁵⁹ Incomplete staining with the anti-perforin, clone $\delta G9$ antibody would make it difficult to completely quantify upregulation of perforin after stimulation. Furthermore, our method of expansion, using autologous monocytes compared to bulk PBMCs for antigen presentation is significantly different from that of other groups, and it has been demonstrated that the type of antigen presenting cell used for stimulation can impact functional development of effector CD8 T-cells.^{60,61}

Studies in SIV-infected rhesus macaques have yielded varying results, unable to clearly define the CD8 T-cell phenotype capable of restricting virus replication. Suppression of SIV replication by CD8 T-cells from infected macaques in earlier studies showed associations with host level of viral control³³ and CD8 T-cell epitope specificity,⁶² while a more recent study could not identify a correlate of SIV suppression despite measuring a number of immune parameters.⁶³ However, vaccine-induced responses in rhesus macaques immunized with a DNA prime/Ad5 boost vaccine demonstrated that CD8 T-cells were able to suppress SIV replication *in vitro*, which trended toward both a lower peak VL during acute infection and a lower VL setpoint.⁶⁴ These inconsistencies may relate to differences in which effector cells were derived. Yet, we report, as others have consistently observed, that HIV-1-specific

CD8 T-cells from controllers have an enhanced ability to inhibit virus replication when compared to CD8 T-cells from patients who lack control, despite varying methods used to isolate effector cells.^{30–32,65}

CD8 T-cell recognition and elimination of infected cells is a critical component of the host immune response to viral infection. However, this effector function is not routinely analyzed in pre-clinical studies of candidate HIV-1 vaccines. We demonstrate that increased *in vitro* suppression of HIV-1 replication is a hallmark of non-progressive disease. This enhanced suppression was associated with a polyfunctional, IL-2⁺ CD8 T-cell response, which may have implications for analysis of vaccine-induced responses. While these studies were completed in the context of chronic HIV-1 infection, and may not translate to protection mediated by vaccine-induced responses, our data suggests that quantification of additional effector functions, especially virus suppression, should be incorporated to evaluate HIV-1 vaccines in a comprehensive manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank staff and patients of the UAB 1917 and Alabama Vaccine Research Clinics. We thank Dr. June Kan-Mitchell for helpful discussions regarding CD8 T-cell expansion. We are grateful to Marion Spell for assistance with flow cytometric acquisition and Juliette Easlick for technical assistance with the *IVSA*.

Support: Supported by the National Institutes of Health (NIH) grants R21 AI073103 and R01 AI064060 (awarded to P.A.G.). This work was also supported by the NIH grant P30AI027767 from the University of Alabama at Birmingham, Center for AIDS Research Clinical and Flow Cytometry Cores.

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Figure 1. ICS analysis of multiple effector functions elicited by *ex vivo* and *in vitro* expanded CD8 T-cells

PBMCs from controllers and progressors were stimulated with HIV-1 peptides and analyzed by ICS for production of CD107a, IFN- γ , IL-2, TNF- α , and perforin. Representative flow cytometry plots depict (A) *ex vivo* and (B) *in vitro* expanded CD8 T-cell responses in subject C5168. Top and bottom panels represent the unstimulated (negative control) and B27-KK10 stimulated response respectively. Lymphocytes were gated by forward and side scatter, viable cells, CD3⁺ cells, and then CD8⁺ cells. Numbers indicate the percent of CD8 T-cells positive for the indicated function. (C) Total *ex vivo* and *in vitro* CD8 T-cell responses detected in HIV-1 infected progressors (*ex vivo*, *n*=6; *in vitro*, *n*=9; open

symbols) and controllers (*ex vivo*, $n=6$; *in vitro*, $n=6$; closed symbols); analysis of *ex vivo* immune response was not completed for subject P5766 due to limited sample availability. Each dot represents a single epitope-specific response. Horizontal bars indicate median response. Significant differences ($p < 0.05$) between *ex vivo* and *in vitro* response are indicated by asterisks.

Figure 2. Polyfunctionality of *ex vivo* and *in vitro* expanded HIV-1 specific CD8 T-cell responses
 Data generated by ICS was analyzed using SPICE software for coincident production of IL-2, perforin, IFN- γ , CD107a, and TNF- α by epitope-specific CD8 T-cells. Pie charts denote the proportion of CD8 T cells producing 1 (gray), 2 (purple), 3 (yellow), 4 (blue), or 5 (red) functions. (A) *Ex vivo* and (B) *in vitro* expanded CD8 T-cell responses were averaged within each group. Arcs identify cell populations that are positive for IL-2 (black arc), perforin (orange), and IFN- γ (green), and CD107a (maroon). Bar graphs depict the relative frequency of responses measured (C) *ex vivo* and (D) after *in vitro* expansion. Responses for all 31 possible subsets are shown for both groups—progressors (pink bars) and controllers (dark blue bars). Plus signs denote a positive response for the effector function listed to the left—2 indicates IL-2, P indicates perforin, G indicates IFN- γ , C indicates CD107a, and T indicates TNF- α .

Figure 3. Enhanced HIV-1 suppression mediated by epitope-specific CD8 T-cells derived from controllers

CD4 T-cells (targets) were infected with HIV-1_{NL4-3} and co-cultured with expanded CD8 T-cell lines (effectors) at multiple E:T ratios for seven days. Supernatant from days 0, 1, 3, 5, and 7 were analyzed for production of luciferase using the TZM-bl reporter cell line.

Background luciferase production in uninfected primary cells was approximately 1000 relative luciferase units (RLU). All experiments were run in duplicate. (A–D) Representative *AVSA* data is shown for a CD8 T-cell line derived from a progressor (cell line P7 in A, B) and a controller (cell line C6 in C, D). Targets cells used were autologous (left panels) or non-autologous (right panels). (E) Antiviral suppression by CD8 T-cell lines derived from

progressors (P, $n=9$) compared to lines derived from controllers (C, $n=6$) at indicated E:T ratios. Each cell line is denoted by a unique symbol. Black bars indicate median percent suppression. Mann-Whitney test was used to compare the median percent suppression between groups at each E:T ratio. NS, not significant.

Table 1

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

Patient ID	Line#	HLA Restriction-Epitope ^a	Protein ^b	AA Sequence
P4470	P1	B07-HA9	Gag p24 (84–92)	HPVHAGPIA
	P2	B07-RL9	Nef (77–85)	RPMTYKAAL
P5766	P3	A02-FK10	p2p7p1p6 (70–79)	FLGKIWPSYK
	P4	<u>B51-TI8</u>	RT (128–135)	TAFTIPSI
	P5	B35-TY9	RT (107–115)	TVLDVGDAY
P1918	P6	A03-RK9	Gag p17 (20–28)	RLRPGGKKK
P2824	P7	<u>B57-IW9</u>	Gag p24 (15–23)	ISPRTLNAW
	P8	<u>B57-KF11</u>	Gag p24 (30–40)	KAFSPEVIPMF
P2883	P9	B53-EW10	Gag p24 (71–80)	ETINEEAAEW
C6602	C1	<u>B58-TF11</u>	Gag p24 (108–118)	TSTLQEQIGWF
C5168	C2	<u>B27-KK10</u>	Gag p24 (131–140)	KRWIILGLNK
C2795	C3	<u>B57-KF11</u>	Gag p24 (30–40)	KAFSPEVIPMF
	C4	<u>B57-QW9</u>	Gag p24 (176–184)	QASQEVKNW
C1655	C5	<u>B57-KF11</u>	Gag p24 (30–40)	KAFSPEVIPMF
	C6	<u>B57-TF11</u>	Gag p24 (108–118)	TSTLQEQIGWF

^aUnderlined epitopes represent those restricted by HLA class I alleles associated with delayed disease progression.

^bNumbers in parentheses indicate epitope alignment to the HIV-1_{HXB2} reference strain.

Table 2

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

	<i>Ex vivo</i> response		<i>In vitro</i> response (Expanded lines)	
	Spearman coefficient (r)	<i>P</i> value	Spearman coefficient (r)	<i>P</i> value
CD107a⁺	0.17	0.58	0.36	0.18
IFN-γ⁺	0.08	0.80	0.27	0.32
IL-2⁺	-0.16	0.62	0.19	0.48
TNF-α⁺	0.19	0.54	0.27	0.32
Perforin⁺	0.32	0.30	0.31	0.25
Polyfunctional (3 functions)	0.26	0.40	0.26	0.34
Polyfunctional + IL-2 production	0.51	0.09	0.56	0.03