Title

Differential Expression of CD8+ T Cell Cytotoxic Effector Molecules in Blood and Gastrointestinal Mucosa in HIV-1 Infection.

Journal

The Journal of Immunology, 200(5)

ISSN

0022-1767

Authors

Kiniry, Brenna E
Hunt, Peter W
Hecht, Frederick M
et al.

Publication Date

2018-03-01

DOI

10.4049/jimmunol.1701532

Peer reviewed

Published in final edited form as:

J Immunol. 2018 March 01; 200(5): 1876–1888. doi:10.4049/jimmunol.1701532.

Differential Expression of CD8+ T Cell Cytotoxic Effector Molecules in Blood and Gastrointestinal Mucosa in HIV-1 Infection[1]

Brenna E. Kiniry[*], **Peter W. Hunt**[+], **Frederick M. Hecht**[‡], **Ma Somsouk**[§], **Steven G. Deeks**[‡], and **Barbara L. Shacklett**[*,†]

[*]Department of Medical Microbiology and Immunology, School of Medicine, University of California, Davis, CA USA

[†]Division of Infectious Diseases, Dept. of Medicine, School of Medicine, University of California, Davis, CA USA

[+]Division of Experimental Medicine, Department of Medicine, Zuckerberg San Francisco General Hospital, UCSF, San Francisco, CA USA

[‡]Positive Health Program, Department of Medicine, Zuckerberg San Francisco General Hospital, UCSF, San Francisco, CA USA

[§]Division of Gastroenterology, Dept. of Medicine, Zuckerberg San Francisco General Hospital, UCSF, San Francisco, CA USA

Abstract

We previously reported that $CD8^+$ T-cells in human gastrointestinal mucosa exhibit reduced perforin expression and weak or impaired cytotoxic capacity compared to their counterparts in blood. Nevertheless, these cells degranulate and express cytokines and chemokines in response to cognate antigen. In addition to weak expression of perforin, earlier studies suggested differential regulation of perforin and granzymes, with granzymes A and B expressed by significantly higher percentages of mucosal CD8+ T-cells than perforin. However, this topic has not been fully explored. The goal of this study was to elucidate the expression and co-expression patterns of granzymes (Gzm) A, B and K in conjunction with perforin in rectosigmoid $CD8^+$ T-cells during HIV-1 infection. We found that expression of both perforin and GzmB, but not GzmA or GzmK, was reduced in mucosa compared to blood. A large fraction of rectosigmoid $CD8^+$ T-cells either

[1]This work was supported by NIH/NIAID R01 AI057020. The UC Davis Flow Cytometry Shared Resource Laboratory (FCSR) was supported by grants from the National Institutes of Health: NCI P30 CA0933730, and NIH NCRR C06-RR12088, S10-RR12964, S10-RR026825 and S10-OD018223-01A1; and from the James B. Pendleton Charitable Trust. The SCOPE cohort was supported by the UCSF/Gladstone Institute of Virology & Immunology CFAR (P30 AI027763). Additional support was provided by the Delaney AIDS Research Enterprise (DARE; AI096109, A127966) and the amfAR Institute for HIV Cure Research (amfAR 109301).

Name and Address for Correspondence: Barbara L. Shacklett, PhD, Dept. of Medical Microbiology and Immunology, UC Davis School of Medicine, 3146 Tupper Hall, Davis CA 95616; Tel: 530 752 6785; Fax: 530 752 8692, blshacklett@ucdavis.edu.

Project concept and direction: BLS. Characterization of participant cohort and procurement of clinical samples: MS, PWH, FMH, SGD. Laboratory assays and data analysis: BEK. Manuscript preparation: BEK, BLS. All authors read and approved the manuscript. BEK. Data analysis: BEK, BLS. Manuscript writing and editing: BEK, BLS. All authors read and approved the manuscript.

Disclosures

The authors report no conflicts of interest that could be perceived to bias this work.

did not express granzymes or were single-positive for GzmA. Rectosigmoid CD8$^+$ T-cells appeared skewed towards cytokine production rather than cytotoxic responses, with cells expressing multiple cytokines/chemokines generally lacking in perforin and granzyme expression. These data support the interpretation that perforin and granzymes are differentially regulated, and display distinct expression patterns in blood and rectosigmoid T-cells. These studies may help inform the development of strategies to combat HIV-1 and other mucosal pathogens.

Introduction

Mucosal tissues are primary targets for human immunodeficiency virus (HIV-1) infection, and the gastrointestinal mucosa is a major site of virus transmission and pathogenesis. Accordingly, understanding mucosal immune responses to HIV-1 is important for the development of strategies to fight infection. CD8$^+$ T-cells are thought to play a crucial role in containing HIV-1 infection, as depletion of these cells results in viral rebound (1, 2). However, the specific effector functions by which CD8$^+$ T-cells mediate viral containment remain unclear. In blood, both perforin-mediated cytotoxicity and polyfunctionality, defined as the simultaneous production of multiple cytokines and chemokines, have been identified as possible correlates of immune-mediated protection from disease progression (3–9). CD8$^+$ T-cell polyfunctionality, including robust production of MIP-1β, has also been identified as a potential correlate of protection in mucosa (10). However, gastrointestinal CD8$^+$ T-cells exhibit strikingly low perforin expression and reduced cytotoxicity compared to blood T-cells, suggesting CD8$^+$ T-cells housed in the gut are primed for cytokine release rather than cytotoxicity (11).

Despite significantly lower perforin levels in gut compared to blood, HIV-1$^+$ individuals not on antiretroviral therapy (ART) display greater proportions of perforin and GzmB-positive CD8$^+$ T-cells in gastrointestinal mucosa compared to those on ART and HIV-1 negative adults (10–12). Whether this indicates expression of cytotoxic effector proteins by polyfunctional HIV-1-specific CD8$^+$ T-cells, the emergence of a cytotoxic HIV-1-specific effector T-cell subset, and/or bystander activation-induced perforin and granzyme B expression in non-HIV-1-specific CD8$^+$ T-cells in the mucosa during chronic HIV-1 infection is unknown.

Interestingly, despite low perforin expression, gastrointestinal CD8$^+$ T-cells express granzyme B, albeit at lower levels compared to blood, and display ample degranulation, measured by expression of CD107, suggesting that these mucosal CD8$^+$ T-cells may produce and release “cytotoxic” granules containing little or no perforin (11, 13). Numerous *in vitro* and mouse *in vivo* studies have demonstrated non-cytolytic, extracellular functions of granzymes A, B, and K, including extracellular matrix degradation and the direct and indirect processing and release of pro-inflammatory cytokines (14–17). The concept of granzymes functioning in a non-cytolytic manner to elicit a pro-inflammatory state appears consistent with the strong propensity of gut CD8$^+$ T-cells to produce chemokines and cytokines in response to antigenic stimulation. Accordingly, the major goal of this study was to more fully explore the expression and coexpression patterns of granzymes A, B and K in

parallel with perforin in HIV-1-specific and non-HIV-1-specific $CD8^+$ T-cells in rectosigmoid mucosa during chronic HIV-1 infection.

Materials and Methods

Participants and Sample Collection

HIV-1 positive and seronegative participants were enrolled through the SCOPE study based at San Francisco General Hospital. Written informed consent for phlebotomy and rectosigmoid biopsy was obtained through protocols approved by the Committee on Human Subjects Research, University of California, San Francisco [Protocols #10-01218, 10-00263 and 10-01330]. Participants were classified in four groups defined by plasma viral load (VL) and antiretroviral therapy (ART) as follows: Controllers (C, n=10) consistently maintained VL <2,000 copies/mL without ART; Viremic (V, n=15) subjects maintained VL ≥ 2,000 copies/mL without ART; ART treated (Tx, n=13) participants had VL < 40 copies/mL; and seronegative (SN, n=10) controls were negative for HIV-1. Seronegative participants were healthy volunteers from whom samples were collected for research purposes only. Exclusion criteria included active sexually transmitted infections other than HIV-1; inflammatory bowel disease or other known inflammatory conditions affecting the gastrointestinal tract; colorectal cancer or other malignancies; and severe anemia or blood clotting disorders. Additional demographic information (gender, race/ethnicity, age, viral load, CD4 count, and time since diagnosis) is supplied in Table I. Twenty to 40 ml of blood was collected by sterile venipuncture into tubes containing EDTA. Approximately 30 rectosigmoid biopsies were obtained by flexible sigmoidoscopy 10–30cm from the anal verge (18). Biopsies were collected in 50mL conical tubes containing 15mL of R-15 medium [RPMI-1640 supplemented with fetal calf serum (15%), penicillin (100U/mL), streptomycin (100 mg/mL), and glutamine (2 mM)]. Blood and biopsy samples were transported at room temperature to the laboratory and processed immediately upon arrival.

Blood and Tissue Processing

Ficoll-Paque™ (Pfizer-Pharmacia, New York, NY) was used to isolate peripheral blood mononuclear cells (PBMCs) from blood. Rectosigmoid mucosal mononuclear cells (RMMCs) were liberated from biopsies using enzymatic and mechanical disruption as previously described (19, 20). Briefly, biopsies underwent shaking incubation at 37°C for 30 minutes in 25mg/mL Liberase DL (Roche, Indianapolis, IN), followed by passage through a 16-gauge blunt end needle to disrupt tissue. Free cells were collected through a sterile 70μm cell strainer. The disruption process was repeated until all biopsy tissue was digested. Free RMMCs were washed three times in 20mL of R-15. All isolated cells were rested overnight at 37°C, 5% CO_2 in R-15 supplemented with 200x Zozyn (Pfizer-Pharmacia).

Antibodies and Peptide Pools

The following fluorochrome-labeled monoclonal antibodies were used in flow cytometry: CD107a (H4A3: PE-Cy5, PE-Cy7), CD8 (SK1: APC-H7, FITC), CCR7 (3D12: PE-Cy7), Granzyme B (GB11: V450, PE-CF594), MIP-1β (D21-1351: APC-H7, PerCP-Cy5.5), IFNγ (B27: PE-Cy7), TNFα (Mab11: Ax700), CD69 (L78: PE) from BD Biosciences (San Jose, CA); Granzyme K (GM6C3: FITC) from Santa Cruz Biotechnology, Inc. (Dallas, Tx);

CD4 (T4D11: ECD) from Beckman Coulter (Brea, CA); CD4 (RPA-TA: BV785), CD45RO (UCHL1: BV785), CD27 (O323: BV650), CD103 (Ber-ACT8: Ax488), Granzyme A (CB9: Ax647, PacBlue), and CD3 (UCHT-1: Ax700) from Biolegend (San Diego, CA). CD3 (S4.1: Qdot655) was purchased from Invitrogen (Carlsbad, CA). S1PR1 (SW4GYPP: eFluor660) was purchased from eBioscience (San Diego, CA); Unlabeled CD28 (L293), and CD49d (L25) were from BD Pharmingen (San Diego, CA). Perforin (B-D48: PE) was from Cell Sciences (Canton, MA). This perforin antibody detects *de novo* as well as pre-formed perforin and is suitable for monitoring perforin expression following TCR stimulation (21). The HIV-1 Gag peptide pool (Consensus Clade B) consisted of 123 15-mers overlapping by 11 residues (JPT Innovative Peptide Solutions, Berlin, Germany). *Staphylococcal* enterotoxin B (SEB) was from Sigma Aldrich.

Antigen stimulation and intracellular cytokine staining

Staining was performed as previously described with slight modifications (12) on *ex vivo* blood and mucosal mononuclear cells rested overnight at 37°C, 5% CO_2. For stimulation assays, cells were incubated at $2x10^6$ cells per 200μL R-15 for 5.5 hours in the presence of anti-CD28 (1μg/mL) and anti-CD49d (1μg/mL), anti-CD107a, 1μM GolgiStop™ (BD Biosciences), brefeldin A (5μg/mL, Sigma Aldrich) and the appropriate antigenic stimulation: Gag-peptide pool (final concentration: 3.5μg/mL of each peptide), or medium containing an equivalent amount of DMSO (peptide solvent) as the negative control. *Staphylococcal* enterotoxin B (5μg/mL) was used as positive control. Following incubation, cells were stained for surface markers and viability (Aqua Dead cell stain kit, Invitrogen) for 20 minutes at room temperature. Cells were then fixed in 4% paraformaldehyde and permeabilized using FACS Perm 2 (BD Biosciences) prior to intracellular staining for CD3, cytotoxic effectors, chemokines, and cytokines. Staining for cytotoxic effectors following antigenic stimulation (Figs. 3, 4 and Supplemental Figs. 2, 3) utilized staining protocols and antibody clones previously demonstrated to reveal perforin and Granzyme B expression following TCR stimulation (21). Cells were re-suspended in 1% paraformaldehyde and stored at 4°C in the dark no longer than 24 hours until analysis.

Data Acquisition and Analysis

An LSR II flow cytometer and FACSDiva™ software (Becton Dickinson Immunocytometry Systems) were used to collect flow cytometry data. Flowjo software (Flowjo LLC, Ashland, OR) was used to perform initial flow cytometric analysis and SPICE was used to visualize multifunctional T-cell populations (22). $CD8^+$ T-cells were identified using the following gating strategy: lymphocytes (SSC-A versus FSC-A) → singlets (FSC-H versus FSC-A) → live, $CD3^+$ (CD3 versus viability) → $CD8^+$, $CD4^{Neg}$ (CD8 versus CD4). Memory subsets within the $CD8^+$ T-cell population were identified by first establishing positive gates for each memory marker within the live, $CD3^+$, $CD4^{Neg}$, $CD8^+$ T-cell population followed by Boolean gating to define memory subsets as follows: naïve (T_{naive} $CD45RO^-CD27^+CCR7^+$), central memory (T_{CM} $CD45RO^+CD27^+CCR7^+$), transitional memory (T_{TM} $CD45RO^+CD27^+CCR7^-$), effector memory (T_{EM} $CD45RO^+CD27^-CCR7^-$), and effector (T_{EFF} $CD45RO^-CD27^-CCR7^-$) (23). Polyfunctional cells and cells co-expressing cytotoxic effector molecules were identified by first establishing positive gates for each response analyte and/or cytotoxic effector molecule within live, $CD3^+$, $CD4^{Neg}$,

$CD8^+$ T-cell populations, followed by Boolean gating to define subsets co-expressing these effector molecules. We then used Boolean gating to define memory marker expression within each cytotoxic effector subset. An in-house statistical algorithm was used to determine whether antigen-specific responses differed significantly from the negative control (12). For responses found to be statistically significant, net responses were then calculated by subtracting the negative control values from antigen-specific responses. Statistical analyses were performed with GraphPad Prism V.6 (GraphPad Software, San Diego, CA) or SPICE using non-parametric statistical test as follows: Wilcoxon matched-pairs signed rank test for paired samples, Mann-Whitney test for unpaired samples, and Spearman correlation and linear regression analysis for correlative analysis.

Results

Ex vivo expression of cytotoxic effector molecules differs between blood and gut and across HIV-1 disease status

Expression of perforin, and to a lesser extent GzmB, is reduced in resting and stimulated $CD8^+$ T-cells in gastrointestinal mucosa compared to blood, but whether this trend extends to other granzymes, or to their coexpression, has not been fully explored (13). It is also unclear whether coexpression of these proteins varies with HIV-1-disease status. To address these questions, we compared intracellular expression of Granzymes A, B, K and perforin in resting $CD8^+$ T-cells freshly isolated from blood and rectosigmoid mucosal biopsies of participants in the following groups: HIV-1^+ controllers (C); HIV-1^+ viremic individuals not on antiretroviral therapy (V); HIV-1^+ individuals on antiretroviral therapy (Tx); and seronegative controls (SN) (Table I).

GzmB, GzmK, and perforin all displayed bi-modal expression patterns with distinctive positive and negative populations (Fig. 1A). GzmA displayed a tri-modal expression pattern, with a small fraction of $CD8^+$ T-cells identified as $GzmA^{Bright}$, expressing GzmA at high fluorescence intensity, and a large subset displaying intermediate intensity, designated as $GzmA^{Int}$ (Fig. 1A). The $GzmA^{Bright}$ population was particularly prominent in mucosa. Irrespective of HIV-1 disease status, GzmA was the most abundantly expressed cytotoxic effector protein in both blood and mucosa, while perforin was the rarest (Fig. 1A, 1B). In agreement with our previous work, the proportion of $CD8^+$ T-cells expressing GzmB and perforin was greater in blood compared to mucosa in both seronegative and HIV-1^+ participants (11, 13). In contrast, the relative abundance of $GzmA^{Int}$ cells in blood and gut differed between HIV-1^+ and seronegative participants. In HIV-1^+ individuals, the proportion of $GzmA^{Int}$ $CD8^+$ T-cells was similar in blood and mucosa; in seronegatives, it was significantly greater in mucosa. No significant differences were detected between blood and gut in the proportion of $GzmK^+$ $CD8^+$ T-cells in either HIV-1^+ or seronegatives (Fig. 1B).

Expression of cytotoxic effectors varied across HIV-1 disease status. With the exception of $GzmA^{Bright}$ cells, which were most abundant in seronegatives, percentages of $CD8^+$ T-cells expressing cytotoxic effectors were typically higher in HIV-1^+ groups compared to seronegatives in both tissues, with the highest percentages observed in HIV-1^+ individuals not on ART (Fig. 1C, Supplemental Fig. 1A). Among HIV-1^+ groups, controllers and viremic individuals not on ART had higher percentages of $GzmA^{Int}$ and GzmK-positive

rectosigmoid $CD8^+$ T-cells compared to ART-treated participants. Viremic individuals not on ART also had greater GzmB expression in gut compared to ART-treated subjects (Fig. 1C). Similar trends were observed in blood although not as pronounced (Supplemental Fig. 1A).

Taken together, these data demonstrate elevated expression of $GzmA^{Int}$, B, K and perforin in rectosigmoid $CD8^+$ T-cells from HIV-1^+ individuals, particularly those not on ART, compared to seronegatives, and highlight for the first time a novel subset of $CD8^+$ T-cells expressing GzmA at high fluorescence intensity in the mucosa.

Co-expression of cytotoxic effectors is reduced in rectosigmoid $CD8^+$ T-cells compared to PBMC but elevated in HIV-1-infection

Differences in the abundance of individual cytotoxic effectors led us to investigate the co-expression patterns of these molecules in mucosa. In blood, HIV-1-specific $CD8^+$ T-cells have been previously characterized as $Perforin^{-/+}$ $GzmB^+$ $GzmA^+$ $GzmK^+$ (24). As expression patterns of cytotoxic effectors were elevated in blood $CD8^+$ T-cells of HIV-1^+ individuals compared to seronegatives, we predicted that their co-expression would likewise be elevated in the mucosa of HIV-1 infected compared to healthy individuals. To test this prediction, we used flow cytometry and SPICE software to analyze co-expression of cytotoxic effectors in unstimulated $CD8^+$ T-cells from rectosigmoid mucosa and blood of chronically HIV-1 infected and seronegative adults (Figure 2). This dataset captured information from all $CD8^+$ T-cells, regardless of specificity.

Irrespective of HIV-1 disease status, we observed significant differences in the percentages of $CD8^+$ T-cells co-expressing certain cytotoxic effectors between blood and mucosa, most notably in the proportions of $Perforin^+GzmA^{Int}GzmB^+$ and $GzmA^{Bright}$ $CD8^+$ T-cells. Although in both tissues, perforin was typically co-expressed with $GzmA^{Int}$ and GzmB, the percentage of $Perforin^+GzmA^{Int}GzmB^+$ $CD8^+$ T-cells was significantly higher in blood compared to mucosa (Fig. 2A, 2B). This putative cytotoxic subset was one of the most abundant in blood ($Blood_{HIV+}$ median 17.1%, $Blood_{SN}$ median 6.17%) but one of the rarest in mucosa ($Rectosigmoid_{HIV+}$ median 1.18%, $Rectosigmoid_{SN}$ median 0.508%). In contrast, the mucosa contained a significantly higher percentage of $GzmA^{Int}$ single positive $CD8^+$ T-cells compared to blood ($Rectosigmoid_{HIV+}$ median 17.6%, $Rectosigmoid_{SN}$ median 28.9%; $Blood_{HIV+}$ median 2.69%, $Blood_{SN}$ median 1.43%). A similar trend was observed with $GzmA^{Bright}$ $CD8^+$ T-cells. Interestingly, in addition to differences in abundance, the co-expression pattern of $GzmA^{Bright}$ also differed between blood and mucosa. In mucosa, $GzmA^{Bright}$ $CD8^+$ T-cells typically lacked expression of any other cytotoxic effectors; in contrast, blood $GzmA^{Bright}$ $CD8^+$ T-cells typically co-expressed perforin and GzmB (Fig. 2A, 2B).

Perhaps not surprisingly, HIV-1^+ participants also had higher median frequencies of $CD8^+$ T-cells co-expressing cytotoxic effectors compared to seronegatives in both blood and gut, including a large proportion of $CD8^+$ T-cells co-expressing $GzmA^{Int}$, B, and K ($Rectosigmoid_{HIV+}$ median 21.7%, $Rectosigmoid_{SN}$ median 6.89%; $Blood_{HIV+}$ median 15.9%, $Blood_{SN}$ median 5.89%) (Fig. 2B). To elaborate on this observation, we compared the abundance of co-expressing subsets across HIV-1-disease status (Fig. 2C, Supplemental

Fig. 1B). In mucosa, the proportions of GzmA^{Int}GzmB^{+}GzmK^{+} and GzmA^{Int}GzmB^{+} CD8^{+} T-cells were significantly greater in all HIV-1^{+} subgroups compared to seronegatives. The proportion of GzmA^{Int}GzmB^{+}GzmK^{+} CD8^{+} T-cells was also greater in viremic individuals not on ART compared to those on ART. Both controllers and viremic individuals not on ART had significantly higher percentages of 'phenotypically cytotoxic' CD8^{+} T-cells, defined as those co-expressing perforin and granzymes, compared to seronegatives. Specifically, controllers and viremic individuals not on ART had higher percentages of Perforin^{+}GzmA^{Int}GzmB^{+}GzmK^{+} CD8^{+} T-cells compared to seronegatives; controllers had higher percentages of Perforin^{+}GzmA^{Int}GzmB^{+} CD8^{+} T-cells compared to seronegatives. In contrast, seronegatives displayed greater frequencies of GzmA^{Int} and GzmA^{Bright} single positive CD8^{+} T-cells compared to all HIV-1^{+} subgroups (Fig. 2C). Similar trends were observed in the blood (Supplemental Fig. 1B).

Taken together, these data reveal that in general, the rectosigmoid mucosa contains a lower percentage of 'phenotypically cytotoxic' CD8^{+} T-cells compared to blood, but an abundance of GzmA single-positive CD8^{+} T-cells. Furthermore, individuals with chronic HIV-1 infection, particularly those not on ART, have greater co-expression of cytotoxic effector proteins in mucosal CD8^{+} T-cells compared to uninfected individuals.

Expression of perforin and GzmB in antigen-specific CD8^{+} T-cells differs with anatomical compartment, antigenic stimulation, and cytokine polyfunctionality

We next turned our attention to antigen-specific CD8^{+} T-cells. To explore the range of functionality exhibited by antigen-specific rectosigmoid CD8^{+} T-cells, lymphocytes isolated from blood and mucosa of HIV-1^{+} individuals during early and chronic infection or seronegative controls (Table I) were stimulated with an HIV-1 Gag peptide pool or *Staphylococcal* enterotoxin B (SEB) in a 5.5-hour *ex vivo* stimulation assay followed by intracellular staining for MIP-1β, IFNγ, and TNFα along with CD107a, GzmB and perforin as surrogates of cytotoxicity. For these experiments we utilized reagents and protocols previously demonstrated to reveal perforin newly produced in response to TCR stimulation (21). Expression of perforin and GzmB was measured within Gag and SEB-responding CD8^{+} T-cells as defined by one or more of the following responses: degranulation (CD107a), MIP-1β, IFNγ, or TNFα production (Fig. 3A).

In both HIV-1-infected and seronegative participant groups, a large fraction of rectosigmoid CD8^{+} T-cells responding to antigenic stimulation did not express detectable perforin or GzmB (median$_{Gag}$ 49.6%, median$_{SEB}$ 74.5%) (Fig. 3B). For both antigens, the proportion of total responding CD8^{+} T-cells co-expressing perforin and GzmB was significantly greater in blood compared to mucosa (Fig. 3B); in contrast, the proportion of total responding CD8^{+} T-cells expressing neither perforin nor GzmB was greater in mucosa compared to blood, reaching statistical significance for SEB (Fig. 3, Supplemental Fig. 2). As no statistically significant differences were observed between HIV-1^{+} subgroups, HIV-1^{+} participant data were consolidated into a single group. Interestingly, in mucosa but not blood, the proportion of responding CD8^{+} T-cells expressing GzmB in the absence of perforin (Perforin^{Neg}GzmB^{+}) was significantly greater for Gag compared to SEB stimulation (medians 38.7% and

19.9% respectively) (Fig. 3B). The Gag-specific Perforin^{Neg}GzmB^{+} CD8^{+} T-cell response was also greater in mucosa compared to blood (Fig. 3B).

We next assessed the expression of perforin and GzmB within CD8^{+} T-cell subsets producing multiple cytokines/chemokines and/or CD107, and again found differences between Gag and SEB-responding CD8^{+} T-cells. Similar to previous observations in blood (7), the proportion of Gag-responding CD8^{+} T-cells expressing perforin and/or GzmB tended to increase as cytokine polyfunctionality decreased in both blood and gut (Fig. 3D). This trend was especially pronounced in mucosa, where the majority of CD8^{+} T-cells responding to Gag with 3 or 4 soluble markers and/or CD107, did not express perforin or GzmB (medians 60.18% and 67.33% for 3- and 4-function cells, respectively) (Fig. 3D). In contrast, single-function mucosal Gag-responding CD8^{+} T-cells displayed the greatest GzmB expression (Fig. 3D). This was not observed for blood SEB-responding CD8^{+} T-cells, in which the proportion of perforin and/or GzmB-expressing cells appeared greater in polyfunctional compared to single-function CD8^{+} T-cells (Supplemental Fig. 2C, 2D). No obvious trend emerged for SEB in the mucosa, as the majority of rectosigmoid SEB-responding CD8^{+} T-cells did not express perforin or GzmB, irrespective of cytokine expression (Supplemental Fig. 2).

Taken together, these data demonstrate an apparent inverse relationship between polyfunctionality (defined as coexpression of multiple cytokines/chemokines and CD107a) and cytotoxic potential (defined as coexpression of perforin and GzmB) in rectosigmoid HIV-1 Gag-specific CD8^{+} T-cells, and highlight discordance in the expression of perforin and GzmB between Gag and SEB-responding CD8^{+} T-cells in the mucosa.

Expression of cytotoxic effectors in antigen-responding CD8^{+} T-cells differs by anatomic location and by antigenic stimulation

The data presented in Figure 3 suggest that most cytokine/chemokine-producing HIV-1-specific rectosigmoid CD8^{+} T-cells do not express perforin and granzyme B. The abundance of mucosal CD8^{+} T-cells expressing granzymes independently of perforin led us to re-examine this result using a broader range of cytotoxic effectors. As before, lymphocytes isolated from blood and rectosigmoid mucosa of chronically HIV-1 infected and seronegative adults were stimulated with an HIV-1 Gag-peptide pool or *Staphylococcal* enterotoxin B (SEB) in a 5.5 hour *ex vivo* stimulation assay. However, in this series of experiments, stimulation was followed by intracellular staining for Perforin, GzmA, GzmB and GzmK, along with MIP-1β and CD107 (Fig. 4). As CD107a and MIP-1β expression were the two strongest responses previously detected in rectosigmoid mucosa in response to HIV-1 Gag and SEB stimulation, they were used here as indicators of antigen responsiveness (10, 12).

For both HIV-1 Gag and SEB stimulation, the mucosa displayed a greater proportion of responding CD8^{+} T-cells not expressing any cytotoxic effectors compared to blood. These Perforin^{-}GzmA^{-}GzmB^{-}GzmK^{-} responses dominated in the mucosa and were particularly striking for SEB-responding CD8^{+} T-cells (Fig. 4C). Rectosigmoid mucosa also exhibited greater proportions of GzmA single positive antigen-responding CD8^{+} T-cells compared to blood. In contrast, blood had greater proportions of Perforin$^{+/-}$GzmA^{+}GzmB^{+} responding

CD8$^+$ T-cells compared to mucosa (Fig. 4C). Differences between antigenic stimulations were also observed. In mucosa but not blood, Gag-responding CD8$^+$ T-cells exhibited a larger proportion of GzmA$^+$GzmB$^+$GzmK$^+$ CD8$^+$ T-cells compared to SEB-responding CD8$^+$ T-cells (Fig. 4C and data not shown). In contrast, rectosigmoid SEB-responding CD8$^+$ T-cells included a greater proportion of cells not expressing any cytotoxic effectors compared to Gag-responding CD8$^+$ T-cells (Fig. 4C and data not shown).

These findings expand upon the data presented in Figures 1 and 3, and further support the interpretation that rectosigmoid CD8$^+$ T-cells are typically primed for cytokine expression rather than cytotoxic effector production. However, this observation may be partly antigen-dependent, as rectosigmoid Gag-specific CD8$^+$ T-cells displayed proportionately greater expression of granzymes A, B, and K compared to SEB-responding cells.

Memory phenotype varies with cytotoxic effector expression and anatomic location

Based on observations in blood, it is thought that CD8$^+$ T-cells acquire expression of cytotoxic effectors as they mature into effector subsets. For example, Perforin$^-$GzmA$^-$GzmB$^-$, GzmA$^+$GzmB$^+$GzmK$^+$, and Perforin$^+$GzmA$^+$GzmB$^+$ subsets are associated with central memory, transitional and effector maturation stages, respectively (24–26). However, despite housing a high proportion of effector-memory cells (27), the mucosa displays an abundance of CD8$^+$ T-cells expressing either only GzmA or not expressing any cytotoxic effectors (Fig. 1). To address this apparent discrepancy, we assessed the intracellular expression of cytotoxic effectors in conjunction with T-cell memory markers CD45RO, CCR7, and CD27 on unstimulated blood and rectosigmoid CD8$^+$ T-cells from chronically HIV-1 infected and seronegative adults.

Not surprisingly, blood CD8$^+$ T-cells not expressing any cytotoxic effectors primarily had a naïve phenotype (Supplemental Fig. 3), whereas in rectosigmoid mucosa most “quadruple negative” Perforin$^-$GzmA$^-$GzmB$^-$GzmK$^-$ CD8$^+$ T-cells were memory cells, with a large fraction displaying a transitional memory phenotype (Fig. 5). Because the proportion of GzmA^{Int}GzmB$^+$GzmK$^+$ was elevated in the mucosa of HIV-1$^+$ individuals not on ART (Fig. 2C), we were interested in the memory phenotype of these cells. Rectosigmoid subsets expressing GzmK, such as GzmA^{Int}GzmB$^+$GzmK$^+$ and GzmA^{Int}GzmK$^+$ CD8$^+$ T-cells, exhibited a more pronounced transitional memory phenotype than their counterparts in blood (Fig. 5, Supplemental Fig. 3). Mucosal single-positive GzmA$^{Int/Bright}$ CD8$^+$ T-cells were predominantly effector-memory cells (Fig. 5). In blood, GzmA^{Bright} cells were absent, and GzmA^{Int} cells were distributed amongst T_{TM}, T_{EM} and T_{EFF} categories. The relatively small subset of rectosigmoid CD8$^+$ T-cells expressing perforin, primarily Perforin$^+$GzmA^{Int}GzmB$^+$ CD8$^+$ T-cells with or without GzmK, was comprised of effector T-cells with some T_{TM} and T_{EM} (Fig. 5B), a distribution similar to that of Perforin$^+$GzmA^{Int}GzmB$^+$ CD8$^+$ T-cells in blood (Supplemental Fig. 3). Taken together, these data reveal differences between blood and gut regarding the memory phenotype of CD8$^+$ T-cells lacking expression of cytotoxic effectors, and identify the GzmA^{Bright} CD8$^+$ T-cell subset, limited to mucosa, as effector-memory cells.

GzmA^{Bright} CD8^{+} T-cells resemble tissue-resident T-cells

The human rectosigmoid mucosa houses a large fraction of CD8^{+} T-cells with a tissue resident phenotype, including in chronic HIV-1 infection (these authors, *in press*). As GzmA^{Bright} CD8^{+} T-cells were abundant in mucosa but not blood, and primarily displayed an effector-memory phenotype, we hypothesized that these cells might also express markers of tissue residency. To test this, we analyzed intracellular expression of GzmA and GzmB in conjunction with the canonical tissue-resident markers CD69, integrin αE (CD103), and sphingosine-1-phosphate receptor (S1PR1) on unstimulated blood and rectosigmoid CD8^{+} T-cells from chronically HIV-1 infected and seronegative adults.

Irrespective of HIV-1-disease status, the overwhelming majority of rectosigmoid GzmA^{Bright} CD8^{+} T-cells were CD69^{+}CD103^{+}S1PR1^{-}, indicative of tissue-residency (Fig. 6). In contrast, the expression of tissue-resident markers on GzmB^{+} and GzmA^{Int} CD8^{+} T-cells varied across HIV-1-disease status. In ART-treated and healthy adults, GzmB^{+} and GzmA^{Int} CD8^{+} T-cells were primarily CD69^{+}CD103^{+}S1PR1^{-}. In contrast, in HIV-1^{+} individuals not on ART, GzmB^{+} and GzmA^{Int} CD8^{+} T-cells were primarily single-positive for CD69 or lacked expression of all three markers (Fig. 6). Together, these data support the interpretation that GzmA^{Bright} CD8^{+} T-cells are likely a subset of tissue-resident CD8^{+} T-cells unique to the mucosa, whose functions and antigenic specificity remain to be determined.

Discussion

The gastrointestinal mucosa is an important site of HIV-1 transmission and viral replication, and is generally considered as one of the “front lines” of defense against mucosal pathogens. Understanding how mucosal CD8^{+} T-cells respond to microbial challenge is important for developing therapeutic and preventative strategies targeted to mucosal tissues. Previous reports have demonstrated that rectosigmoid CD8^{+} T-cells are robust producers of chemokines and cytokines but have limited perforin expression, supporting the interpretation that gut CD8^{+} T-cells are primed for cytokine/chemokine secretion rather than cytotoxicity (11–13, 28). However, missing from earlier studies of mucosal T-cells were detailed analyses of cytolytic effector proteins granzymes A, B and K and their patterns of expression/coexpression with cytokines and chemokines. In the present study we provide a more comprehensive view of the effector molecules produced by rectosigmoid CD8^{+} T-cells, which may be of particular interest in light of recent literature demonstrating novel extracellular, pro-inflammatory functions of granzymes (14, 15, 17, 29, 30).

As previously reported, the percentages of CD8^{+} T-cells expressing perforin and GzmB were significantly reduced in rectosigmoid mucosa compared to blood (11–13, 28). In contrast, the percentages of GzmK and GzmA-positive CD8^{+} T-cells were similar between the two tissues. These observed differences in expression levels of cytotoxic effectors suggest differential regulation at the transcriptional and/or post-transcriptional level. Interestingly, the gene for human GzmB is located on chromosome 14 whereas genes for GzmA and GzmK, whose expression was not deficient in the gut, are located in close proximity to one another on chromosome 5 (29, 31). Little is known about the transcriptional regulation of GzmA and GzmK but their spatial separation from GzmB might suggest differing

transcriptional regulation (32). Differences in post-transcriptional regulatory mechanisms may also account for the variations in expression levels. For example, although resting murine NK cells express mRNA for perforin, GzmB and GzmA, they must be activated to express perforin and GzmB protein; in contrast, both resting and activated NK cells express high levels of GzmA protein (33).

Although GzmA was the most abundantly expressed cytotoxic effector in both blood and gut, we observed discordance in its expression pattern between tissues. In mucosa, a large percentage of $CD8^+$ T-cells were single-positive for GzmA; a subset of these expressed granzyme A at high levels ($GzmA^{Bright}$) and displayed an effector/memory, tissue-resident phenotype ($CD103^+CD69^+S1PR1^-$) (34, 35). In blood, however, $GzmA^{Bright}$ $CD8^+$ T-cells were scarce and GzmA was nearly always co-expressed with other cytotoxic effectors. These data suggest that similar to perforin and GzmB, GzmA expression may be regulated differently in rectosigmoid $CD8^+$ T-cells compared to blood.

In contrast to the abundance of GzmA, the percentage of $CD8^+$ T-cells co-expressing perforin and granzymes was sharply reduced in gut compared to blood, further evidence of the diminished cytotoxic potential of rectosigmoid $CD8^+$ T-cells (11). We previously speculated that perforin and GzmB are tightly regulated in mucosa to avoid ‘bystander’ damage to the fragile mucosal epithelium (11). As granzymes are believed to be unable to induce target cell apoptosis without perforin (36), one interpretation of these data is that limited perforin expression enables rectosigmoid $CD8^+$ T-cells to utilize granzymes for non-cytolytic functions. Consistent with this interpretation, while the cytotoxicity of GzmB and perforin are well established, controversy remains as to whether other granzymes contribute significantly to target cell apoptosis *in vivo* (15, 37). Rather, GzmA and GzmK may function in extracellular, non-cytotoxic capacities in the mucosa. For example, GzmA and GzmK have been shown to facilitate cytokine responses to the microbial cell wall component lipopolysaccharide (LPS) (16, 38). Additionally, GzmA, B, and K have demonstrated extracellular activities that promote cellular migration and inflammation (14, 15, 17, 29, 30). Pro-inflammatory functions of granzymes appear consistent with the polyfunctional nature of rectosigmoid $CD8^+$ T-cells, whose expression of cytokines and beta-chemokines likely have similar pro-inflammatory effects (39, 40).

Disparities in the expression levels of cytotoxic effectors varied with HIV-1-disease status. The percentages of $CD8^+$ T-cells expressing $GzmA^{Int}$, GzmB, GzmK, and perforin were higher in HIV-1 infected compared to seronegative adults, with the highest percentages observed in $HIV\text{-}1^+$ individuals not on ART. Interestingly, the opposite trend was observed for single-positive $GzmA^{Bright}$ cells. One interpretation is that HIV-1 infection results in up-regulation of other cytotoxic effectors in $GzmA^{Bright}$ $CD8^+$ T-cells. The overwhelming majority of $GzmA^{Bright}$ $CD8^+$ T-cells expressed αE integrin (CD103), which is associated with proximity to epithelial cells expressing the CD103 ligand, E-cadherin (41–43). A characteristic of HIV-1 infection is loss of epithelial barrier integrity (44, 45). Accordingly, an alternative explanation is that lower proportions of $GzmA^{Bright}$ $CD8^+$ T-cells in HIV-1 infection reflects a loss of $CD103^+$ $CD8^+$ T-cells, due in part to reduced availability of epithelial-derived E-cadherin.

Notably, the proportion of $CD8^+$ T-cells co-expressing GzmA, B, and K was significantly greater in HIV-1 infected compared to seronegative participants in both blood and gut. In blood, co-expression of GzmA, B, and K is associated both with an intermediate memory maturation status and HIV-1-specificity (24–26). Similarly, rectosigmoid $GzmA^+GzmB^+GzmK^+$ $CD8^+$ T-cells primarily displayed a transitional memory phenotype and a substantial fraction of rectosigmoid Gag-responding $CD8^+$ T-cells co-expressed GzmA, B, and K. Whether co-expression of GzmA, B, and K in Gag-specific $CD8^+$ T-cells is a reflection of maturation status or a consequence of HIV-1-specific priming of the immune response will require further exploration. However previous work has demonstrated that blood HIV-1-specific $CD8^+$ T-cells are skewed towards an intermediate maturation stage, suggesting the former (46).

Consistent with the hypothesis that rectosigmoid $CD8^+$ T-cells are skewed towards cytokine production rather than cytotoxicity, the proportions of Gag and SEB-responding $CD8^+$ T-cells expressing cytotoxic effectors were significantly lower in rectosigmoid mucosa compared to blood. Furthermore, we observed that perforin and GzmB expression increased in Gag-specific $CD8^+$ T-cells as polyfunctionality decreased, suggesting that cytotoxic and cytokine/chemokine-producing Gag-specific $CD8^+$ T-cells are to some extent separate subsets. In the mucosa, responding $CD8^+$ T-cells often lacked expression of perforin and granzymes. As postulated elsewhere (11), degranulation in the absence of cytotoxic effectors may reflect the release of other granule components like MIP-1α and MIP-1β (47). Alternatively, it may reflect differences in the kinetics of cytotoxic effector up-regulation between blood and gut $CD8^+$ T-cells. In addition to differences between tissues, we also observed differences in expression of cytotoxic effectors between HIV-1 Gag and SEB-responding $CD8^+$ T-cells in the mucosa. The proportion of rectosigmoid Gag-responding $CD8^+$ T-cells expressing cytotoxic effectors was greater compared to SEB-responding $CD8^+$ T-cells, indicating that expression of cytotoxic effectors is, to a certain degree, influenced by antigen-specificity. It is currently unclear whether elevated cytotoxic effector expression in Gag-responding rectosigmoid $CD8^+$ T-cells reflects the up-regulation of cytotoxic effectors by tissue resident $CD8^+$ T-cells or, alternatively, an influx of cytotoxic effector-expressing $CD8^+$ T-cells from circulation. Addressing this question will require further work, likely involving experimental infection studies in nonhuman primate models.

Interestingly, unlike $GzmA^{Bright}$ $CD8^+$ T-cells that were overwhelmingly tissue-resident, a large fraction of $GzmB^+$ and $GzmA^{Int}$ rectosigmoid $CD8^+$ T-cells did not express the phenotypic markers of tissue-residency, suggesting they might be recent immigrants. Notably, Gag-specific polyfunctional $CD8^+$ T-cells were not completely devoid of perforin and granzyme B, suggesting a small fraction of Gag-specific $CD8^+$ T-cells may be capable of both cytotoxic and polyfunctional responses. Determining whether the co-expression of cytotoxic effectors with cytokines/chemokines confers a protective advantage against HIV-1 will require further analysis. No differences were observed between HIV-1 Controllers and Non-controllers in the proportion of Gag-specific $CD8^+$ T-cells expressing perforin and/or granzymes, although this analysis was limited by small sample size.

Taken together, these data demonstrate that rectosigmoid $CD8^+$ T-cells produce granzymes independently of perforin, likely complementing dominant cytokine and chemokine

responses to promote an inflammatory response. The work described here supports a model in which rectosigmoid $CD8^+$ T-cells are skewed towards pro-inflammatory responses rather than direct cytotoxicity, suggesting new insights into the mucosal adaptive immune response that may inform the development of therapeutics to treat mucosal infections such as HIV-1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Rebecca Hoh, Montha Pao, Monika Deswal and the clinical staff at San Francisco General Hospital for their assistance with participant recruitment and sample collection. We thank the participants for their willingness to contribute to this study. We thank the UC Davis Flow Cytometry Shared Resource Laboratory staff members Ms. Bridget McLaughlin and Mr. Jonathan Van Dyke, for assistance with flow cytometry.

Abbreviations used in this article

Gzm	granzyme
ART	antiretroviral therapy
SEB	*Staphylococcal* Enterotoxin B
MIP	macrophage inflammatory protein
CD	cluster of differentiation
C	controllers
V	viremic untreated
Tx	treated
SN	seronegative
S1PR1	sphingosine-1-phosphate receptor
VL	viral load
Perf	perforin

References

1. Cartwright EK, Spicer L, Smith SA, Lee D, Fast R, Paganini S, Lawson BO, Nega M, Easley K, Schmitz JE, Bosinger SE, Paiardini M, Chahroudi A, Vanderford TH, Estes JD, Lifson JD, Derdeyn CA, Silvestri G. CD8(+) Lymphocytes Are Required for Maintaining Viral Suppression in SIV-Infected Macaques Treated with Short-Term Antiretroviral Therapy. Immunity. 2016; 45:656–668. [PubMed: 27653601]
2. Di Stefano M, Favia A, Monno L, Lopalco P, Caputi O, Scardigno AC, Pastore G, Fiore JR, Angarano G. Intracellular and cell-free (infectious) HIV-1 in rectal mucosa. J Med Virol. 2001; 65:637–643. [PubMed: 11745925]
3. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. Journal of virology. 1994; 68:6103–6110. [PubMed: 8057491]

4. Shankar P, Xu Z, Lieberman J. Viral-specific cytotoxic T lymphocytes lyse human immunodeficiency virus-infected primary T lymphocytes by the granule exocytosis pathway. Blood. 1999; 94:3084–3093. [PubMed: 10556193]

5. Migueles SA, Laborico AC, Shupert WL, Sabbaghian MS, Rabin R, Hallahan CW, Van Baarle D, Kostense S, Miedema F, McLaughlin M, Ehler L, Metcalf J, Liu S, Connors M. HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. Nature immunology. 2002; 3:1061–1068. [PubMed: 12368910]

6. Saez-Cirion A, Lacabaratz C, Lambotte O, Versmisse P, Urrutia A, Boufassa F, Barre-Sinoussi F, Delfraissy JF, Sinet M, Pancino G, Venet A. E. P. H. I. V. C. S. G. Agence Nationale de Recherches sur le Sida. HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype. Proc Natl Acad Sci U S A. 2007; 104:6776–6781. [PubMed: 17428922]

7. Hersperger AR, Pereyra F, Nason M, Demers K, Sheth P, Shin LY, Kovacs CM, Rodriguez B, Sieg SF, Teixeira-Johnson L, Gudonis D, Goepfert PA, Lederman MM, Frank I, Makedonas G, Kaul R, Walker BD, Betts MR. Perforin expression directly ex vivo by HIV-specific CD8 T-cells is a correlate of HIV elite control. PLoS pathogens. 2010; 6:e1000917. [PubMed: 20523897]

8. Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J, Lederman MM, Benito JM, Goepfert PA, Connors M, Roederer M, Koup RA. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. Blood. 2006; 107:4781–4789. [PubMed: 16467198]

9. Almeida JR, Price DA, Papagno L, Arkoub ZA, Sauce D, Bornstein E, Asher TE, Samri A, Schnuriger A, Theodorou I, Costagliola D, Rouzioux C, Agut H, Marcelin AG, Douek D, Autran B, Appay V. Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. J Exp Med. 2007; 204:2473–2485. [PubMed: 17893201]

10. Ferre AL, Hunt PW, Critchfield JW, Young DH, Morris MM, Garcia JC, Pollard RB, Yee HF Jr, Martin JN, Deeks SG, Shacklett BL. Mucosal immune responses to HIV-1 in elite controllers: a potential correlate of immune control. Blood. 2009; 113:3978–3989. [PubMed: 19109229]

11. Kiniry BE, Ganesh A, Critchfield JW, Hunt PW, Hecht FM, Somsouk M, Deeks SG, Shacklett BL. Predominance of weakly cytotoxic, T-betLowEomesNeg CD8+ T-cells in human gastrointestinal mucosa: implications for HIV infection. Mucosal Immunol. 2017; 10:1008–1020. [PubMed: 27827375]

12. Critchfield JW, Young DH, Hayes TL, Braun JV, Garcia JC, Pollard RB, Shacklett BL. Magnitude and complexity of rectal mucosa HIV-1-specific CD8+ T-cell responses during chronic infection reflect clinical status. PLoS One. 2008; 3:e3577. [PubMed: 18974782]

13. Shacklett BL, Cox CA, Quigley MF, Kreis C, Stollman NH, Jacobson MA, Andersson J, Sandberg JK, Nixon DF. Abundant expression of granzyme A, but not perforin, in granules of CD8+ T cells in GALT: implications for immune control of HIV-1 infection. Journal of immunology. 2004; 173:641–648.

14. Metkar SS, Menaa C, Pardo J, Wang B, Wallich R, Freudenberg M, Kim S, Raja SM, Shi L, Simon MM, Froelich CJ. Human and mouse granzyme A induce a proinflammatory cytokine response. Immunity. 2008; 29:720–733. [PubMed: 18951048]

15. Susanto O, Trapani JA, Brasacchio D. Controversies in granzyme biology. Tissue antigens. 2012; 80:477–487. [PubMed: 23137319]

16. Wensink AC, Kemp V, Fermie J, Garcia Laorden MI, van der Poll T, Hack CE, Bovenschen N. Granzyme K synergistically potentiates LPS-induced cytokine responses in human monocytes. Proc Natl Acad Sci U S A. 2014; 111:5974–5979. [PubMed: 24711407]

17. Wensink AC, Hack CE, Bovenschen N. Granzymes regulate proinflammatory cytokine responses. Journal of immunology. 2015; 194:491–497.

18. Shacklett BL, Ferre AL. Mucosal immunity in HIV controllers: the right place at the right time. Curr Opin HIV AIDS. 2011; 6:202–207. [PubMed: 21399497]

19. Shacklett BL, Critchfield JW, Lemongello D. Isolating mucosal lymphocytes from biopsy tissue for cellular immunology assays. Methods Mol Biol. 2009; 485:347–356. [PubMed: 19020836]

20. Shacklett BL, Yang O, Hausner MA, Elliott J, Hultin L, Price C, Fuerst M, Matud J, Hultin P, Cox C, Ibarrondo J, Wong JT, Nixon DF, Anton PA, Jamieson BD. Optimization of methods to assess

human mucosal T-cell responses to HIV infection. Journal of immunological methods. 2003; 279:17–31. [PubMed: 12969544]

21. Hersperger AR, Makedonas G, Betts MR. Flow cytometric detection of perforin upregulation in human CD8 T cells. Cytometry Part A : the journal of the International Society for Analytical Cytology. 2008; 73:1050–1057. [PubMed: 18615597]
22. Roederer M, Nozzi JL, Nason MC. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. Cytometry Part A : the journal of the International Society for Analytical Cytology. 2011; 79:167–174. [PubMed: 21265010]
23. Hamann D, Baars PA, Rep MH, Hooibrink B, Kerkhof-Garde SR, Klein MR, van Lier RA. Phenotypic and functional separation of memory and effector human CD8+ T cells. J Exp Med. 1997; 186:1407–1418. [PubMed: 9348298]
24. Harari A, Bellutti Enders F, Cellerai C, Bart PA, Pantaleo G. Distinct profiles of cytotoxic granules in memory CD8 T cells correlate with function, differentiation stage, and antigen exposure. Journal of virology. 2009; 83:2862–2871. [PubMed: 19176626]
25. Chattopadhyay PK, Betts MR, Price DA, Gostick E, Horton H, Roederer M, De Rosa SC. The cytolytic enzymes granyzme A, granzyme B, and perforin: expression patterns, cell distribution, and their relationship to cell maturity and bright CD57 expression. Journal of leukocyte biology. 2009; 85:88–97. [PubMed: 18820174]
26. Takata H, Takiguchi M. Three memory subsets of human CD8+ T cells differently expressing three cytolytic effector molecules. Journal of immunology. 2006; 177:4330–4340.
27. Thome JJ, Yudanin N, Ohmura Y, Kubota M, Grinshpun B, Sathaliyawala T, Kato T, Lerner H, Shen Y, Farber DL. Spatial map of human T cell compartmentalization and maintenance over decades of life. Cell. 2014; 159:814–828. [PubMed: 25417158]
28. Andersson J, Behbahani H, Lieberman J, Connick E, Landay A, Patterson B, Sonnerborg A, Lore K, Uccini S, Fehniger TE. Perforin is not co-expressed with granzyme A within cytotoxic granules in CD8 T lymphocytes present in lymphoid tissue during chronic HIV infection. Aids. 1999; 13:1295–1303. [PubMed: 10449281]
29. Anthony DA, Andrews DM, Watt SV, Trapani JA, Smyth MJ. Functional dissection of the granzyme family: cell death and inflammation. Immunological reviews. 2010; 235:73–92. [PubMed: 20536556]
30. Joeckel LT, Wallich R, Martin P, Sanchez-Martinez D, Weber FC, Martin SF, Borner C, Pardo J, Froelich C, Simon MM. Mouse granzyme K has pro-inflammatory potential. Cell Death Differ. 2011; 18:1112–1119. [PubMed: 21311565]
31. Vahedi F, Fraleigh N, Vlasschaert C, McElhaney J, Hanifi-Moghaddam P. Human granzymes: related but far apart. Med Hypotheses. 2014; 83:688–693. [PubMed: 25459135]
32. Chowdhury D, Lieberman J. Death by a thousand cuts: granzyme pathways of programmed cell death. Annu Rev Immunol. 2008; 26:389–420. [PubMed: 18304003]
33. Fehniger TA, Cai SF, Cao X, Bredemeyer AJ, Presti RM, French AR, Ley TJ. Acquisition of murine NK cell cytotoxicity requires the translation of a pre-existing pool of granzyme B and perforin mRNAs. Immunity. 2007; 26:798–811. [PubMed: 17540585]
34. Schenkel JM, Masopust D. Tissue-resident memory T cells. Immunity. 2014; 41:886–897. [PubMed: 25526304]
35. Thome JJ, Farber DL. Emerging concepts in tissue-resident T cells: lessons from humans. Trends Immunol. 2015; 36:428–435. [PubMed: 26072286]
36. Thiery J, Lieberman J. Perforin: a key pore-forming protein for immune control of viruses and cancer. Subcell Biochem. 2014; 80:197–220. [PubMed: 24798013]
37. Joeckel LT, Bird PI. Are all granzymes cytotoxic in vivo? Biol Chem. 2014; 395:181–202. [PubMed: 24002663]
38. Sharma M, Merkulova Y, Raithatha S, Parkinson LG, Shen Y, Cooper D, Granville DJ. Extracellular granzyme K mediates endothelial activation through the cleavage of protease-activated receptor-1. FEBS J. 2016; 283:1734–1747. [PubMed: 26936634]
39. Schenkel JM, Fraser KA, Beura LK, Pauken KE, Vezys V, Masopust D. T cell memory. Resident memory CD8 T cells trigger protective innate and adaptive immune responses. Science. 2014; 346:98–101. [PubMed: 25170049]

40. Schenkel JM, Fraser KA, Vezys V, Masopust D. Sensing and alarm function of resident memory CD8(+) T cells. Nat Immunol. 2013; 14:509–513. [PubMed: 23542740]

41. Cepek KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL, Brenner MB. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. Nature. 1994; 372:190–193. [PubMed: 7969453]

42. Agace WW, Higgins JM, Sadasivan B, Brenner MB, Parker CM. T-lymphocyte-epithelial-cell interactions: integrin alpha(E)(CD103)beta(7), LEEP-CAM and chemokines. Curr Opin Cell Biol. 2000; 12:563–568. [PubMed: 10978890]

43. Sheridan BS, Pham QM, Lee YT, Cauley LS, Puddington L, Lefrancois L. Oral infection drives a distinct population of intestinal resident memory CD8(+) T cells with enhanced protective function. Immunity. 2014; 40:747–757. [PubMed: 24792910]

44. Burgener A, McGowan I, Klatt NR. HIV and mucosal barrier interactions: consequences for transmission and pathogenesis. Curr Opin Immunol. 2015; 36:22–30. [PubMed: 26151777]

45. Mudd JC, Brenchley JM. Gut Mucosal Barrier Dysfunction, Microbial Dysbiosis, and Their Role in HIV-1 Disease Progression. J Infect Dis. 2016; 214(Suppl 2):S58–66. [PubMed: 27625432]

46. Champagne P, Ogg GS, King AS, Knabenhans C, Ellefsen K, Nobile M, Appay V, Rizzardi GP, Fleury S, Lipp M, Forster R, Rowland-Jones S, Sekaly RP, McMichael AJ, Pantaleo G. Skewed maturation of memory HIV-specific CD8 T lymphocytes. Nature. 2001; 410:106–111. [PubMed: 11242051]

47. Demers KR, Reuter MA, Betts MR. CD8(+) T-cell effector function and transcriptional regulation during HIV pathogenesis. Immunological reviews. 2013; 254:190–206. [PubMed: 23772621]

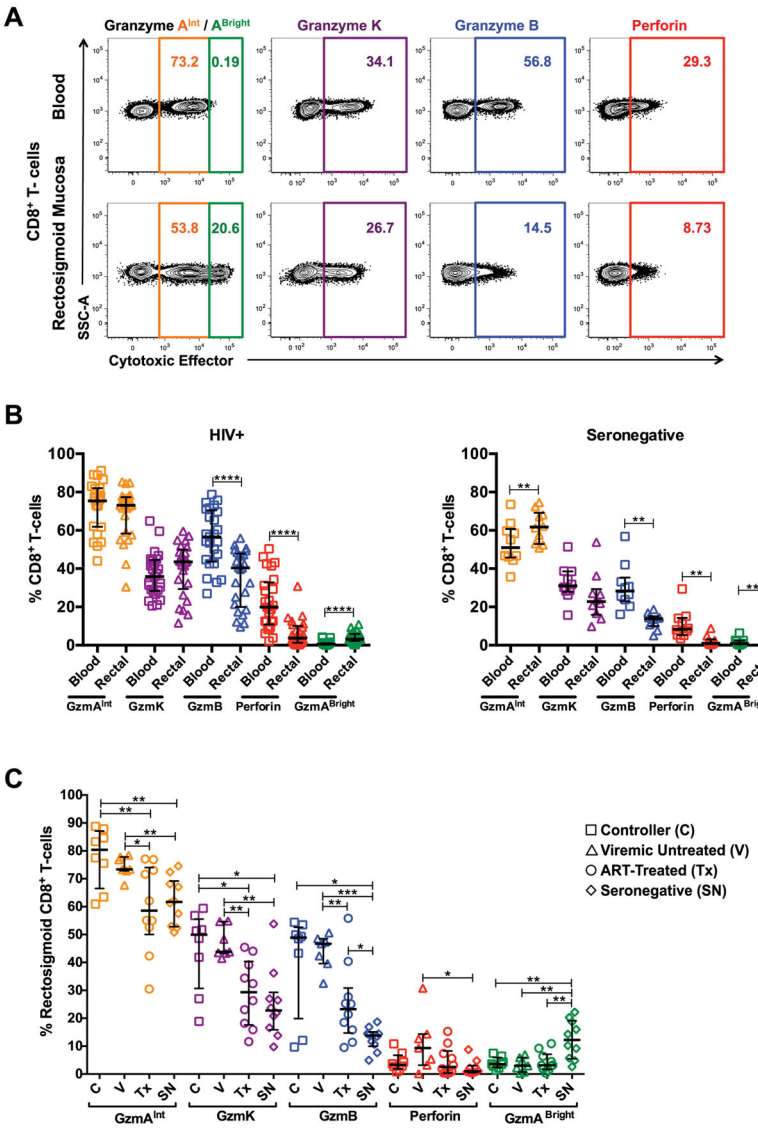

FIGURE 1. Expression of cytotoxic effector molecules in unstimulated blood and rectosigmoid CD8$^+$ T-cells across HIV-1-disease status

(**A**) Representative flow cytometry plot displaying intracellular protein staining for GzmA$^{Int/Bright}$, GzmB, GzmK, and perforin in unstimulated blood and rectosigmoid CD8$^+$ T-cells CD8$^+$ T-cells. (**B**) Differences in the intracellular expression of GzmA$^{Int/Bright}$, GzmB, GzmK, and perforin between unstimulated blood and rectosigmoid CD8$^+$ T-cells in HIV-1-infected (HIV-1$^+$) and seronegative participant groups (n=25 and 10, respectively). (**C**) Differences in the intracellular expression of GzmA$^{Int/Bright}$, GzmB, GzmK, and perforin in unstimulated rectosigmoid CD8$^+$ T-cells across HIV-1-disease status as follows: C (Controllers) n=8, V (viremic untreated) n=7, Tx (ART-suppressed) n=10, SN (seronegatives) n=10. In **B** and **C**, wide horizontal bars represent medians; narrow whiskers indicate interquartile ranges; asterisks show level of significance as follows: * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001.

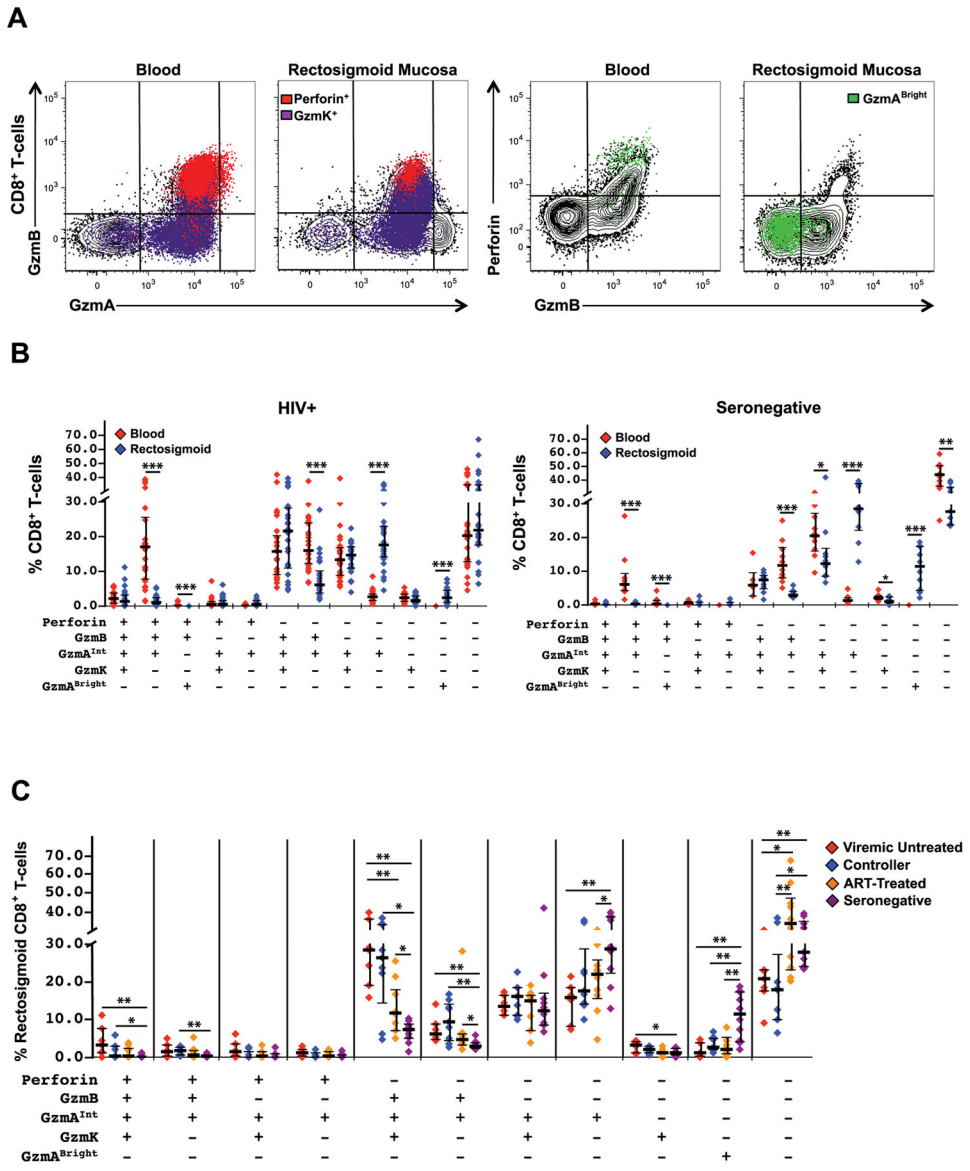

FIGURE 2. Co-expression patterns of cytotoxic effectors vary with anatomical compartment and HIV-1 disease status

(**A**) Representative flow cytometry plot displaying the intracellular co-expression patterns of GzmA$^{Int/Bright}$, GzmB, GzmK, and perforin in unstimulated blood and rectosigmoid CD8$^+$ T-cells. (**B**) Differences in the proportions of CD8$^+$ T-cells co-expressing GzmA$^{Int/Bright}$, GzmB, GzmK, and perforin between blood and rectosigmoid mucosa in HIV-1 infected (HIV-1$^+$) and seronegative participants (n=25 and 10, respectively). Wide horizontal bars represent medians; narrow whiskers indicate interquartile ranges; asterisks show level of significance as follows: * P <0.05, ** P <0.01, *** P <0.001. (**C**) Differences in the percentages of rectosigmoid CD8$^+$ T-cells co-expressing cytotoxic effectors across HIV-1-disease status as follows: Viremic Untreated (V) n=7, Controllers (C) n=8, ART-suppressed (Tx) n=10, and seronegatives (SN) n=10. Wide horizontal bars represent medians; narrow

whiskers indicate interquartile ranges; horizontal bars between participant subgroups indicate significance as follows: * P <0.05, ** P <0.01.

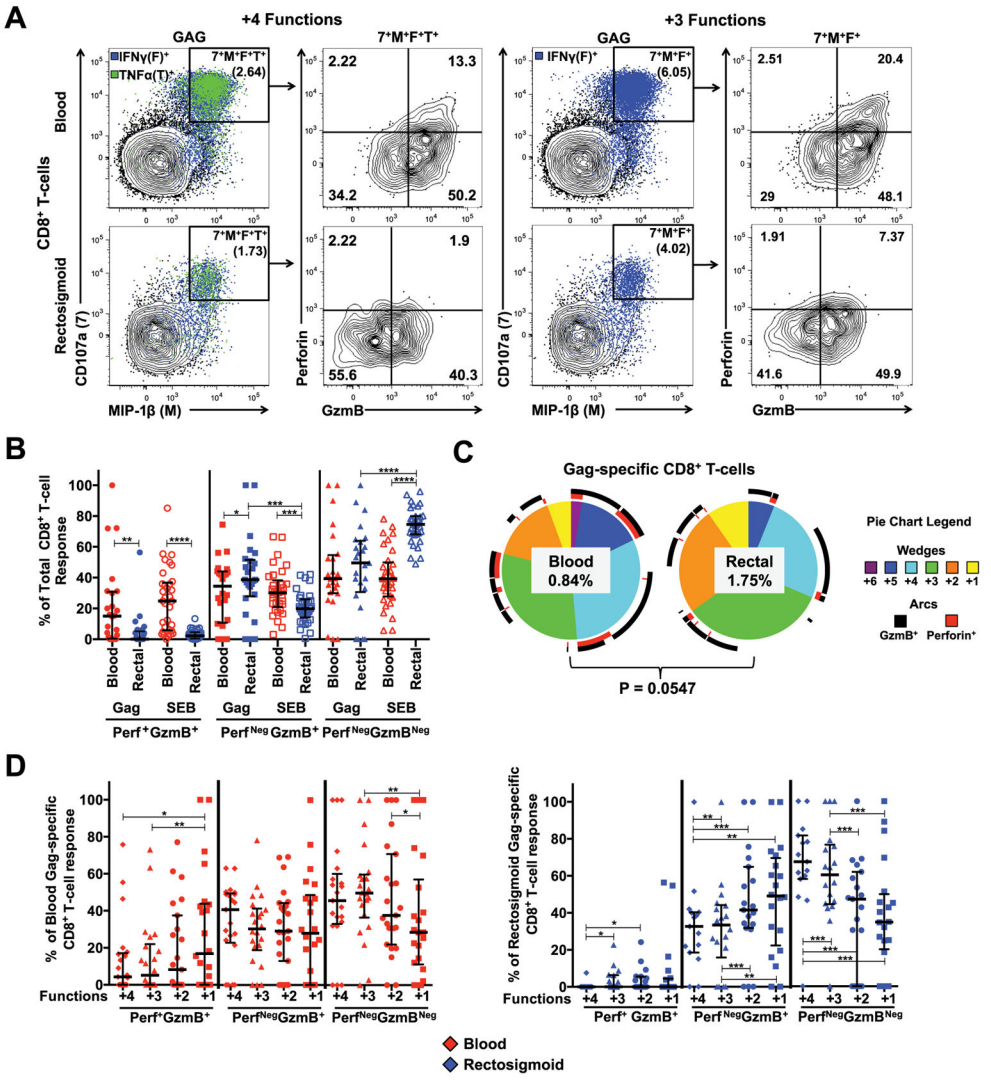

FIGURE 3. Expression of cytokines, chemokines, perforin and GzmB in blood and rectosigmoid CD8$^+$ T-cells responding to *ex vivo* stimulation

(**A**) Representative flow cytometry plot displaying intracellular expression of perforin and GzmB in polyfunctional HIV-1 Gag-responding CD8$^+$ T-cells (4 functions: CD107a$^+$MIP-1β$^+$IFNγ$^+$TNFα$^+$ and 3 functions: CD107a$^+$MIP-1β$^+$IFNγ$^+$) in blood and rectosigmoid mucosa. (**B**) Expression of perforin (Perf) and/or GzmB in HIV-1 Gag or SEB-responding CD8$^+$ T-cells showing differences related to stimulus and anatomical compartment. n=27. (**C**) Differences in intracellular expression of perforin and GzmB within mono- and polyfunctional HIV-1 Gag-responding CD8$^+$ T-cells in blood and rectosigmoid mucosa visualized with a SPICE pie chart. Responsiveness was defined as the expression of one or more of the following effectors: CD107a, MIP-1β, IFNγ, and TNFα. Percentages indicate the median total HIV-1 Gag CD8$^+$ T-cell response. Wedge colors correspond to the number of simultaneous functions, as indicated in the legend (perforin, GzmB, CD107a, MIP-1β, IFNγ, and TNFα). n=27. (**D**) Differences in expression of perforin and/or GzmB between mono- and polyfunctional HIV-1 Gag-specific CD8$^+$ T-cells in blood and mucosa.

Numbers on the x-axis correspond to the number of simultaneous functions (CD107a, MIP-1β, IFNγ, and TNFα). n=27. In **B** and **D,** wide horizontal bars represent medians; narrow whiskers indicate interquartile ranges; asterisks show level of significance as follows: * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001.

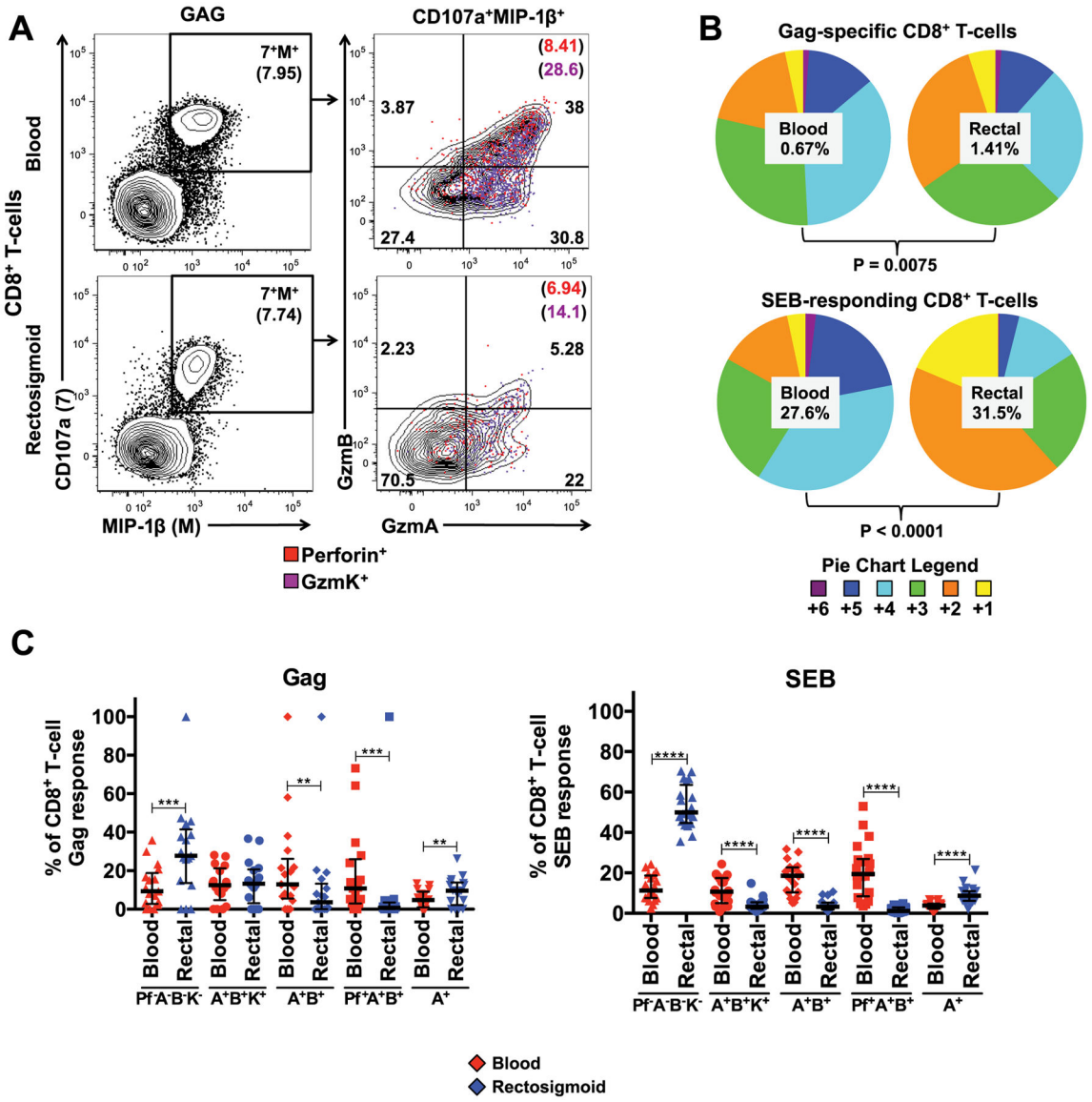

FIGURE 4. Expression/co-expression patterns of cytotoxic granule proteins in *ex vivo* stimulated CD8$^+$ T-cells from blood and rectosigmoid mucosa

(**A**) Representative flow cytometry plot displaying expression of GzmA, B, K, and perforin in CD8$^+$ T-cells responding to *ex vivo* Gag-peptide stimulation as indicated by degranulation (CD107a) and MIP-1β expression. (**B**) Differences in expression of cytotoxic effectors between blood and rectosigmoid mucosal CD8$^+$ T-cells responding to an HIV-1 Gag-peptide pool or SEB, summarized with SPICE pie charts. Responsiveness was defined as the expression of one or more of the following: CD107a and Mip-1β. n=18. (**C**) Differences in the expression of cytotoxic effectors between blood and rectosigmoid mucosal CD8$^+$ T-cells responding to an HIV-1 Gag-peptide pool or SEB, detailing individual response combinations. n=18. Wide horizontal bars represent medians; narrow whiskers indicate interquartile ranges; asterisks show level of significance as follows: ** P <0.01, *** P <0.001, **** P <0.0001.

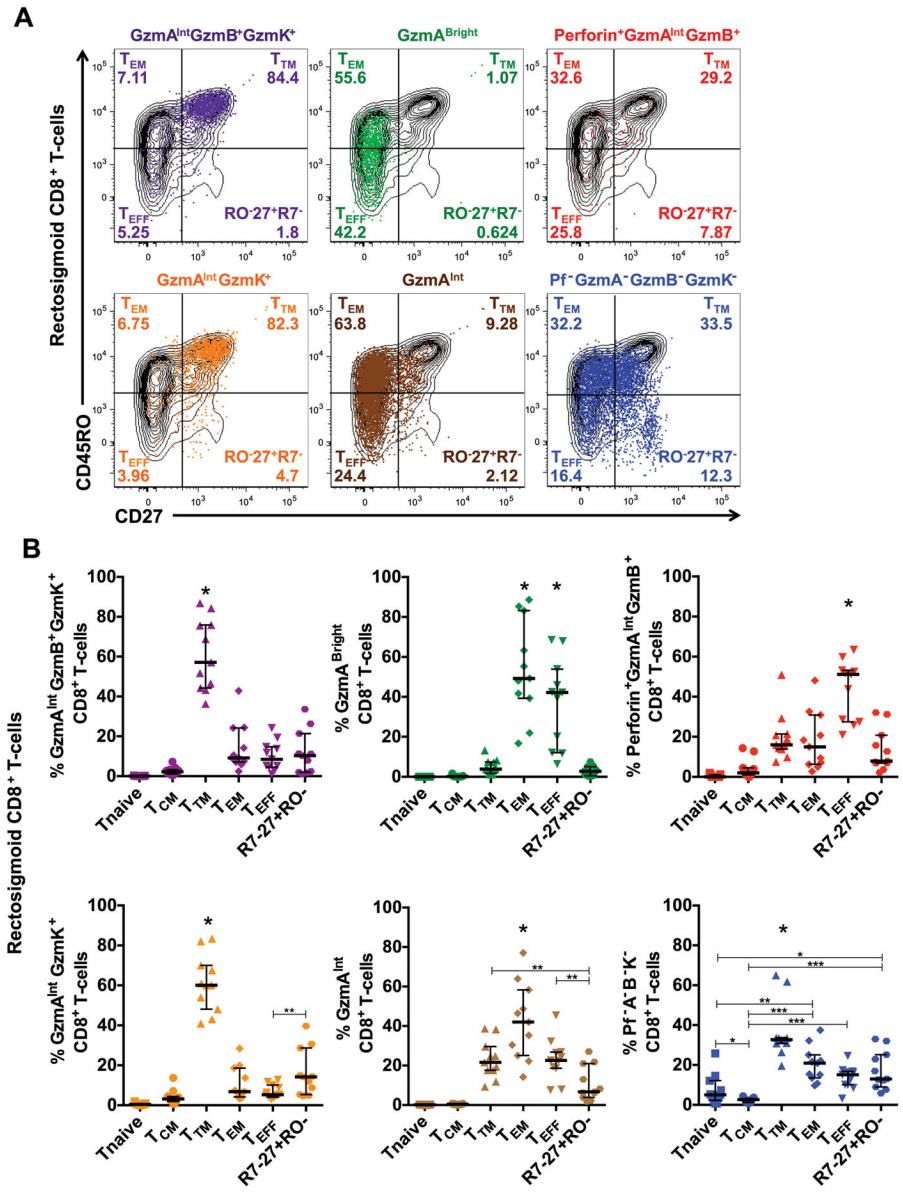

FIGURE 5. Expression of memory/effector markers by prominent CD8$^+$ T-cell subsets in rectosigmoid mucosa

(**A**) Representative flow cytometry plot displaying expression of memory markers CD45RO and CD27 on the following rectosigmoid CD8$^+$ T-cells subsets: GzmA^{Int}GzmB$^+$GzmK$^+$ (purple), GzmA^{Int}GzmK$^+$ (orange), GzmA^{Bright} (green), GzmA^{Int} (brown), Perforin $^+$GzmA^{Int}GzmB$^+$ (red), and cells lacking expression of cytotoxic effectors (Perforin$^-$GzmB $^-$GzmA$^-$GzmK$^-$)(blue). The proportions of transitional memory (T_{TM}), effector-memory (T_{EM}), effector (T_{EFF}), and CD45RO$^-$CD27$^+$CCR7$^-$ cells within each subset are displayed within the quadrants. (**B**) Differences in the percentages of naïve (T_{naive}), central-memory (T_{CM}), transitional memory (T_{TM}), effector-memory (T_{EM}), effector (T_{EFF}), and CD45RO $^-$CD27$^+$CCR7$^-$ cells within GzmA^{Int}GzmB$^+$GzmK$^+$, GzmA^{Int}GzmK$^+$, GzmA$^{Int/Bright}$ single-positive, Perforin$^+$GzmA^{Int}GzmB$^+$, and Perforin$^-$GzmB$^-$GzmA$^-$GzmK$^-$

rectosigmoid $CD8^+$ T-cells. Large asterisk identifies memory phenotypes with a significantly greater median frequency compared to all other memory subsets. n=11. Wide horizontal bars represent medians; narrow whiskers indicate interquartile ranges; small asterisks show level of significance as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

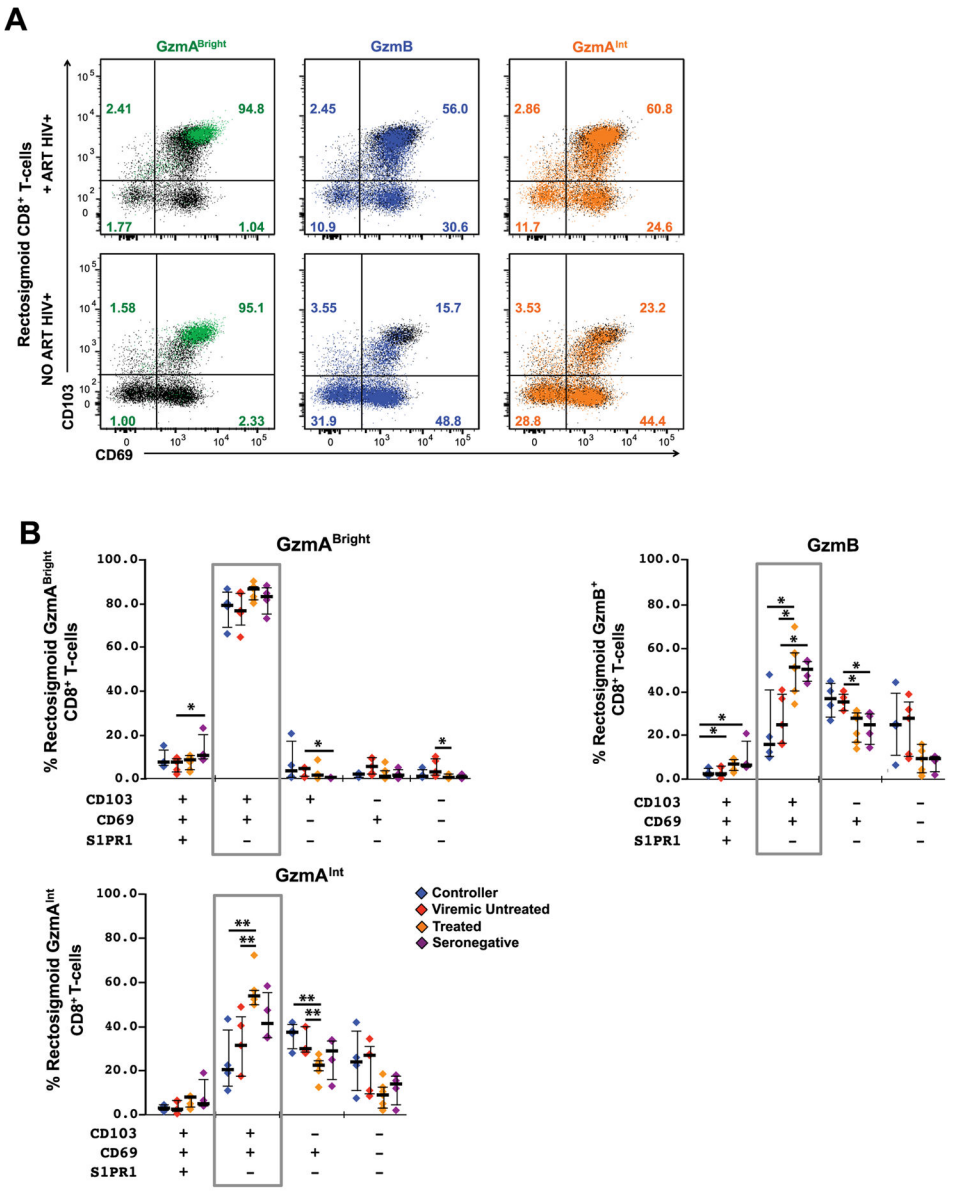

FIGURE 6. Expression of tissue-resident markers on GrzA^{Bright}, GrzA^{Int}, and GrzB-expressing CD8^{+} T-cells in rectal mucosa

(A) Representative flow cytometry plot showing expression of the tissue-resident markers early leukocyte activation marker CD69 and integrin αE (CD103) on GrzA^{Bright}, GrzA^{Int}, and GrzB-expressing CD8^{+} T-cells in rectal mucosa. (B) Proportion of rectal GrzA^{Bright}, GrzA^{Int}, and GrzB-positive CD8^{+} T-cells expressing the tissue residency markers CD69, CD103, and sphingosine-1-phosphate receptor (S1PR1) in HIV-1 Controllers n=4, Viremic Untreated n=5, ART-treated n=7, and Seronegatives n=4. Gray box outlines the canonical tissue residency phenotype. Co-expression analysis was generated using SPICE software. Wide horizontal bars represent medians; narrow whiskers indicate interquartile ranges; symbols indicate level of significance as follows: * P <0.05, ** P <0.01.

J Immunol. Author manuscript; available in PMC 2019 March 01.

Table I

Participant Characteristics

	Gender	Race/Ethnicity	Plasma viral load, RNA copies/mL* [Median] [Range]	CD4 count, Cells/mm^3 [Median] [Range]	Time Post HIV+Diagnosis, years [Median] [Range]
Controller (n = 10)	M, 5 F, 3 T, 2	AA, 7 C, 2 Mx, 1	844 <40 – 1,727	716 353 – 2,542	20.5 4 – 29.8
Viremic Untreated (n = 15)	M, 15	AA, 7 C, 4 HL, 3 Mx, 1	20,544 393 – 186,307	506 236 – 1075	2.75 1 – 22.67
ART-Treated (n = 13)	M, 11 F, 1 T, 1	AA, 3 C, 7 HL, 3	<40 NA	603 193 – 811	18 2.7 – 27.1
Seronegative ** **(n = 10)**	M, 10	AA, 1 C, 6 HL, 2 Mx, 1	NA NA	950 600 – 1,211	NA NA[1]

[1]M, Male; F, Female; T, Transgender (M to F)

AA, African American; C, Caucasian; HL, Hispanic/Latino; Mx, Mixed; NA, Not Applicable.

*Median calculated using only detectable viral load data points.

**CD4 count unavailable for one subject.