Elevated Expression of CD160 and 2B4 Defines a Cytolytic HIV-Specific CD8⁺ T-Cell Population in Elite Controllers

Carolina Pombo,¹ E. John Wherry,^{1,2} Emma Gostick,⁴ David A. Price,^{3,4} and Michael R. Betts¹

¹Department of Microbiology, and ²Institute for Immunology, Perelman School of Medicine, University of Pennsylvania, Philadelphia; ³Human Immunology Section, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland; and ⁴Institute of Infection and Immunity, Cardiff University School of Medicine, United Kingdom

During chronic human immunodeficiency virus (HIV) infection, virus-specific CD8⁺ T cells become functionally exhausted. Unlike most chronically infected individuals, elite controllers of HIV retain CD8⁺ T-cell polyfunctionality and cytolytic capacity. It remains unclear whether elite controllers manifest T-cell exhaustion similar to subjects with chronic progression of HIV infection. Here we assessed coexpression of PD-1, Lag-3, CD160, and 2B4 as a measure of T-cell exhaustion in a cohort of elite controllers and in chronic progressors. We found that elite controllers have a high proportion of potentially exhausted (PD1⁺CD160⁺2B4⁺) HIV-specific CD8⁺ T cells that is comparable to the proportion in chronic progressors. However, elite controllers also harbor a population of HIV-specific CD160⁺2B4⁺ CD8⁺ T cells that correlates with cytolytic capacity, as measured by perforin expression, a population not commonly present in chronic progressors. We therefore propose that coexpression of CD160 and 2B4 delineates a population of cytolytic CD8⁺ T cells important for the control of HIV.

Keywords. HIV; elite controllers; T-cell exhaustion; PD-1; CD160; 2B4; CD8⁺ T cells.

Human immunodeficiency virus (HIV) infection is typically characterized as a chronic progressive disease associated with high viral loads and a steady decline in CD4⁺ T-cell counts that results in the eventual collapse of the immune system. During the early stages of infection, CD8⁺ T cells play an important role in controlling viral replication by eliminating infected cells [1]. However, CD8⁺ T cells lose their functional ability as the disease progresses into the chronic stage, a state known as T-cell exhaustion [2]. If left untreated, the majority of HIV-infected individuals, known as chronic progressors (hereafter, “progressors”), will develop gradual immunodeficiency that results in an increased risk of opportunistic infections and tumors 7–10 years, on

average, after infection. However, a small subset of HIV-infected individuals, referred to as elite controllers (hereafter, “controllers”), are capable of suppressing viral replication to very low levels without the need for antiretroviral therapy. Understanding differences in the CD8⁺ T-cell response between progressors and controllers may provide insights into the correlates of immune protection, thereby informing the development of HIV vaccines and immunotherapeutic strategies.

Previous work from our laboratory has shown that CD8⁺ T-cell responses can be classified into polyfunctional memory (interleukin 2 [IL-2] and interferon γ [IFN- γ] coexpression and other functions) or polyfunctional effector (perforin and IFN- γ coexpression and other functions) on the basis of viral specificity [3]. In the context of elite control, highly functional effector HIV-specific CD8⁺ T cells are maintained and can contribute to the containment of HIV replication [4–8]. Additional evidence suggests that the number of T cells with highly polyfunctional effector responses correlates with immune protection during viral infections [9–11]. Furthermore, data from nonhuman primate models support the role of CD8⁺ T cells in

Received 16 February 2015; accepted 6 April 2015; electronically published 15 April 2015.

Correspondence: Michael R. Betts, PhD, Microbiology, University of Pennsylvania, 522E Johnson Pavilion, 3610 Hamilton Walk, Philadelphia, PA 19104 (betts@mail.med.upenn.edu).

The Journal of Infectious Diseases® 2015;212:1376–86

© The Author 2015. Published by Oxford University Press on behalf of the Infectious Diseases Society of America. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com.
DOI: 10.1093/infdis/jiv226

controlling viral replication, with depletion of these cells leading to increased simian immunodeficiency virus loads and rapid disease progression [12, 13].

During chronic disease, HIV-specific CD8⁺ T cells become exhausted, a state characterized by the loss of proliferative capacity, cytolysis, and polyfunctionality [14–16]. T-cell exhaustion has been studied in mice [17–20], nonhuman primates [21, 22], and human subjects with chronic diseases [16, 23–26]. Although there are differences among the various models used, a common characteristic of T-cell exhaustion is that it is antigen specific and associated with translational, metabolic, and bioenergetic deficiencies, as well as with the cumulative expression of inhibitory receptors [16, 17, 23–28]. Typical markers associated with exhaustion include PD-1, Lag-3, CD160, and 2B4. Additionally, studies have shown a strong association between high levels of PD-1, HIV load, and disease progression [15, 29, 30]. Different patterns of inhibitory receptor expression have also been shown to identify exhausted populations defined by decreased production of IL-2, tumor necrosis factor (TNF), and IFN- γ [14, 16, 17, 24, 31, 32]. While the majority of previous work has been done in the context of chronic disease, the level of T-cell exhaustion in elite control of HIV has not been explored. To better understand the relationship between inhibitory receptors and T-cell function, we measured patterns of PD-1, Lag-3, CD160, and 2B4 expression on HIV-specific and Epstein-Barr virus (EBV)-specific CD8⁺ T cells from controllers and progressors. In addition, we assessed the ability of CD8⁺ T cells expressing multiple inhibitory receptors to produce various and essential effector functions. Our results show that a large proportion of Gag-specific CD8⁺ T cells from controllers coexpress PD-1, CD160, and 2B4, suggesting a level of potential exhaustion similar to that of progressors. However, controllers also harbor a large population of Gag-specific CD8⁺ T cells coexpressing CD160 and 2B4 that is associated with cytolytic potential. Together, these data suggest that the coexpression of inhibitory markers on HIV-specific CD8⁺ T cells does not always indicate exhaustion, but rather that expression must be interpreted within the context of T-cell function and phenotype and HIV disease status.

MATERIALS AND METHODS

Samples

Peripheral blood mononuclear cells (PBMCs) were obtained from HIV-negative subjects at the Penn Center for AIDS Research Immunology Core and were cryopreserved in fetal bovine serum (FBS; ICS Hyclone, Logan, Utah) containing 10% dimethyl sulfoxide (DMSO; Fisher Scientific, Pittsburgh, Pennsylvania). PBMCs from HIV-positive individuals were obtained in compliance with the guidelines set by the respective institutional review boards from the Penn Center for AIDS Research Human Clinical Core Facility and clinics

associated with Harvard University and the University of Alabama–Birmingham.

Progressors were defined as untreated individuals with HIV plasma RNA levels of >2000 copies/mL (range, 2319–484 699 copies/mL; median, 10 193 copies/mL) infected for >2 years. Controllers were defined as therapy-naive individuals with viral loads of <75 HIV RNA copies/mL at multiple time points who had been infected for >2 years. All subjects had CD4⁺ T-cell counts of >200 cells/mm³ and lacked evidence of AIDS-defining illness.

Antibodies

Antibodies for surface staining included anti-CD4 PE-Cy5.5 (clone S3.5; Invitrogen), anti-CD8 Qdot605 (clone 3B5; Invitrogen), or BV605 (clone RPA-T8; Biolegend); anti-CD27 Qdot 655 (clone CLB-27/1; Invitrogen) or BV650 (clone O323; Biolegend); anti-CD45RO ECD (clone UCHL1; Beckman Coulter), anti-PD-1 PE (clone EH12.2 H7; Biolegend), anti-CD160 FITC [33], anti-CD244 (2B4) PE-Cy5 (clone C1.7; Beckman Coulter), anti-CD14 APC-AF750 (clone Tük4, Invitrogen), anti-CD19 APC-AF750 (clone SJ25-C1; Invitrogen), anti-Lag-3 biotin (polyclonal; R&D Systems), streptavidin-APC (Invitrogen), and anti-APC biotin (clone eBioAPC-62A; eBioscience). Antibodies for intracellular staining included anti-CD3 Qdot 585 [34] or BV570 (clone UCHL1; Biolegend), anti-perforin PacBlue (clone B-D48 [34]) or BV421 (clone B-D48; Biolegend), anti-IFN- γ Alexa-700 (clone B27; Invitrogen), and anti-TNF PE-Cy7 (clone MAb11; BD Biosciences). Peptide/major histocompatibility complex class I complexes of HLA-A*0201:Gag-SL9, HLA-A*A0201:Gag-KF10, HLA-B*5701:Gag-KF11, and HLA-B*5701:Gag-IW9 were produced as described previously [35].

Cell Stimulation

Cryopreserved PBMCs were thawed and rested overnight at 37°C and 5% CO₂ in complete medium (Roswell Park Memorial Institute 1640 medium; Mediatech, Manassas, Virginia) supplemented with 10% FBS, 1% L-glutamine (Mediatech), and 1% penicillin-streptomycin (Lonza; Walkersville, Maryland) at 2×10^6 cells/mL. The cells were then washed and resuspended at a concentration of 1×10^6 cells/mL with costimulatory antibodies (anti-CD28 and anti-CD49d; 1 μ g/mL final concentration; BD Biosciences, San Jose, California), in the presence of brefeldin A (1 μ g/mL final concentration; Sigma-Aldrich, St. Louis, Missouri). Next, cells were stimulated with exogenous peptide pools for HIV Gag-PTE (NIH AIDS Reference Reagent Program) or EBV proteins (BCRF1, BMLF1, BMRF1, BRLF1, BZLF1, gp85, gp110, gp350, and EBNA4) at final concentration of 2 μ g/mL for each peptide. Stimulation with *Staphylococcus enterotoxin B* (1 μ g/mL; Sigma-Aldrich) was used as a positive control, and DMSO (5 μ L/mL) was used as a negative control. PBMCs were stimulated at 37°C in 5% CO₂ for 5 hours.

Flow Cytometric Staining

After stimulation, cells were washed once with fluorescence-activated cell-sorting (FACS) buffer and stained serially for Lag-3 as follows, with further washes between each step: anti-Lag-3 biotin (R&D Systems) for 15 minutes, streptavidin-APC (Invitrogen) for 15 minutes, anti-APC biotin (eBioscience) for 15 minutes, and streptavidin-APC for 15 minutes. After washing with phosphate-buffered saline (PBS) and staining with Aqua amine-reactive viability dye (Invitrogen) for 10 minutes to exclude nonviable events, the cells were stained for surface markers with an antibody cocktail for an additional 30 minutes. Following a further wash with FACS buffer, cells were permeabilized with Cytofix/Cytoperm (BD Biosciences) as per the manufacturer's instructions. Next, a cocktail of antibodies against intracellular markers was added and incubated for 1 hour. Finally, the cells were washed with Perm Wash Buffer (BD Biosciences) and fixed in PBS containing 1% paraformaldehyde. All incubations were done at room temperature in the dark. Fixed cells were stored at 4°C until the time of collection.

Flow Cytometric Analysis

For each sample, between 5×10^5 and 1×10^6 total events were acquired on a modified flow cytometer (LSRII; BD Immunocytometry Systems) equipped for the detection of 18 fluorescent parameters and longitudinally standardized for signal consistency, using previously described calibration methods [36]. Antibody-capture beads (BD Biosciences) were used to prepare individual fluorophore-matched compensation tubes for each antibody used in the experiments. Data analysis was performed using FlowJo, version 9.6.4 (TreeStar). Reported functional data have been corrected for background. Statistical analysis was performed with Prism, version 5.0. Comparison of inhibitory receptor expression among cohorts was analyzed using the Mann–Whitney *U* test. Correlation coefficients were calculated using the Spearman rank sum test. All tests were 2-tailed, and *P* values of $<.05$ were considered statistically significant.

RESULTS

Controllers Express Less PD-1 but More CD160 Than Progressors

To determine the level of potential T-cell exhaustion present in controllers, compared with that in progressors, we analyzed the expression patterns of the inhibitory markers PD-1, Lag-3, CD160, and 2B4 by polychromatic flow cytometry. Representative gating schemes for analysis of PD-1, Lag-3, CD160, and 2B4 expression are shown in [Supplementary Figure 1A](#). Differential expression of CD27 and CD45RO was used to distinguish between naive and memory populations. Naive cells were then used as reference to determine surface expression of individual exhaustion markers. Flow cytometry plots showing differential expression of PD-1, Lag-3, CD160, and 2B4 on naive and

memory CD8⁺ T cells for HIV-negative individuals, progressors, and controllers are shown in [Supplementary Figure 1B](#). We also evaluated individual surface expression of these exhaustion markers on total CD8⁺ T cells ([Supplementary Figure 2A](#)). Overall, differences in expression of PD-1, Lag-3, CD160, and 2B4 observed between total and memory populations were due to the proportion of naive CD8⁺ T cells present ([Supplementary Figure 2B](#)). Therefore, to better compare the controller, progressor, and HIV-negative groups, we continued our analysis by focusing on bulk memory cells. Representative flow cytometry plots showing gating of individual inhibitory receptors on memory CD8⁺ T cells from controllers, progressors, and HIV-negative subjects are shown in [Figure 1A](#). In accordance with previous reports [37], memory CD8⁺ T cells from controllers expressed PD-1 at significantly lower frequencies (mean, 30%) than those from progressors (mean, 45%; $P < .01$) and HIV-negative subjects (mean, 40%; $P < .01$; [Figure 1B](#)). Lag-3 was expressed at low frequencies on memory CD8⁺ T cells (mean, 5%–10%), regardless of infection status ([Figure 1B](#)). Memory CD8⁺ T cells in the controller cohort expressed significantly more CD160 (mean, 58%), compared with those in the progressor cohort (mean, 27%; $P < .01$) and the HIV-negative cohort (mean, 32%; $P < .05$; [Figure 1B](#)). However, controller memory CD8⁺ T cells expressed 2B4 at frequencies comparable to those from progressors (mean, 84% and 86%, respectively) but higher than those from HIV-negative individuals (mean, 72%; $P < .001$; [Figure 1B](#)).

Controllers Express a High Frequency of CD160⁺2B4⁺ CD8⁺ T Cells

Oneway T-cell exhaustion is characterized by the coexpression of inhibitory receptors on the cell surface [17, 27]. Accordingly, we simultaneously measured coexpression of PD-1, Lag-3, CD160, and 2B4 on total ([Supplementary Figure 2C](#)) and memory ([Figure 2](#)) CD8⁺ T cells. A majority of total and memory CD8⁺ T cells were grouped into 4 populations: PD-1⁺Lag-3⁺CD160⁺2B4⁺, PD-1⁺Lag-3⁺CD160⁺2B4⁺, PD-1⁺Lag-3⁺CD160⁺2B4⁺, and PD-1⁺Lag-3⁺CD160⁺2B4⁺. Differences in frequencies between total and memory populations in controllers, progressors, and HIV-negative subjects were due to the fraction of naive CD8⁺ T cells (data not shown). Therefore, we continued our analysis by focusing on memory CD8⁺ T cells. Controllers had a higher frequency of PD-1⁺Lag-3⁺CD160⁺2B4⁺ memory CD8⁺ T cells than progressors ($P < .0001$) and HIV-negative individuals ($P < .0001$). In contrast, controllers expressed less PD-1⁺Lag-3⁺CD160⁺2B4⁺ than progressors and HIV-negative subjects ($P < .0001$) and fewer PD-1⁺Lag-3⁺CD160⁺2B4⁺ single-positive cells than progressors ($P < .05$; [Figure 2](#)). We found a trend toward a higher frequency of the triple-positive (PD-1⁺Lag-3⁺CD160⁺2B4⁺) population previously defined as exhausted [16] in HIV-positive subjects, compared with HIV-negative subjects, but this trend did not reach statistical significance ([Figure 2](#)).

Figure 1. Individual expression of PD-1, Lag-3, CD160 and 2B4 on memory CD8⁺ T cells. *A*, Representative flow cytometry plots showing expression of PD-1, Lag-3, CD160 and 2B4 on memory CD8⁺ T cells from human immunodeficiency virus (HIV)-negative subjects (HIV-), chronic progressors (CP) and elite controllers (EC). Gates were set based on the naïve population (CD27⁺CD45RO⁻). *B*, The percentage of memory CD8⁺ T cells from HIV-negative, CP and EC individuals expressing each receptor. Error bars represent mean + SEM. ***P* < .01, and ****P* < .001.

Expression of PD-1, Lag-3, CD160, and 2B4 on HIV- and EBV-Specific CD8⁺ T Cells

To further investigate potential T-cell exhaustion within controllers, we measured expression of PD-1, Lag-3, CD160, and 2B4 on HIV-specific CD8⁺ T cells. For comparison, we also measured expression of these inhibitory receptors on EBV-specific CD8⁺ T cells. Virus-specific CD8⁺ T cells were identified by intracellular staining for IFN- γ and TNF after 6 hours of stimulation with an overlapping peptide pool spanning the HIV Gag protein or with an EBV peptide pool containing previously defined CD8⁺

T-cell epitopes derived from BCRF1, BMLF1, BMRF1, BRLF1, BZLF1, gp85, gp110, gp350, and EBNA4 (Supplementary Figure 1C). Figure 3A and 3B shows representative flow cytometry plots of PD-1, Lag-3, CD160, and 2B4 expression on HIV-specific and EBV-specific cells from controllers and progressors and on EBV-specific CD8⁺ T cells from HIV-negative subjects, overlaid onto the corresponding memory populations. Similar to the memory compartment, HIV-specific CD8⁺ T cells from controllers expressed less PD-1 (mean, 50%) and more CD160 (mean, 65%) than progressors (mean, 75% [*P* < .0001] and 20%

Figure 2. PD-1, Lag-3, CD160 and 2B4 co-expression on memory CD8⁺ T cells. Single expression gates were used in a Boolean analysis to get the relative expression of each possible inhibitory receptor expression profile of memory CD8⁺ T cells from human immunodeficiency virus (HIV)-negative (HIV-) (black bars), chronic progressors (dark grey bars) and elite controllers (light grey bars). Bars represent mean of expression. Dots indicate individual subjects. **P* < .05, ***P* < .01, and ****P* < .001.

Figure 3. PD-1, Lag-3, CD160 and 2B4 expression on human immunodeficiency virus (HIV) and Epstein-Barr virus (EBV)-specific CD8⁺ T cells. PBMCs were stimulated for 6 hours with a Gag-PTE peptide pool or an EBV pool containing peptides derived from lytic proteins. Responding CD8⁺ T cells were identified by interferon γ (IFN- γ) and tumor necrosis factor (TNF) production. *A*, Representative flow cytometry plots of PD-1, Lag-3, CD160 and 2B4 expression on HIV-specific (red) and *B*, EBV-specific (blue) CD8⁺ T cells. *C*, Percentage of Gag-specific CD8⁺ T cells expressing individual inhibitory receptors. *D*, Percent of EBV-specific CD8⁺ T cells expressing individual inhibitory receptors. Error bars represent mean + SEM. ** $P < .01$ and *** $P < .001$.

[$P < .0001$], respectively; Figure 3C). Gag-specific CD8⁺ T cells from controllers and progressors had a higher frequency of PD-1 and Lag-3 expression but not CD160 or 2B4 expression, compared with bulk memory cells (Supplementary Figures 3A and 3B). In comparison, EBV-specific CD8⁺ T cells from controllers and progressors had similar levels of PD-1, Lag-3, and 2B4 expression; but CD160⁺ EBV-specific cells were significantly higher in controllers (mean, 60%) compared with progressors (mean, 20%; $P < .001$; Figure 3D). Unlike HIV-specific CD8⁺ T cells, the frequency of PD-1, Lag-3, and CD160 expression on EBV-specific CD8⁺ T cells from controllers and progressors did not differ significantly from that of the corresponding memory populations. However, a higher proportion of EBV-specific CD8⁺ T cells from HIV-negative individuals expressed PD-1 and Lag-3 but a lower proportion expressed CD160, compared with the total memory population (Supplementary Figure 3C).

Gag-Specific CD8⁺ T Cells From Controllers Express Multiple Inhibitory Receptors

We next evaluated coexpression of PD-1, Lag-3, CD160, and 2B4 on HIV-specific and EBV-specific CD8⁺ T cells. HIV-specific CD8⁺ T cells from controllers were mainly distributed among three major populations: PD-1⁺Lag-3⁻CD160⁺2B4⁺, PD-1⁻Lag-3⁻CD160⁺2B4⁺ and PD-1⁺Lag-3⁻CD160⁻2B4⁺ with a small percentage expressing only 2B4 (Figure 4A). In comparison, HIV-specific CD8⁺ T cells from progressors were restricted to 2 major populations: PD-1⁺Lag-3⁻CD160⁺2B4⁺ and, at a higher proportion than controllers, PD-1⁺Lag-3⁻CD160⁻2B4⁺, with a minor proportion expressing 2B4 alone (Figure 4A). Similar patterns were observed for Gag-specific tetramers (Supplementary Figure 4). Consistently, EBV-specific CD8⁺ T cells from controllers, progressors, and HIV-negative subjects expressed the same key combinations of inhibitory receptors seen in HIV-specific

Figure 4. PD-1, CD160 and 2B4 co-expression on human immunodeficiency virus (HIV) and Epstein-Barr virus (EBV)-specific CD8⁺ T cells. Single expression gates on IFN γ ⁺ and/or TNF⁺ cells were used in a Boolean analysis to get the relative expression of each possible inhibitory receptor expression profile of (A) Gag-specific and (B) EBV-specific CD8⁺ T cells from HIV-negative, chronic progressors (CP), and elite controllers (EC). Responding CD8⁺ T cells were then grouped by the number of receptors co-expressed. (C) Percent of Gag-specific CD8⁺ T cells co-expressing inhibitory receptors. (D) Percent of EBV-specific CD8⁺ T cells co-expressing inhibitory receptors. HIV-negative (black bars, triangles), CP (dark grey bars, squares), and EC (light grey bars, circles). Error bars represent mean + SEM. * $P < .05$, ** $P < .01$, and *** $P < .001$.

cells (Figure 4B). Previous work linked the coexpression of ≥ 3 inhibitory receptors on antigen-specific CD8⁺ T cells to exhaustion [16, 17, 24, 25]. Accordingly, we examined the number of receptors coexpressed by HIV-specific and EBV-specific CD8⁺ T cells. Approximately 30% of HIV-specific CD8⁺ T cells from controllers expressed any combination of 3 inhibitory receptors. Surprisingly, a significantly smaller proportion of HIV-specific CD8⁺ T cells from progressors expressed 3 inhibitory receptors (19%; $P < .01$; Figure 4C). Of interest, a considerable percentage

of HIV-specific CD8⁺ T cells from controllers (46%) and progressors (52%) expressed only 2 inhibitory receptors (Figure 4C), indicating that a large portion of the HIV-specific CD8⁺ T-cell response falls within this group. In contrast, EBV-specific CD8⁺ T cells from controllers, progressors, and HIV-negative subjects were equally divided between coexpression of 2 inhibitory receptors and expression of a single inhibitory receptor (Figure 4D). Together, these data suggest that, even though previous work has associated the triple-positive population

Figure 5. Functional profile of human immunodeficiency virus (HIV) and Epstein-Barr virus (EBV)-specific CD8⁺ T cells. Proportion of cytokine and perforin production by CD8⁺ T cells in response to peptide pool stimulation of PBMCs with a Gag-PTE peptide pool or an EBV pool containing peptides derived from lytic proteins. HIV-negative (HIV-, black bars), chronic progressors (CP, dark grey bars) and elite controllers (EC, light grey bars). (A) HIV Gag-specific response. (B) EBV-specific response. Bars represent mean of expression: each dot represents a subject. **P* < .05, ***P* < .01, and ****P* < .001.

PD-1⁺Lag-3⁻CD160⁺2B4⁺ with T-cell exhaustion, the presence of this population alone does not explain differences between controllers and progressors. Instead, the presence or absence of the

PD-1⁻Lag-3⁻CD160⁺2B4⁺ and PD-1⁺Lag-3⁻CD160⁻2B4⁺ populations could contribute to the differences between controllers and progressors.

Figure 6. PD-1, CD160 and 2B4 co-expression on perforin⁺ and perforin⁻ virus-specific CD8⁺ T cells. Responding CD8⁺ T cells identified by interferon γ (IFN-γ) and/or tumor necrosis factor (TNF) production were divided into perforin⁺ and perforin⁻ responses. (A) Relative expression of each possible inhibitory receptor expression profile of perforin⁺ (dark gray) and perforin⁻ (light gray) human immunodeficiency virus (HIV)-specific responses from elite controllers (EC) (top) and chronic progressors (CP) (bottom). (B) Relative expression of each possible inhibitory receptor expression profile of perforin⁺ (dark gray) and perforin⁻ (light gray) EBV-specific responses from HIV negative (top), EC (middle) and CP (bottom). Bars represent mean of expression: each dot represents a subject. ***P* < .01. Abbreviation: EBV, Epstein-Barr virus.

Figure 7. Association between perforin and co-expression of CD160 and 2B4. Correlation between the percent of the (A) human immunodeficiency virus (HIV)-specific and (B) Epstein–Barr virus (EBV)-specific CD8⁺ T cell response that is perforin⁺ and the percent of the response co-expressing inhibitory receptors. HIV-negative (triangles), chronic progressors (CP) (squares), elite controllers (EC) (circles). Spearman *r* and *P* value of correlations are depicted in the upper right corner of each graph. Lines represent linear regression. Abbreviation: NS, not significant.

Coexpression of CD160 and 2B4 Is Associated With Cytolytic Capacity of CD8⁺ T Cells

Highly functional effector HIV-specific CD8⁺ T cells are preferentially maintained in controllers and contribute to the control of HIV replication [4, 5, 11]. Effector responses can be characterized by the presence of the cytolytic molecule perforin, along with IFN- γ and/or TNF, as well as by the lack of IL-2 expression [3]. In addition, PD-1 expression has been associated with decreased cytolytic effector function, whereas CD160 and 2B4 have been shown both to stimulate and inhibit cytolytic activity [16, 31, 32, 38–43]. To examine the relationship between PD-1, CD160, and 2B4 in the context of CD8⁺ T-cell functionality, we first assessed the functional profile of HIV-specific (Figure 5A) and EBV-specific (Figure 5B) CD8⁺ T cells by staining for the cytolytic marker perforin in combination with IFN- γ and TNF. In accordance to previous reports, controllers had a higher proportion of perforin-expressing HIV-specific responses ($P < .01$), compared with progressors, whose responses consisted mainly of IFN- γ expression ($P < .001$). Next, we divided responding cells into 2 subsets: effector responses, characterized by perforin expression (as well as IFN- γ and/or TNF expression), and memory responses, characterized by no perforin expression (as well as IFN- γ and/or TNF expression), and assessed the coexpression of PD-1, CD160, and 2B4 (Figure 6). A significantly high proportion of perforin-expressing HIV-specific and

EBV-specific T cells were grouped within the PD-1–negative subsets. In contrast, a significant portion of the perforin-negative (but IFN- γ and/or TNF expressing) HIV-specific and EBV-specific responses were grouped within the PD-1⁺ subsets (Figure 6A and 6B). We next assessed the relationship between perforin and PD-1, CD160, and 2B4 expression, individually or in combination, by HIV-specific CD8⁺ T cells. We found an inverse correlation between perforin and PD-1 (Supplementary Figure 5A) but not with CD160 or 2B4 (Supplementary Figure 5B and 5C). However, there was a negative correlation between the fraction of perforin-expressing effector responses and the proportion of responding cells coexpressing PD-1⁺CD160⁺2B4⁺ and a positive correlation with coexpression of PD-1⁺CD160⁺2B4⁺ in both HIV-specific and EBV-specific CD8⁺ T cells (Figure 7A and 7B). Together, these data suggest that specific inhibitory receptor coexpression patterns delineate CD8⁺ T cells with different functional capacities and that certain inhibitory receptor combinations may be associated with functions involved in the control of HIV.

DISCUSSION

Elite control of HIV infection is generally associated with an increased presence of polyfunctional and effector-like CD8⁺ T-cell responses [1, 4, 5, 11, 44]. Given the chronic nature of

infection and the continual need for effective immunosurveillance to prevent HIV recrudescence, one could hypothesize that CD8⁺ T cells in controllers display some indications of exhaustion. Conversely, it is possible that CD8⁺ T-cell exhaustion does not manifest in HIV controllers, thus permitting superior longitudinal control of HIV viremia. Here, we began to address these issues by comparing the expression of previously identified exhaustion markers [15, 29, 37, 45, 46] on HIV-specific CD8⁺ T cells from controllers, progressors, and HIV-negative individuals.

Previous work has shown that coexpression of PD-1, CD160, and 2B4 identifies exhausted CD8⁺ T cells [16]. Our results suggest that T-cell exhaustion is not affecting the memory pool as a whole in controllers or progressors. However, controllers had a significantly higher proportion of HIV-specific CD8⁺ T cells expressing 3 inhibitory receptors simultaneously, compared with progressors, with the majority of these expressing the combination PD-1, CD160, and 2B4. If these markers truly define exhaustion, then our data would suggest the presence of a large population of exhausted HIV-specific CD8⁺ T-cells in controllers. Such a population might indicate ongoing low-level viral replication and general immune activation in these subjects.

We have previously shown that perforin expression by HIV-specific CD8⁺ T cells is a correlate of protection against disease progression in controllers [4]. Interestingly, our current data indicate that perforin expression in HIV-specific CD8⁺ T cells is highly associated with the expression of CD160 and 2B4, regardless of disease status. These 2 coreceptors have both inhibitory and stimulatory roles with respect to effector functions in T cells and natural killer cells. [16, 31, 32, 38–43]. CD160 is expressed in 2 isoforms, either anchored by GPI (CD160-GPI) or with a transmembrane domain (CD160-TM). Cross-linking of CD160 and GPI has an activating effect on CD4⁺ and CD8⁺ T-cell function [47]. In contrast, the CD160-TM signaling pathway has not been characterized, and further studies are necessary to elucidate a possible role in T-cell exhaustion. The duality of 2B4 has been associated with expression levels of the adaptor proteins SAP and EAT-2 or recruitment of FynT [41]. Our results imply that CD160 and 2B4 coexpression, rather than signifying exhaustion, may identify a population of activated cytolytic CD8⁺ T cells important in the response against HIV. In this context, CD160 and 2B4 may be serving as activating coreceptors, not as inhibitory receptors. We hypothesize that PD-1 signaling in the exhausted triple-positive population tempers the association between perforin, CD160, and 2B4. Further studies are necessary to elucidate whether a downstream target of PD-1 signaling modulates CD160-TM signal transduction and/or regulates 2B4 via SAP, EAT-2, or FynT.

Differences between HIV controllers and progressors have been studied extensively to identify correlates of protection that can be used to improve current therapies and develop potential vaccines. Efforts to revitalize antigen-specific CD8⁺ T-

cell responses in chronic infections and various cancers by blocking the PD-1 inhibitory pathway are ongoing, and clinical trials involving solid tumors show promising results (AIDS Clinical Trials Group protocol A5326: Anti-PD-L1 Antibody in HIV-1; ClinicalTrials.gov identifier NCT01629758: Safety Study of IL-21/Anti-PD-1 Combination in the Treatment of Solid Tumors [available at: <http://www.clinicaltrials.gov/ct2/show/NCT01629758?term=PD-1&rank=4>]) [48]. The present study shows that not all inhibitory receptors are detrimental and that, in fact, some may play a positive role in the antiviral response. In the context of HIV infection, studies attempting to link inhibitory receptor expression and T-cell exhaustion would benefit from measuring effector functions such as proliferation and IL-2 and perforin production, especially as these functions delineate different but important T-cell populations not always expressed by the same cell. It is important to note in addition that inhibitory receptor expression is not only linked to T-cell exhaustion but also to differentiation/activation status, antigen specificity, and anatomical localization [49, 50]. Our findings therefore have important implications for the development of new therapeutics and caution against the use of inhibitory marker expression alone as an indicator of exhaustion in CD8⁺ T cells.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank Paul A. Goepfert (Department of Microbiology, University of Alabama–Birmingham), for providing us with samples from progressors; Bruce Walker (Ragon Institute of MGH, MIT, and Harvard and Harvard University Center for AIDS Research [CFAR]), for providing us with samples from controllers; and Sarah Ratcliff (Penn CFAR Biostatistics Core).

Financial support. This work was supported by the National Institutes of Health (grant R01 A1076066 to M. R. B. and C. P. and HIV training grant T32 AI 07632 to C. P.) and the Wellcome Trust (to D. A. P.).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Gulzar N, Copeland K. CD8⁺ T-cells: function and response to HIV infection. *Curr HIV Res* **2004**; 2:23–37.
2. El-Far M, Halwani R, Said E, et al. T-cell exhaustion in HIV infection. *Curr HIV/AIDS Rep* **2008**; 5:13–9.
3. Makedonas G, Hutnick N, Haney D, et al. Perforin and IL-2 upregulation define qualitative differences among highly functional virus-specific human CD8 T cells. *PLoS Pathog* **2010**; 6:e1000798.

4. Hersperger AR, Pereyra F, Nason M, et al. Perforin expression directly ex vivo by HIV-specific CD8 T-cells is a correlate of HIV elite control. *PLoS Pathog* **2010**; 6:e1000917.
5. Betts MR, Nason MC, West SM, et al. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* **2006**; 107:4781–9.
6. Migueles SA, Osborne CM, Royce C, et al. Lytic granule loading of CD8+ T cells is required for HIV-infected cell elimination associated with immune control. *Immunity* **2008**; 29:1009–21.
7. Gea-Banacloche JC, Migueles SA, Martino L, et al. Maintenance of large numbers of virus-specific CD8+ T cells in HIV-infected progressors and long-term nonprogressors. *The J Immunol* **2000**; 165: 1082–92.
8. Sáez-Cirión A, Lacabartz C, Lambotte O, et al. 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**; 104:6776–81.
9. Schellens IMM, Borghans JAM, Jansen CA, et al. Abundance of early functional HIV-specific CD8+ T cells does not predict AIDS-free survival time. *PLoS One* **2008**; 3:e2745.
10. Betts MR, Ambrozak DR, Douek DC, et al. Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection. *J Virol* **2001**; 75:11983–91.
11. Almeida JR, Price DA, Papagno L, et al. Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. *J Exp Med* **2007**; 204:2473–85.
12. Schmitz JE, Kuroda MJ, Santra S, et al. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* **1999**; 283:857–60.
13. Jin X, Bauer DE, Tuttleton SE, et al. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* **1999**; 189:991–8.
14. Peretz Y, He Z, Shi Y, et al. CD160 and PD-1 co-expression on HIV-specific CD8 T cells defines a subset with advanced dysfunction. *PLoS Pathog* **2012**; 8:e1002840.
15. Trautmann L, Janbazian L, Chomont N, et al. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med* **2006**; 12:1198–202.
16. Yamamoto T, Price DA, Casazza JP, et al. Surface expression patterns of negative regulatory molecules identify determinants of virus-specific CD8+ T-cell exhaustion in HIV infection. *Blood* **2011**; 117:4805–15.
17. Blackburn SD, Shin H, Haining WN, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* **2008**; 10:29–37.
18. Bucks CM, Norton JA, Boesteanu AC, Mueller YM, Katsikis PD. Chronic antigen stimulation alone is sufficient to drive CD8+ T cell exhaustion. *J Immunol* **2009**; 182:6697–708.
19. Jackson SR, Berrien-Elliott MM, Meyer JM, Wherry EJ, Teague RM. CD8+ T cell exhaustion during persistent viral infection is regulated independently of the virus-specific T cell receptor. *Immunol Invest* **2013**; 42:204–20.
20. Utzschneider DT, Legat A, Fuertes Marraco SA, et al. T cells maintain an exhausted phenotype after antigen withdrawal and population reexpansion. *Nat Immunol* **2013**; 14:603–10.
21. Petrovas C, Price DA, Mattapallil J, et al. SIV-specific CD8+ T cells express high levels of PD1 and cytokines but have impaired proliferative capacity in acute and chronic SIVmac251 infection. *Blood* **2007**; 110:928–36.
22. Velu V, Kannanganat S, Ibegbu C, et al. Elevated expression levels of inhibitory receptor programmed death 1 on simian immunodeficiency virus-specific CD8 T cells during chronic infection but not after vaccination. *J Virol* **2007**; 81:5819–28.
23. Kroy DC, Ciuffreda D, Cooperrider JH, et al. Liver environment and HCV replication affect human T-cell phenotype and expression of inhibitory receptors. *Gastroenterology* **2014**; 146:550–61.
24. Bengsch B, Seigel B, Ruhl M, et al. Coexpression of PD-1, 2B4, CD160 and KLRG1 on exhausted HCV-specific CD8+ T cells is linked to antigen recognition and T cell differentiation. *PLoS Pathog* **2010**; 6: e1000947.
25. Riches JC, Davies JK, McClanahan F, et al. T cells from CLL patients exhibit features of T-cell exhaustion but retain capacity for cytokine production. *Blood* **2013**; 121:1612–21.
26. Fourcade J, Sun Z, Pagliano O, et al. CD8(+) T cells specific for tumor antigens can be rendered dysfunctional by the tumor microenvironment through upregulation of the inhibitory receptors BTLA and PD-1. *Cancer Res* **2012**; 72:887–96.
27. Wherry EJ, Ha S-J, Kaech SM, et al. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* **2007**; 27:670–84.
28. Aldy KN, Horton NC, Mathew PA, Mathew SO. 2B4+ CD8+ T cells play an inhibitory role against constrained HIV epitopes. *Biochem Biophys Res Commun* **2011**; 405:503–7.
29. Day CL, Kaufmann DE, Kiepiela P, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* **2006**; 443:350–4.
30. Petrovas C, Casazza JP, Brenchley JM, et al. PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *J Exp Med* **2006**; 203:2281–92.
31. Rey J, Giustiniani J, Mallet F, et al. The co-expression of 2B4 (CD244) and CD160 delineates a subpopulation of human CD8+ T cells with a potent CD160-mediated cytolytic effector function. *Eur J Immunol* **2006**; 36:2359–66.
32. Viganò S, Banga R, Bellanger F, et al. CD160-Associated CD8 T-Cell Functional Impairment Is Independent of PD-1 Expression. *PLoS Pathog* **2014**; 10:e1004380.
33. Cai G, Anumanthan A, Brown JA, et al. CD160 inhibits activation of human CD4+ T cells through interaction with herpesvirus entry mediator. *Nat Immunol* **2008**; 9:176–85.
34. Chattopadhyay PK, Price DA, Harper TF, et al. Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry. *Nat Med* **2006**; 12:972–7.
35. Price DA, Brenchley JM, Ruff LE, et al. Avidity for antigen shapes clonal dominance in CD8+ T cell populations specific for persistent DNA viruses. *J Exp Med* **2005**; 202:1349–61.
36. Perfetto SP, Ambrozak D, Nguyen R, Chattopadhyay PK, Roederer M. Quality assurance for polychromatic flow cytometry using a suite of calibration beads. *Nat Protoc* **2012**; 7:2067–79.
37. Zhang JY, Zhang Z, Wang X, et al. PD-1 up-regulation is correlated with HIV-specific memory CD8+ T-cell exhaustion in typical progressors but not in long-term nonprogressors. *Blood* **2007**; 109:4671–8.
38. Nikolova MH, Muhtarova MN, Taskov HB, et al. The CD160+ CD8high cytotoxic T cell subset correlates with response to HAART in HIV-1+ patients. *Cell Immunol* **2005**; 237:96–105.
39. Šedý JR, Bjordahl RL, Bekiaris V, et al. CD160 activation by herpesvirus entry mediator augments inflammatory cytokine production and cytolytic function by NK cells. *J Immunol* **2013**; 191:828–36.
40. Nikolova M, Cardine AM, Boumsell L. BY55/CD160 acts as a co-receptor in TCR signal transduction of a human circulating cytotoxic effector T lymphocyte subset lacking CD28 expression. *Int Immunol* **2002**; 14:445–51.
41. Meinke S, Watzl C. NK cell cytotoxicity mediated by 2B4 and NTB-A is dependent on SAP acting downstream of receptor phosphorylation. *Front Immunol* **2013**; 4:3.
42. Schlaphoff V, Lunemann S, Suneetha PV, et al. Dual function of the NK cell receptor 2B4 (CD244) in the regulation of HCV-specific CD8+ T cells. *PLoS Pathog* **2011**; 7:e1002045.
43. Sinha SK, Gao N, Guo Y, Yuan D. Mechanism of induction of NK activation by 2B4 (CD244) via its cognate ligand. *J Immunol* **2010**; 185:5205–10.
44. Bailey JR, Brennan TP, O'Connell KA, Siliciano RF, Blankson JN. Evidence of CD8+ T-cell-mediated selective pressure on human

- immunodeficiency virus type 1 nef in HLA-B*57+ elite suppressors. *J Virol* **2008**; 83:88–97.
45. Barber DL, Wherry EJ, Masopust D, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* **2005**; 439:682–7.
 46. Doering TA, Crawford A, Angelosanto JM, et al. Network analysis reveals centrally connected genes and pathways involved in CD8+ T cell exhaustion versus memory. *Immunity* **2012**; 37:1130–44.
 47. El-Far M, Pellerin C, Pilote L, et al. CD160 isoforms and regulation of CD4 and CD8 T-cell responses. *J Transl Med* **2014**; 12:217.
 48. Harvey RD. Immunologic and clinical effects of targeting PD-1 in lung cancer. *Clin Pharmacol Ther* **2014**; 96:214–23.
 49. Baitsch L, Legat A, Barba L, et al. Extended co-expression of inhibitory receptors by human CD8 T-Cells depending on differentiation, antigen-specificity and anatomical localization. *PLoS One* **2012**; 7: e30852.
 50. Legat A, Speiser DE, Pircher H, Zehn D, Furtak SA. Inhibitory receptor expression depends more dominantly on differentiation and activation than ‘exhaustion’ of human CD8 T cells. *Front Immunol* **2013**; 4:455.