

HIV-specific CD8 T cells from Elite Controllers Rapidly Upregulate Perforin

Hersperger AR^{1*}, Pereyra F², Sheth P⁴, Shin LYY⁴, Kaul R⁴, Walker BD^{2,3}, and Betts MR¹

¹ Department of Microbiology, University of Pennsylvania, Philadelphia, PA ² Partners AIDS Research Center, Massachusetts General Hospital, Harvard Medical School, Boston, MA ³ Howard Hughes Medical Institute, Chevy Chase, MD ⁴ Department of Medicine, University of Toronto, Canada

* Contact information: 3610 Hamilton Walk, 518 Johnson Pavilion, Philadelphia, PA 19104; adamrh@mail.med.upenn.edu; 215-746-6526

ABSTRACT

Background: Evidence suggests that CD8 T cells are important to the control of HIV replication, but the mechanism by which this occurs is unclear. In fact, few differences in CD8 T cell cytotoxic function have been observed to date in HIV-infected subjects that control viral replication compared to individuals that do not.

Methods: We have recently discovered the novel ability of human CD8 T cells to rapidly upregulate perforin following antigen-specific stimulation. Using polychromatic flow cytometry, we measured perforin expression, cytokine production, and degranulation following stimulation of CD8 T cells from several HIV-infected groups: elite controllers, viremic controllers, chronically-infected progressors, and Sooty Mangabey-like long-term nonprogressors (LTNP).

Results: We observe that on average 40% of the total CD8 T cell response in elite controllers is perforin-positive following HIV-specific stimulation versus about 20% in the other cohorts. However, there is no significant difference in the proportion of the response that produces IFN- γ between groups. Elite controllers also had a significantly larger proportion of responding CD8 T cells that both degranulated and appeared perforin-positive, which suggests the presence of newly upregulated perforin. Additionally, the cells that upregulate perforin are mainly of an effector/effector memory phenotype.

Conclusions: The rapid perforin upregulation displayed by CD8 T cells in elite controllers may contribute to the superior control of HIV replication in these subjects.

BACKGROUND

- CD8 T cells are capable of eliminating intracellular pathogens, such as human immunodeficiency virus (HIV) or human cytomegalovirus (HCMV), by secreting cytolytic effector proteins into the immunological synapse formed with a target cell. The predominant killing pathway makes use of pre-formed granules containing lytic proteins - mainly perforin and granzymes - that are secreted by exocytosis following target recognition.
- Granule-mediated killing occurs shortly after target cell recognition, but the reconstitution or upregulation of intracellular perforin after activation has historically been detected only after cellular proliferation (1,2).
- In the past, human perforin has been detected by means of an antibody (clone δ G9) that was made by using purified cytotoxic granules from CD8 T cells as an immunogen in mice.
- By virtue of a new antibody (clone B-D48) that was raised against recombinant perforin, its *de novo* synthesis can, in fact, be detected by flow cytometry after a 6 hour intracellular cytokine-staining (ICS) assay (Figure 1).

FIGURE 1

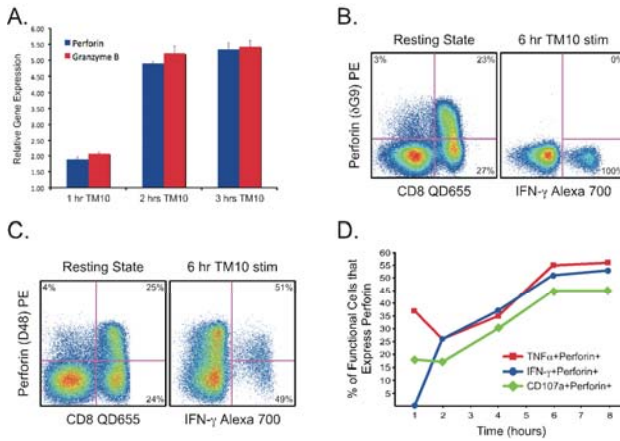


Figure 1: New perforin production is detectable after antigen-specific activation. (A) PBMC from donor ND172 were stimulated with TM10 peptide derived from HCMV for 1, 2 or 3 hours. For each time point, mRNA abundance was normalized to GAPDH and expressed as relative fold change from the no peptide control. (B,C) Left: Resting PBMC from ND172 were stained with anti-perforin antibodies. Events shown are gated upon CD3+ cells. Percentages represent the frequency of total CD3+ cells. Right: PBMC from ND172 were stimulated with peptide TM10 for 6 hours. Events shown are gated upon CD3+CD8+ cells. Percentages represent the proportion of IFN- γ + cells that stain either positive or negative for perforin. (D) PBMC from ND172 were stimulated with peptide TM10 in the presence of BFA and monensin for various periods of time and stained for perforin (D48 clone) in conjunction with TNF α (red; squares), IFN- γ (blue; circles), and CD107a (green; diamonds). The proportions of TNF α +, IFN- γ +, or CD107a+ cells (CD3+CD8+) that stained positive for perforin are plotted.

RESULTS

- We next wanted to extend the analysis of perforin upregulation to HIV-specific CD8 T cell responses. We hypothesized that elite controllers would display a greater ability to express perforin following stimulation because perforin is a critical molecule in CD8 T cell-mediated cytotoxicity and these subjects do control HIV replication to undetectable levels.
- Our cohort consists of the following groups of treatment naïve, HIV-infected patients:
 - 1) **Elite controllers:** n=33; viral load (VL) < 50 copies/mL; average duration of infection > 10 yrs
 - 2) **Viremic controllers:** n=26; VL between 50 and 2,500 copies/mL; average duration of infection > 10 yrs
 - 3) **Progressors:** n=11; VL between 50 and 10⁵ copies/mL; CD4 T cell counts > 500 cells/mm³; infected < 10 yrs
 - 4) **Sooty Mangabey-like LTNP:** n=4; VL > 10⁴ copies/mL; CD4 T cell counts > 500 cells/mm³; infected > 10 yrs
- We conducted standard 6 hour ICS assays on PBMCs from each subject using 5 pools of overlapping HIV peptides from Gag, Pol, Env, Nef, and the accessory proteins (TRVVV).
- Using polychromatic flow cytometry, we could simultaneously measure phenotype (CD3, CD4, CD8, CD27, CD45RO, CD57) and function (CD107a, IL-2, IFN- γ , TNF α , MIP1 α , and perforin).
- A number of HIV-specific responses from elite controllers expressed perforin in cytokine producing cells that were mainly of a terminally differentiated effector/effector memory phenotype (Figure 2).

FIGURE 2

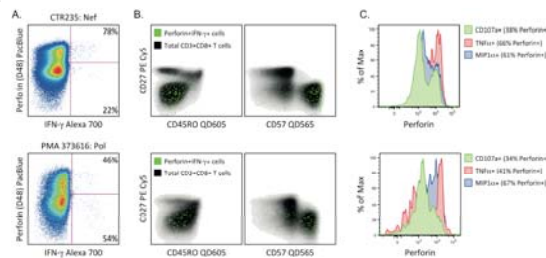


Figure 2: HIV-specific CD8 T cells from elite controllers upregulate perforin upon stimulation. (A) PBMC from elite controller CTR235 and PMA 373616 were stimulated with the indicated peptide pools. Events shown are gated upon CD3+CD8+ cells. Percentages represent the proportion of IFN- γ + cells that stain either positive or negative for perforin. (B) The perforin+IFN- γ + cells from (A) were superimposed onto plots of the memory phenotype of total CD3+CD8+ T cells (shown as black density plots) from each subject. (C) The amount of perforin positivity is shown for other functional cells in histogram form.

- Since we examined 6 functions in our assay, there exist 64 combinations of these functional outputs. This arises because each function can be either positive or negative during our analysis: 2⁶ = 64. The end result is a functional profile of an entire antigen-specific CD8 T cell response.
- Figure 3 depicts the average functional profile for each group of HIV-infected subjects to Nef stimulation. The black arcs around each pie depict the proportion of each slice that is perforin-positive. It is clear that perforin comprises a much larger fraction of the average response in the elite controllers versus the other groups. This finding also holds true for the other HIV antigens except with Env-specific responses.

FIGURE 3

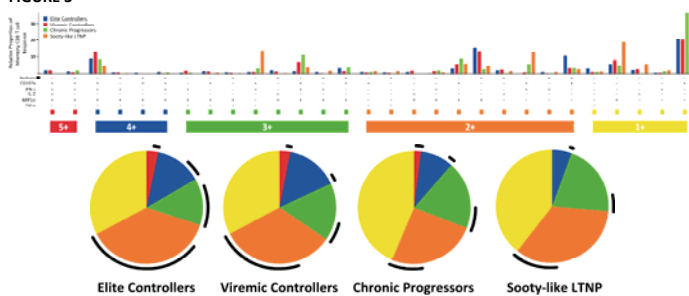


Figure 3: A significant proportion of the Nef-specific CD8 T cell response in elite controllers is perforin-positive. The average functional profile of CD8 T cells that respond to Nef stimulation across the groups of HIV-infected subjects is depicted. The entire response, based upon perforin, CD107a, IFN- γ , IL-2, MIP1 α , and TNF α , was broken down into the relative contribution of each functional combination. Combinations that did not contribute to the functional profile are not shown. Responses are grouped according to the amount of positivity and matched to the colors in the pie graph. The relative frequency of perforin positivity within each functional group is depicted as black arcs around the pie graph.

- Recent findings suggest that Gag-specific CD8 T cell responses are crucial to a favorable disease course in HIV-infected patients (3). As shown in Figure 4A, a significantly higher proportion of the entire Gag-specific response in elite controllers is perforin-positive in our study. However, this finding is not true for all the measured functional parameters as the percentage of the Gag-specific cytokine response that is IFN- γ + is not significantly different across the groups (Figure 4B).

FIGURE 4

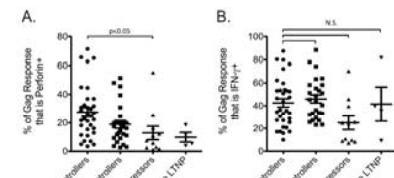
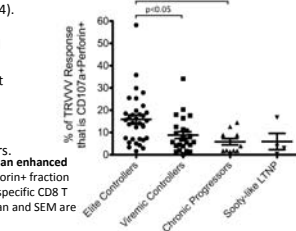


Figure 4: A significant proportion of the Gag-specific CD8 T cell response in elite controllers is perforin-positive. Within each group, the perforin+ (A) or IFN- γ + (B) fraction of each Gag-specific CD8 T cell response is plotted. Each symbol represents a single subject. The mean and standard error of the mean (SEM) are shown superimposed on the data points. For each data set, a one-way ANOVA test was performed not assuming a Gaussian distribution. As a post-test, a Dunns multiple comparisons test was carried out to compare each subject group to every other group. Statistical significance was not achieved for the Sooty-like LTNP group due to low numbers of these rare subjects.

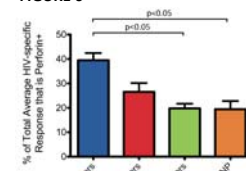
- A very useful flow cytometric assay measures the presence of CD107a (LAMP-1) on the surface of activated CD8 T cells (4). A CD107a+ cell has degranulated and presumably lost all (or nearly all) of its stored perforin. If a cell is both CD107a+ and perforin+, it suggests true upregulation of new perforin.
- We calculated the percentage of each cytokine response that is

FIGURE 5



- at a minimum positive for both CD107a and perforin. As depicted in Figure 5, a significantly higher fraction of HIV-specific responses upregulate new perforin in elite controllers.
- Figure 5: TRVVV-specific CD8 T cells from elite controllers demonstrate an enhanced ability to upregulate new perforin. Within each group, the CD107a+perforin+ fraction (plus any combination of IL-2, IFN- γ , TNF α , or MIP1 α) of each TRVVV-specific CD8 T cell response is plotted. Each symbol represents a single subject. The mean and SEM are shown. Statistical analyses were performed as described for Figure 4.

FIGURE 6



- As demonstrated in Figure 6, elite controllers display a higher proportion of perforin positivity on average across all HIV antigen-specific responses.
- Viremic controllers, chronically-infected progressors, and Sooty Mangabey-like LTNP do not significantly differ from each other in the

proportion of their HIV-specific responses that are perforin-positive. **Figure 6:** A significant fraction of the total average HIV-specific CD8 T cell response in elite controllers is perforin-positive. The average proportion of perforin positivity in each response was found across all HIV antigens in each cohort. The mean and SEM are shown. Statistical analyses were performed as described for Figure 4.

SUMMARY & CONCLUSIONS

- CD8 T cells in patients who are able to control HIV viral replication to below the limit of detection demonstrate a superior ability to rapidly upregulate new perforin, an important molecule in anti-viral defense.
- On average, these elite controllers exhibit a higher proportion of perforin as part of their entire HIV-specific CD8 T cell response versus subjects who have detectable viral load.
- It is possible that rapid perforin upregulation provides CD8 T cells in elite controllers an enhanced capacity to kill HIV-infected targets - especially multiple targets in a short period of time.
- These findings may be useful in the HIV vaccine field as a marker for an effective anti-viral CD8 T cell response.

REFERENCES

- Meng Y, Harlin H, O'Keefe JP, Gajewski TF. Induction of cytotoxic granules in human memory CD8+ T cell subsets requires cell cycle progression. *J Immunol* 2006;177(3):1981-7.
- Migueles SA, Laborico AC, Shupert WL, Sabbaghian MS, Rabin R, Hallahan CW, Van Baarle D, Kostense S, Miedema F, McLaughlin M and others. HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol* 2002;3(11):1061-8.
- Kiepiela P, Ngumbela K, Thobakgale C, Ramuthu D, Honeyborne I, Moodley E, Reddy S, de Pierres C, Mncube Z, Mkhwanazi N and others. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nat Med* 2007;13(1):46-53.
- Betts MR, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M, Koup RA. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J Immunol Methods* 2003;281(1-2):65-78.