

Differential Impact of Magnitude, Polyfunctional Capacity, and Specificity of HIV-Specific CD8⁺ T Cell Responses on HIV Set Point

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Defining the characteristics of HIV-specific CD8⁺ T cell responses that lead to viral control is crucial for vaccine development. We evaluated the differential impact of magnitude, polyfunctional capacity, and specificity of the CD8⁺ response at approximately 6 months postinfection on the viral set point at 12 months in a cohort of HIV-infected individuals. High frequencies of Gag and Nef responses endowed with four functions were the best predictors of a low viral set point.

Understanding the attributes of an immune response that results in control of HIV replication is critical for designing an HIV vaccine capable of eliciting such a response (1). A robust predictor of viral control would accelerate vaccine development significantly, since we currently rely on infection outcomes in lengthy, large clinical trials. Simple measures such as the magnitude of CD8⁺ gamma interferon (IFN- γ) T cell responses to HIV failed to associate with control of viral replication (2, 3). However, several quantitative or qualitative characteristics of HIV-specific CD8⁺ T cells have been correlated with HIV control, including their capacity to (i) develop breadth with respect to Gag responses (4, 5); (ii) target conserved regions of the HIV proteome (6); (iii) secrete multiple cytokines simultaneously (polyfunctionality) (7); (iv) maintain enhanced proliferative or killing ability (8–10); (v) recognize peptide with high avidity (11, 12); and (vi) exhibit particular memory phenotypes (13, 14). The majority of these correlates have been identified in HIV long-term nonprogressors (LTNPs) or in large cross-sectional studies of chronic HIV infection. We examined the characteristics of HIV-specific CD8⁺ cells in a prospective cohort of individuals during early HIV infection with viral loads at various set points.

The purpose of this study was to identify the attributes of early CD8⁺ responses that predicted a reduced viral set point (VSP), a well-established indicator of the rate of HIV disease progression (15). In order to dissect the relative contribution to viral control of the quantity as opposed to the quality of the CD8⁺ T cell response, we analyzed the influence of the magnitude, specificity, and polyfunctional profile of HIV-specific cells on HIV load at 12 months postinfection as an estimate of the VSP. T cell responses were measured at approximately 6 months postinfection in a cohort of individuals infected with HIV-1 clade C (CAPRISA 002 acute infection cohort; see Table S1 in the supplemental material for clinical characteristics) (16). Peripheral blood mononuclear cells (PBMC), collected at a median of 30 weeks postinfection (range, 18 to 42 weeks), were stimulated for 6 h in the presence of Gag ($n = 21$ individuals), Nef ($n = 15$), or Env ($n = 15$) peptide pools spanning HIV-1 clade C. Using polychromatic flow cytometry, HIV-specific CD8⁺ T cell responses were assessed by measuring five distinct functions, namely, those corresponding to tumor necrosis factor alpha (TNF- α), IFN- γ , interleukin-2 (IL-2), MIP-1 β ,

and CD107a (Fig. 1A). We first compared the VSPs between individuals with and without a detectable CD8⁺ response at 6 months for each of the peptide pools tested. We found no significant differences in the VSP between responders and nonresponders for any of the peptide pools (Fig. 1B). When we investigated the overall relationship between the combined magnitudes of Gag-, Nef-, and Env-specific CD8⁺ T cell responses with the VSP, no association was found ($P = 0.09$, $r = -0.45$; data not shown). However, when HIV responses were analyzed based on their individual protein specificities, the frequencies of Gag- and Nef-specific CD8⁺ T cell responses inversely correlated with VSP ($P = 0.0005$ [$r = -0.7$] and $P = 0.006$ [$r = -0.69$], respectively; Fig. 1C). The combined magnitudes of Gag- and Nef-specific CD8⁺ T cell values also correlated inversely with VSP ($P = 0.011$, $r = -0.63$; data not shown). No such association was observed with the frequencies of Env-specific responses. Of note, there was no association between the concomitant HIV load and the frequency of HIV-specific responses to any of the peptide pools tested (data not shown). Collectively, these data indicate that the effect of the magnitude of HIV-specific CD8⁺ T cell responses (as measured by five functions) on viral replication control is dependent on the specificity of the targeted HIV protein, where high frequencies of Gag and Nef responses were associated with a lower VSP whereas Env responses had no impact.

To evaluate the relationship between the functional quality of HIV-specific CD8⁺ T cells and the VSP, we next assessed the polyfunctional quality of Gag-specific T cell responses at 6 months postinfection, by grouping responding individuals with different VSPs: VSP < 10,000 HIV RNA copies/ml (median, 1,060 HIV

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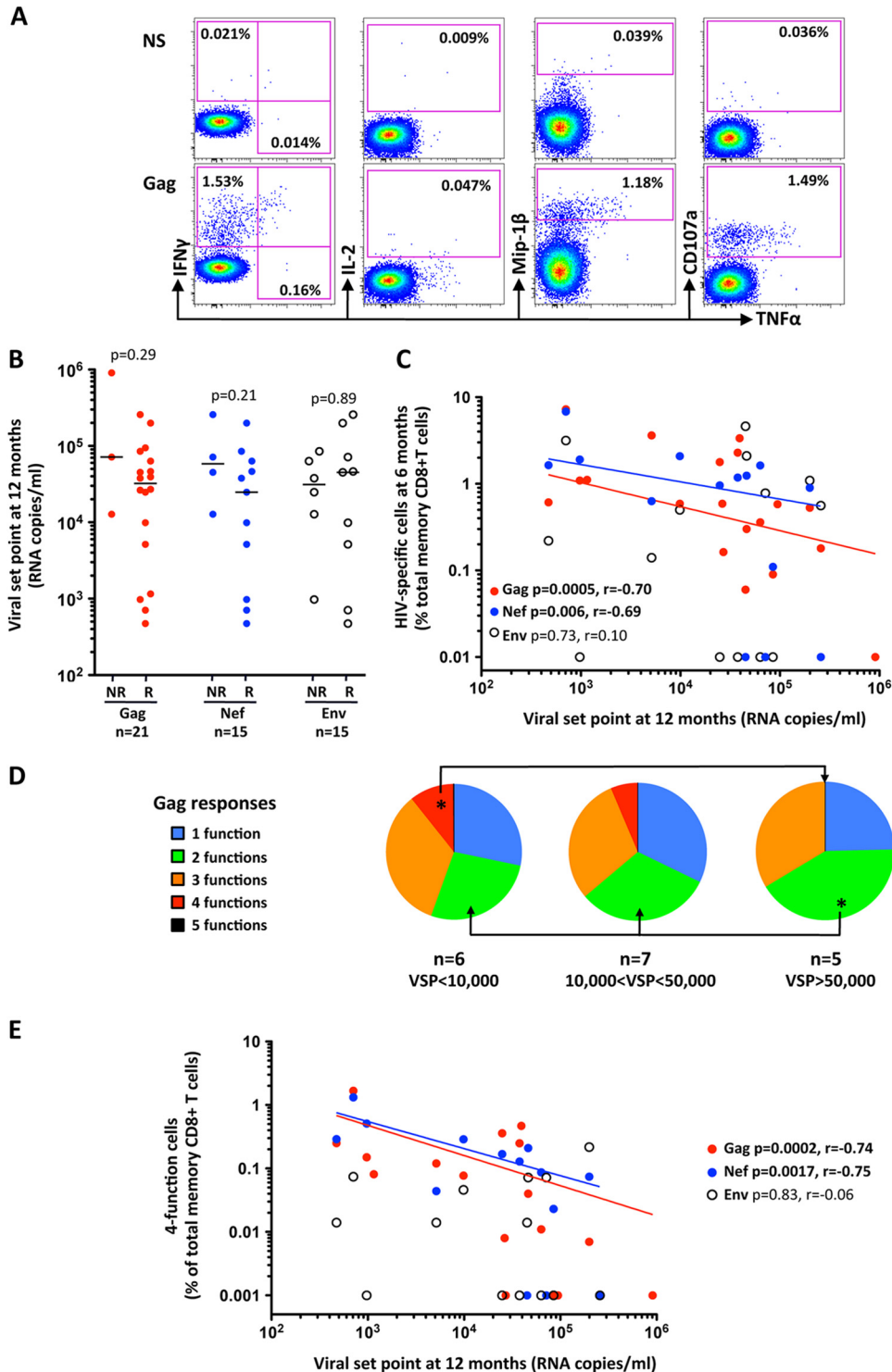


FIG 1 Frequencies and polyfunctional capacities of HIV-specific CD8⁺ T cells in response to Gag, Nef, and Env peptide pools at 6 months postinfection. (A) Representative flow cytometry plots of a Gag-specific CD8⁺ T cell response as measured by the production of TNF- α , IFN- γ , IL-2, and MIP-1 β and expression of CD107a. The top panel corresponds to unstimulated PBMC (NS) and the bottom panel to cells stimulated with a Gag peptide pool. Numbers represent the frequencies of cytokine-producing cells expressed as percentages of the total memory CD8⁺ T cell population. Total memory CD8⁺ T cells were gated based on the exclusion of naive CD8⁺ T cells (CD45RO⁻ CD27⁺). (B) Comparison between responders (R) and nonresponders (NR) of HIV set points (VSP) at 12 months for each of the tested peptide pools (Gag responses are depicted with red circles [$n = 21$], Nef responses are depicted with blue circles [$n = 15$], and Env responses are displayed with open circles [$n = 15$]). A positive cytokine response was defined as being at least twice the background (no antigen, only costimulatory antibodies) and $>0.05\%$ after background subtraction. Medians are shown as horizontal bars. The viral set point was calculated as the geometric mean of viral load measurements at three time points at week 52 ± 6 weeks postinfection (46 to 58 weeks) in order to minimize possible spurious viral load measurements. (C) Association of the magnitude of HIV-specific responses producing any of the five functions measured (i.e., TNF- α , IFN- γ , IL-2, CD107a, and

RNA copies/ml; $n = 6$), $10,000 < \text{VSP} < 50,000$ HIV RNA copies/ml (median, 37,690 HIV RNA copies/ml; $n = 7$), and $\text{VSP} > 50,000$ HIV RNA copies/ml (median, 94,940 HIV RNA copies/ml; $n = 5$). The proportion of Gag-specific CD8⁺ T cells endowed with 4 functions (TNF- α positive [TNF- α ⁺] IFN- γ ⁺ CD107a⁺ MIP-1 β ⁺) was significantly higher in responders with the lowest VSP than in individuals with the highest VSP (median, 10.3% versus 0.2%; $P = 0.008$) (Fig. 1D). The proportion of Gag-specific CD8⁺ T cells endowed with 2 functions (IFN- γ ⁺ CD107a⁺, CD107a⁺ MIP-1 β ⁺, or IFN- γ ⁺ MIP-1 β ⁺), in turn, was significantly higher in individuals with the highest VSP than in those with $\text{VSP} < 10,000$ and $10,000 < \text{VSP} < 50,000$ HIV RNA copies/ml. We performed the same analysis for Nef and found a greater proportion of 4-function cells in the group with the lowest VSP (although the proportion was not significantly different from that determined for the group with the highest VSP) and a trend toward a higher proportion of Nef-specific CD8⁺ T cells endowed with 2 functions in individuals with the highest VSP ($P = 0.07$; data not shown). The smaller number of responders for Nef ($n = 11$) may explain why these values do not reach statistical significance.

In addition to assessing the proportions of Gag- and Nef-specific cells with 4 functions (as a proportion of the total Gag or Nef response), we measured the magnitude of the 4-function population (as a frequency of total memory CD8⁺ response). We found a significant association between the frequency of 4-function CD8⁺ T cells and VSP in response to Gag and Nef ($P = 0.0002$ [$r = -0.74$] and $P = 0.0017$ [$r = -0.75$], respectively; Fig. 1E). As with the total-magnitude results, no such association was observed with Env-specific 4-function responses. In addition, there was no association between the presence of protective HLA alleles (see Table S1 in the supplemental material) and the magnitude of response, proportion, or frequency of 4-function cells (data not shown). Thus, these data extend our current understanding of the importance of maintaining highly polyfunctional CD8⁺ T cells for viral control (7, 11) and highlight the fact that this association may depend on the targeting of specific HIV proteins.

In order to define the specificity and sensitivity of Gag- and Nef-specific CD8⁺ T cell response measurements at 6 months postinfection for 12-month VSP prediction, we calculated receiver operating characteristics (ROC) curves comparing the frequency of total Gag- or Nef-specific CD8⁺ T cell responses, the proportion of 4-function Gag- or Nef-specific responses, and the frequency of 4-function Gag- or Nef-specific responses. All three measurements were associated with significant performance characteristics for the prediction of VSP for Gag (Fig. 2), while this was true for the frequency of total and 4-function cells for Nef (Fig. 2A to C). However, the frequency of 4-function Gag- and Nef-specific CD8⁺ T cells best predicted VSP (AUC of 0.89 [$P = 0.0065$] and 95% confidence interval [CI] of 0.74 to 1.04 for Gag and AUC of 0.9 [$P = 0.014$] and 95% CI of 0.7 to 1.09 for Nef; Fig. 2C). The

threshold values of the frequencies of total and 4-function Gag-specific CD8⁺ T cells, identified to maximize discrimination between individuals with VSPs lower or higher than 10,000 HIV RNA copies/ml, were 0.7% and 0.08%, respectively (Fig. 2F). For Nef, these values were 1.2% and 0.15%, respectively (data not shown).

In conclusion, both the frequency and the polyfunctional capacity of Gag- and Nef-specific CD8⁺ T cell responses during early HIV infection were associated with a lower VSP, and we identified the frequency of a specific subset of Gag- and Nef-specific CD8⁺ T cells, namely, cells simultaneously expressing TNF- α , IFN- γ , CD107a, and MIP-1 β , as the best predictor. Although many early studies did not find an association between the magnitude of HIV-specific responses and viral load (2, 3), their frequencies were assessed using the IFN- γ enzyme-linked immunosorbent spot (ELISpot) assay. However, five functions were measured in this study, enabling a more accurate quantification of the overall magnitude of HIV-specific responses. In fact, measuring IFN- γ only would have missed 60% of the responses to HIV (data not shown), with MIP-1 β being the most abundant cytokine produced, in agreement with other studies (7). Our findings accord with recent data (17) showing that viral control of HIV-2 was significantly associated with a high-magnitude, polyfunctional Gag-specific CD8⁺ T cell response. This further confirms the prevailing notion that immune responses to Gag possess enhanced antiviral potency compared to other specificities, likely as a result of its conservation and the higher rate of viral escape that exacts a fitness cost for HIV (18–20). More intriguing is the association we found with Nef-specific responses and lower VSP. Recently, Nef responses have been implicated in mediating control of simian immunodeficiency virus (SIV) in macaques (21, 22) and HIV in viral controllers (23, 24). In addition, there are convincing data that HLA-selected polymorphisms in Nef revert to the wild type at a high rate upon transmission to non-HLA-matched hosts, strongly suggesting that these are escape mutations resulting from immune selective pressure that result in a fitness cost for HIV (25). Thus, our finding is consistent with mounting evidence of a role for CD8⁺ T cell responses to Nef in viral control, which has implications for T cell vaccine design.

This study had several limitations. We were limited by sample availability to measuring CD8⁺ responses using peptide pools rather than individual peptides. We thus cannot differentiate between epitope responses known to result in a fitness cost to HIV and conserved epitopes within the protein-specific pools. In addition, we had insufficient sample to determine polymerase (Pol) responses and their relationship with the viral set point. Given the observation that Pol responses can be frequent and dominant and can target highly conserved epitopes (26–28), this warrants further investigation. Furthermore, while we can offer predictive values for frequencies of highly functional Gag and Nef-specific CD8⁺ T cells for a lower viral set point, we cannot establish cau-

MIP-1 β) at 6 months postinfection with VSP at 12 months. One individual who was a nonresponder for all three proteins was excluded from the analysis. (D) Comparison of the polyfunctional profiles of Gag-specific responses measured at 6 months postinfection in the 18 responding individuals with $\text{VSP} < 10,000$ RNA copies/ml ($n = 6$), $10,000 < \text{VSP} < 50,000$ RNA copies/ml ($n = 7$), and $\text{VSP} > 50,000$ RNA copies/ml ($n = 5$). The slices of the pies correspond to the proportions of Gag-specific CD8⁺ T cells (as a frequency of the total Gag response) expressing 1, 2, 3, 4, or 5 functions, calculated using Boolean gating. Statistical comparisons were determined using a nonparametric one-way analysis of variance (ANOVA; Kruskal-Wallis test). *, $P < 0.05$. (E) Association between the magnitude of HIV-specific CD8⁺ T cells endowed with four functions (as a frequency of total memory CD8⁺ T cells) at 6 months postinfection and VSP at 12 months. The individual who was a nonresponder for all three proteins was excluded from this analysis. Statistical comparisons were determined using nonparametric Mann-Whitney U-tests and two-tailed nonparametric Spearman rank correlations for statistical associations.

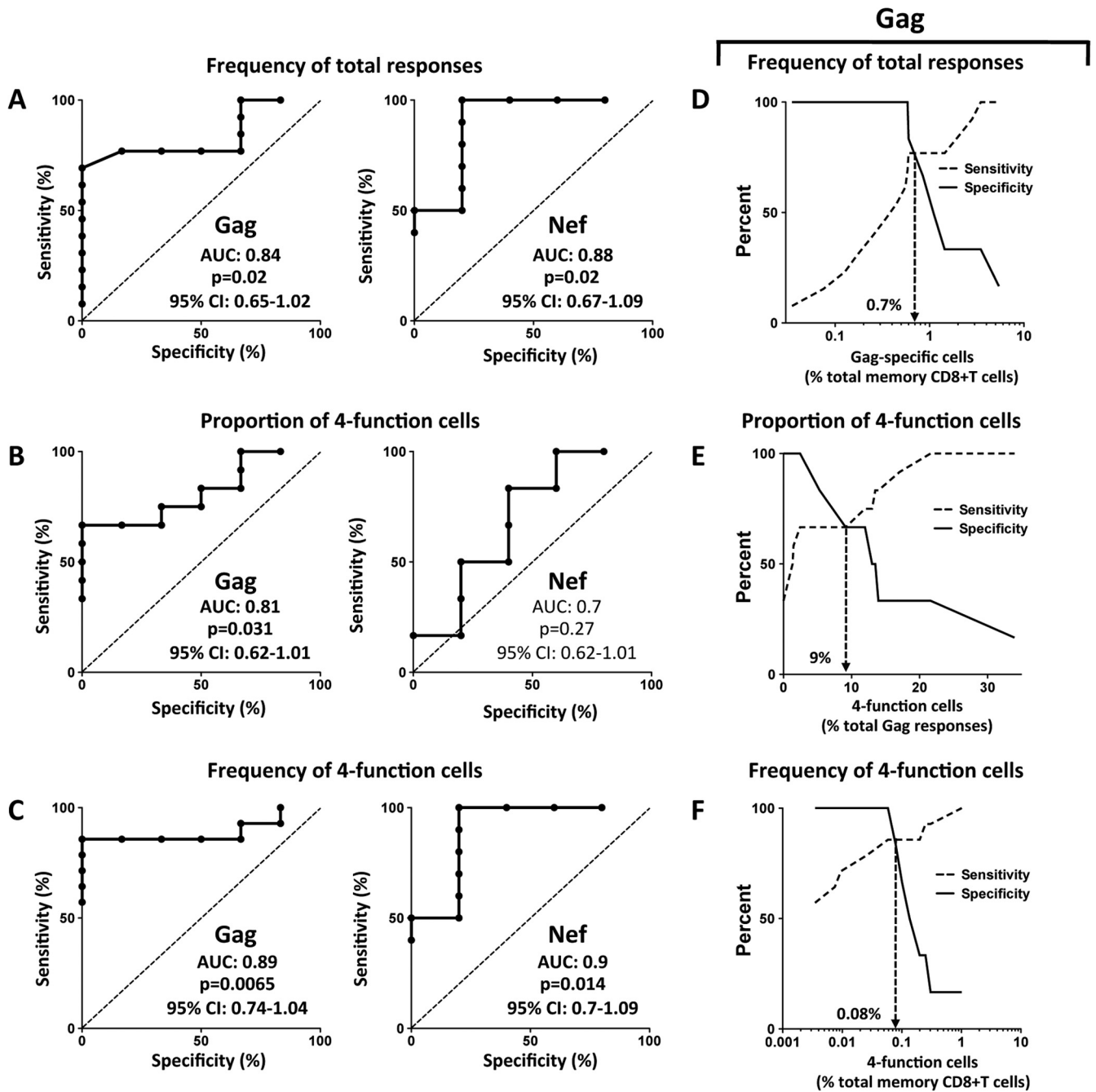


FIG 2 Sensitivity and specificity of Gag- and Nef-specific CD8⁺ T cell measurements at 6 months postinfection for the prediction of a VSP of <10,000 HIV RNA copies/ml at 12 months. Receiver operator characteristics (ROC) curves for the total magnitude of the Gag- and Nef-specific CD8⁺ T cell response (as a frequency of total memory CD8⁺ T cells) (A), the proportion of 4-function cells specific for Gag and Nef (as a frequency of the total HIV responses) (B), and the magnitude of 4-function Gag- and Nef-specific CD8⁺ T cells (as a frequency of total memory CD8⁺ T cells) (C) with VSP at 12 months are shown. The area-under-the-curve (AUC) values, P values, and confidence intervals (CI) are shown. The dotted line depicts an AUC of 0.5, representing a random test. (D, E, and F) Specificity/sensitivity crossover plots showing the optimal threshold (vertical arrow) at which each measurement in response to Gag predicts a VSP of <10,000 HIV RNA copies/ml, minimizing discrepant results. This value is the threshold that limits the number of false positives (sensitivity) or false negatives (specificity). For example, in panel F, a frequency of 4-function Gag-specific CD8⁺ T cells of >0.08% cells at 6 months postinfection predicts a VSP of <10,000 copies/ml with maximum specificity and sensitivity.

salinity. Lastly, all participants in our cohort were African females infected with HIV-1 clade C; testing the generalizability of our findings in additional populations is warranted.

Thus, while the total CD8⁺ T cell response to all HIV antigens

does not correlate with VSP, a particular component of this response does correlate. It is notable that measuring other characteristics of responding cells may further delineate those with the best functional control, such as perforin expression, recently high-

lighted as a measure of elite control of HIV (29, 30). In line with this, Yang and colleagues demonstrated that the ability of CD8⁺ T cells to mediate inhibition of HIV *in vitro* associated inversely with viral set point in a cohort of viremic individuals (31).

In summary, by combining measures of magnitude, specificity, and function of the HIV-specific CD8⁺ T cell response, we identified a subset of highly polyfunctional cells directed at Gag and Nef which, when occurring at a high frequency, were associated with a lower HIV set point. This finding not only substantiates mechanistic hypotheses of immunological control (32) but also highlights the importance of eliciting such cells by vaccination to enable the best outcome following breakthrough infection.

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