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Relative Dominance of Gag p24-Specific Cytotoxic T Lymphocytes Is Associated with Human Immunodeficiency Virus Control

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Conflicting data on the role of total virus- and protein-specific cytotoxic-T-lymphocyte (CTL) responses in the control of human immunodeficiency virus (HIV) disease progression exist. We present data generated from a Peruvian cohort of untreated, clade B-infected subjects, demonstrating that the proportion of Gag-specific, and in particular p24-reactive, CTL responses among the total virus-specific CTL activity is associated with individuals' CD4 counts and viral loads. Analyses in a second cohort in the United States confirm these findings and point towards a dominant role of Gag-specific immunity in effective control of HIV infection, providing important guidance for HIV vaccine development.

True immune correlates of controlled human immunodeficiency virus (HIV) infection remain elusive, despite a wealth of studies and data supportive of one aspect or another of virus-specific immune responses. In particular, the cellular arm of the host immune system has been associated with relative control of viral infection, most impressively shown in studies of cytotoxic-T-lymphocyte (CTL) depletion and CD8 T-cell immune escape and by the link between specific HLA class I alleles and favorable HIV disease outcome (reviewed in references 4 and 21). While such studies support the important role of specific CTL epitopes in controlling HIV infection, they provide little guidance for selecting immunogens for vaccine design in the setting of diverse HLA alleles and extensive sequence diversity.

A number of studies, performed with adults and children of diverse ethnicities, in the past have suggested that Gag-specific, T-cell-mediated immunity could be especially important in viral control (6, 7, 18, 20, 22, 23). Possible explanations put forward to explain the importance of Gag include viral gene expression profiles, functional constraints preventing rapid immune escape, density of conserved CTL epitopes within this protein, and other factors that would render the Gag-specific immunity more effective than CTL targeting other HIV proteins (4). However, there are also a number of reports, some based on large cohorts of several hundred individuals, that did not confirm these findings, questioning the superior role of Gag-specific CTL immunity (1, 3, 9). Importantly, in these studies, viral loads and/or CD4 T-cell counts were generally compared to total virus- or total Gag-specific responses, with only one study comparing relative magnitudes of protein-specific pool responses to viral load (17). Here, we have addressed the question of potentially beneficial Gag-specific CTL responses in an alternative way, namely by not only assessing the total breadth and magnitude of immune responses directed against individual overlapping peptides spanning all viral proteins but also by determining the relative contribution of Gag-specific responses to the total virus-specific CTL activity.

To assess total HIV- and individual protein-specific immune responses, 45 HIV clade B-infected individuals were enrolled in Lima, Peru, after signing informed consent and tested by ex vivo gamma interferon (IFN-γ) ELISpot assays using a peptide set spanning the entire expressed HIV viral genome. The peptide set used was based on a consensus clade B sequence as described in the past (9, 10). The cohort consisted of 8 female participants and 37 male participants, all antiretroviral-treatment naïve and infected for at least 12 months (Table 1). High-resolution HLA typing was also performed for all subjects and indicated wide HLA class I and class II diversity (data not shown). Based on previously described cutoffs for positive responses (9) and using freshly isolated peripheral blood mononuclear cells (PBMC), ELISpot responses were detected against all viral proteins, with the majority of responses targeting overlapping peptides (OLPs) in Gag, Pol, and Nef (Fig. 1 and Table 2), in line with previously reported data generated on several different cohorts, including groups of clade B- and C-infected subjects (1, 9, 14, 19).

Given previous reports describing a superior role of the Gag-specific CTL activity in controlling HIV replication compared to responses to other parts of the viral genome, the total immune responses were broken down by viral protein. Responses were recorded as either the breadth of responses (i.e., the number of individual OLPs targeted) or the magnitude of responses (expressed as the total number of spot-forming cells [SFC]/10^6 input PBMC) (Table 2) and compared to CD4 T-cell counts and HIV viral loads. When breadth and magnitude of the total virus-specific responses were compared to viral loads or CD4 counts, no significant associations were observed

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TABLE 1. Demographics, viral loads, and CD4 counts in the study cohort

Parameter	Value[a]
Total no. enrolled	45
Gender (no. of males/no. of females)	37/8
Median viral load (log_{10} copies/ml)	4.53 (<1.7–6.0)
Median CD4 count (cells/μl)	417 (170–823)

[a] Numbers in parenthesis indicate ranges of viral load (log_{10}) and CD4 counts.

($P > 0.6$). Similarly, comparisons between the breadth of single-protein-specific responses and viral load or CD4 T-cell counts did not yield significant associations, with the exception of HIV Gag-specific breadth, which was directly correlated with the CD4 T-cell count but not with viral load ($P = 0.0036$). Similarly, when the single-protein-specific magnitude of responses was compared to CD4 count and viral loads, only a direct association between the Gag-specific magnitude and CD4 T-cell count emerged ($P = 0.0045$). However, after Bonferroni correction for multiple comparisons ($n = 40$), none of these associations remained statistically significant.

In contrast, when the protein-specific responses were expressed as the proportion of the entire virus-specific response (i.e., by comparing the protein-specific total magnitude of responses to the total virus-specific magnitude or by comparing the number of targeted OLP in a protein to the total number of recognized OLPs), a direct association between the relative breadth of the IFN-γ-producing, Gag-specific CTL response and the CD4 T-cell count was observed ($P = 0.0002$) (Fig. 2). Also, a statistically significant inverse correlation that withstood correction for multiple comparisons was observed between the magnitude of the relative Gag response and CD4 T-cell counts ($P = 0.0001$). No other total or relative protein-specific response was associated with CD4 T-cell counts, indicating that a dominance of Gag-specific responses in relation to the remainder of the virus-specific CTL activity is an indicator of relative control of HIV infection. Further analyses

TABLE 2. Distribution of CTL responses between HIV proteins

Protein	No. of peptides[a]	% of peptides targeted[b]	No. of subjects with response
p17	17	82	21
p24	31	97	32
p15	18	61	16
Nef	27	74	37
Rev	15	47	9
Tat	12	50	3
Vpu	9	11	1
Pro	20	55	16
RT	76	64	36
Int	37	70	19
Vpr	11	55	3
gp120	67	36	21
gp41	46	37	20
Vif	24	50	9

[a] Number of overlapping peptides spanning protein sequence.
[b] Fraction of OLP targeted at least once in the cohort.

based on Gag p17-, p24-, and p15-specific reactivity demonstrated that the relative breadth of HIV-Gag p24-specific responses ($P = 0.0004$) (but less so with Gag p17 [$P = 0.047$] and not with Gag p15 [$P = 0.93$]) were associated with CD4 T-cell counts. Similarly, the relative magnitude of the HIV Gag p24 but not of Gag p17 or p15 was directly associated with CD4 T-cell counts, indicating that the Gag-specific effects observed were largely mediated by immune responses to the p24 subunit of the Gag protein.

When viral loads were compared to the relative protein-specific T-cell responses, there was an inverse association between viral loads and the breadth ($P = 0.0105$) and magnitude ($P = 0.022$) of the relative Gag response (data not shown). Interestingly, while total Nef-specific responses were not associated with either CD4 counts or viral loads, the relative Nef response showed a direct correlation between the relative Nef-specific magnitude of responses and viral load ($P = 0.0031$), suggesting that Nef-specific CTL activity may be driven by viral load.

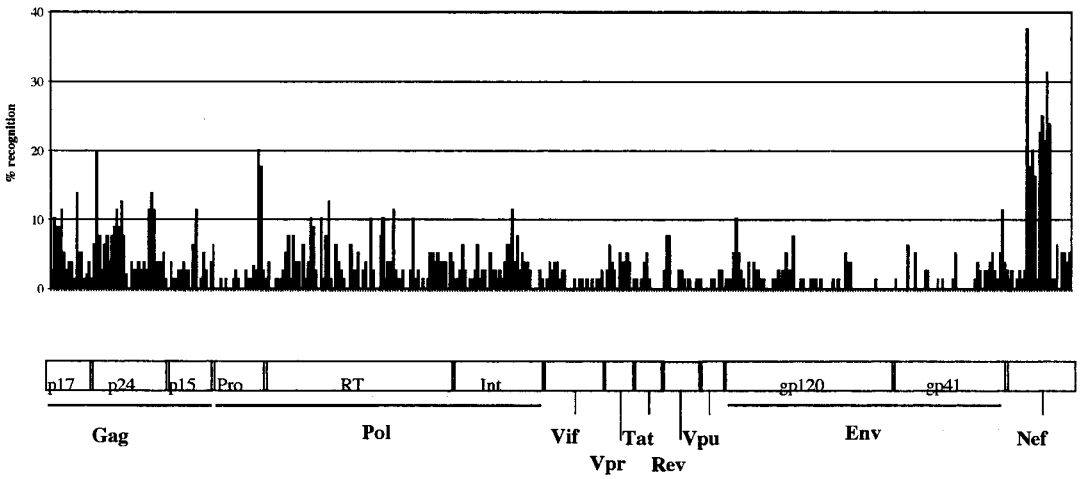

FIG. 1. CTL responses are distributed over the entire HIV genome and cluster in immunodominant regions. A total of 45 Peruvian subjects were tested for their total HIV-specific $CD8^+$ T-cell activity by using an overlapping peptide set of 410 peptides. The figure shows the frequency of recognition for each single peptide among the 45 subjects tested (*y* axis). The horizontal box indicates the HIV proteins spanned by the overlapping peptides.

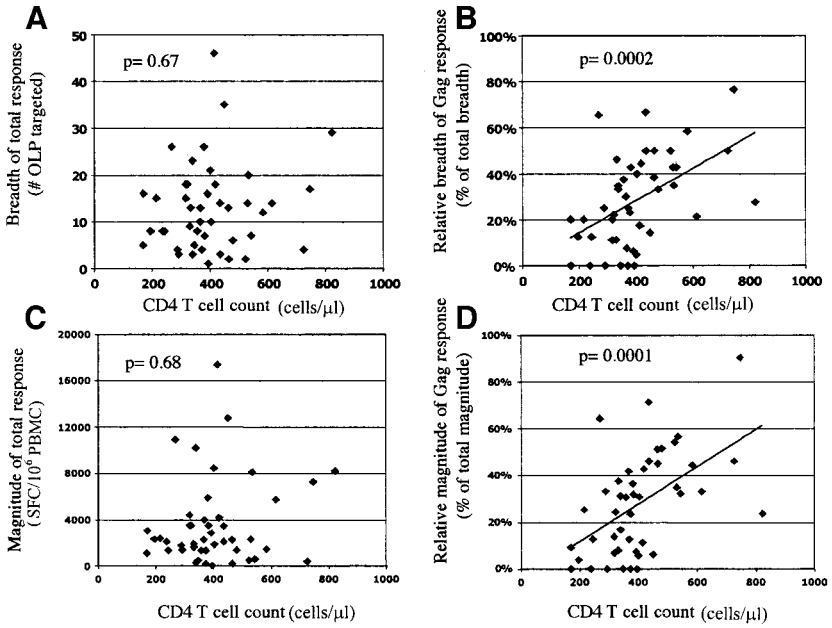

FIG. 2. Relative Gag-specific magnitude and breadth are associated with increased CD4 T-cell counts; ELISpot data from 45 untreated and chronically infected subjects were recorded as breadth (number of OLP targeted) or magnitude (SFC/10^6 PBMC) of responses and compared to individuals' CD4 counts. Responses were calculated either for the total virus-specific response (A and C) or the relative proportion of the Gag-specific activity only (B and D). *P* values are based on Spearman's rank test (two-sided) and are shown before correction for multiple comparisons.

To ensure that the detected responses were indeed mediated by CD8 T cells, virus-specific T-cell responses were assessed by intracellular cytokine staining (ICS) analyses (8). Of the 45 untreated, chronically infected subjects, cells were available for 24 individuals and showed CD4 T-cell-mediated responses to Gag, the most frequently targeted protein, in only 5 of these subjects, indicating that the observed associations were mediated by CD8 T-cell responses. Although this analysis could have missed CD4 T-cell responses that did not produce IFN-γ (12), these data are in line with previous observations where cell depletion studies showed that in chronically infected subjects, CD4 T-cell-mediated responses contributed only marginally to the observed total ELISpot responses (9).

To confirm the apparent beneficial role of focused Gag responses in the control of HIV infection, data from a previously described cohort tested in an identical manner and using the same peptide test sets as the Peruvian cohort were reanalyzed for the relative Gag- and Nef-specific responses (9). This cohort, established at hospitals in the Boston area and also consisting of HIV clade B-infected subjects, included 69 untreated subjects for which viral loads and CD4 T-cell counts were available. In particular, the Boston cohort included more subjects with advanced HIV disease, as CD4 T-cell counts were below 200 in 18 of 69 subjects, compared to the Peruvian cohort, in which 3 of 45 individuals had CD4 T-cell counts below 200. Indeed, significant positive and negative associations between the relative breadth of the Gag-specific response and CD4 counts ($P = 0.0012$) and viral load ($P = 0.014$), respectively, were observed. The relative magnitude of the Gag-specific response was directly associated with CD4 counts ($P = 0.0147$) and inversely correlated with viral loads ($P = 0.046$). In addition, the relative Nef-specific magnitude and breadth were inversely associated with CD4 counts ($P = 0.008$) and positively associated with viral load ($P = 0.029$). Altogether, the Boston-based cohort confirmed the results seen in the Peruvian study, further indicating that the relative Gag-specific CTL activity mediates relative HIV control. Combining the two cohorts further increased the statistical significance of the associations between the relative Gag magnitude/breadth and individuals' CD4 T-cell counts and viral loads (all *P* values were <0.0002, except for that of the viral load versus relative Gag magnitude, which was 0.001) and strongly suggests that more focused Gag-specific response patterns are associated with elevated CD4 T-cell counts and reduced HIV viral loads. Although these findings could be biased by different times after infection for individuals with strong or weak relative Gag-specific responses, the conclusions are in agreement with the study by Masemola et al., who reported an association between the hierarchy of responses to Gag peptide pools and viral loads but not CD4 T-cell counts (17). However, in that clade C study, no single peptides were used and, thus, no comparison between the breadth of responses and control of HIV was possible, nor were p24-specific responses assessed separately. In addition, reported *P* values were not corrected for multiple comparisons, making it difficult to estimate the statistical significance of those findings. Interestingly, though, that study also showed a trend for an inverse correlation between CTL responses to HIV Nef and viral load. Although this does not necessarily imply that Nef-specific CTLs are bad responses, they may be a surrogate for uncontrolled viral replication and high Nef protein expression, effectively mediating its immunorefractory effects on the cellular immune response.

As the HIV *gag* gene is relatively conserved, the test peptide set may generally be more closely matched to the individual's

autologous virus sequence compared to other, more variable regions of the viral genome, for which the present study may have underestimated the true breadth of responses. However, it is unlikely that the observed association between the relative breadth of Gag-specific responses and elevated CD4 T-cell counts and lower viral loads are affected by this potential bias, as relative and not absolute numbers of responses are being compared. These findings are also in line with recently presented analyses showing that an increased response rate against HIV Gag p24-derived CTL epitopes compared to non-Gag epitopes was associated with better disease outcome (13); furthermore, the findings are in line with previous reports linking CTL escape in HLA-B27- and -B57-restricted CTL epitopes in Gag with loss of viral control (11, 16). A likely explanation for this observation is that CTL escape mutations in Gag p24 are only poorly tolerated by the virus and are associated with significant reduction in viral replicative capacity (5). Thus, the data presented here, generated in two independent cohorts, strongly suggest that the more immune pressure the host's immune system can mount against HIV Gag, and in particular against Gag p24, the better the virus can be controlled. Alternatively, the data also suggest that in case the virus manages to escape the Gag-specific CTL immune surveillance, it may suffer significant losses in viral replicative capacity (2, 5, 15). Either way, the data presented here may call for a reanalysis of data sets that have not identified a relative protective role of Gag-specific CTL immunity and which have not been analyzed with regards to the relative dominance of the Gag- and p24-specific CTL immunity in HIV controllers. Emerging data from such analyses may further support the notion of shifting the focus of the HIV-specific immunity by therapeutic or prophylactic vaccination towards the viral Gag protein or at least its p24 subunit.

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