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# Gag-Specific CD4$^+$ T-Cell Frequency Is Inversely Correlated with Proviral Load and Directly Correlated with Immune Activation in Infection with Human Immunodeficiency Virus Type 2 (HIV-2) but Not HIV-1$^\nabla$

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**Human immunodeficiency virus type 2 (HIV-2) infection, unlike HIV-1 infection, is normally characterized by low rates of CD4 depletion and low-to-undetectable viremia. We found that the frequency of Gag-specific CD4$^+$ T cells featured positive correlations with the expression of markers of CD4 activation and a negative correlation with peripheral blood mononuclear cell-associated proviral load in infection with HIV-2, in contrast with HIV-1. Moreover, HIV-2-infected individuals exhibited a greater ability to respond to HIV-1 Gag peptides (heterologous responses). Our data suggest a potential link between HIV-2-specific CD4 responses, immune activation, and viral control, which may in turn relate to the better prognosis associated with HIV-2 infection.**

Infection with human immunodeficiency virus type 2 (HIV-2) is associated with slow disease progression and, in the majority of infected adults, a limited impact on mortality (12, 18, 24). CD4 depletion occurs at a much lower rate with HIV-2 than with HIV-1 disease (5, 12). Despite plasma viremia remaining low to undetectable throughout HIV-2 infection (1, 14, 23), levels of proviral DNA have been shown to be similar in both HIV-1 and -2 infections (23, 25). A possible explanation for the phenotypic differences between these two diseases is that they reflect better control of viral replication by virus-specific immune responses in HIV-2-infected individuals.

Although no major differences in the frequencies of HIV-specific CD8$^+$ T cells have been reported in HIV-1- and HIV-2-infected individuals (2, 4, 8, 9, 13, 15), HIV-2-specific CD8$^+$ T cells have been shown to recognize a broader range of viral proteins (30) and to have a higher functional flexibility (16). HIV-2-specific CD4$^+$ T cells have been less well characterized (6, 7, 21, 22, 30), though a better preserved proliferative capacity has been shown (6).

Here, we show for the first time that HIV-2 Gag-specific CD4$^+$ T-cell frequency correlated positively with CD4 activation and negatively with proviral load, raising the possibility of a closer relationship between the state of immune hyperactivation and virus-specific CD4 responses in HIV-2 infection.

We investigated homologous and heterologous Gag-specific CD4 T-cell responses in 19 HIV-2-infected and 19 HIV-1-infected individuals living in Portugal, matched for CD4 depletion. All individuals were antiretroviral therapy naïve and without evidence of ongoing opportunistic infections or tumors. Their epidemiological and clinical features are detailed in Table 1. The research was approved by the Ethical Committee of the Faculdade de Medicina da Universidade de Lisboa.

Responses were assessed in terms of frequency and magnitude at the level of cytokine production. The relative contribution of each cytokine-producing HIV-specific CD4 subset to each individual's response was also determined. The following peptide sets were used: 20-mers, overlapping by 10 residues and spanning residues 1 to 386 of HIV-2 ROD Gag (prepared by MRC, United Kingdom), and 15-mers, overlapping by 11 residues and spanning the equivalent region of HIV-1 HXB2 Gag (AIDS Reagent Program, National Institutes of Health). Peptide sets were combined in two separate pools, such that the concentration of each peptide within a pool was 400 μg/ml and used at a final concentration of 2 μg/ml/peptide. A total of $1 \times 10^6$ thawed peripheral blood mononuclear cells (PBMC) were resuspended in 1 ml of complete medium (29) with 1 μg/ml anti-CD28 (BD Biosciences, San Jose, CA) and cultured for 6 h alone or together with the HIV-1 or HIV-2 Gag peptide pools with the addition of 10 μg/ml of brefeldin A (Sigma-Aldrich, St. Louis, MO) after 1 hour of culture. Intracellular cytokine staining for interleukin-2 (IL-2) and gamma interferon (IFN-γ) was performed after surface staining for CD4 within 24 h of cell fixation as previously described (29). Results were expressed as percentages of cytokine-positive CD4$^+$ T cells within total CD4$^+$ T cells after background subtraction (anti-CD28 stimulation alone). Phorbol myristate acetate (Sigma-Aldrich)-plus-ionomycin (Calbiochem, Merck Biosciences, Nottingham, United Kingdom) stimulation was used as a positive control (29). Each peptide pool was tested against thawed PBMC from nine seronegative controls to establish an average background response for each cytokine, alone or in combination, and cutoffs were set accordingly (range, 0 to 0.06%).

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TABLE 1. Characterization of HIV-2- and HIV-1-infected cohorts

| Characteristic | Value for cohort infected with[a]: | |
|---|---|---|
| | HIV-2 | HIV-1 |
| Total no. of subjects (males/females) | 19 (11/8) | 19 (5/14) |
| Age (yr) | 47 ± 3.2 (26–62) | 41 ± 2.3 (21–68) |
| Caucasians/others | 8/11 | 12/7 |
| $CD4^+$ T cell count (cells/μl) | 686 ± 79 (188–1,436) | 667 ± 113 (133–2,174) |
| % $CD4^+$ T cells | 30 ± 3 (14–46) | 27 ± 3 (5–52) |
| Viremia (RNA copies/ml) | <200 (200–4,006)[b] | $8.6 \times 10^4 \pm 4.1 \times 10^4$ ($50 \times 10^4$–$55 \times 10^4$) |
| Proviral DNA (copies/$10^6$ PBMC) | 367 ± 107 (20–1,296) | 473 ± 173 (61–2,016) |
| % $HLA\text{-}DR^+$ within CD4 | 3.02 ± 0.95 (0.16–2.66) | 3.17 ± 0.62 (0.26–9.8) |
| % $HLA\text{-}DR^+$ $CD38^+$ within CD4 | 2.01 ± 0.66 (0.07–8.45) | 2.23 ± 0.45 (0.13–2.75) |

[a] Results are expressed as mean ± standard error of the mean (range), except for the total number of subjects and for ethnicity. Two group comparisons were made using unpaired *t* tests or the Mann-Whitney test, as appropriate. No statistical differences were found except for viremia, $P = 0.0005$, after applying the appropriate assay cutoff value to individuals with an undetectable viral load.

[b] Results are for 18/19 subjects.

Responses less than or equal to the corresponding assay cutoff were assigned a value of 0.

The numbers of individuals mounting homologous responses were the same in both cohorts (15/19 [79%]). The total magnitudes of these responses were similar for both groups, which was reflected in the comparable frequencies of each of the cytokine-producing subsets (Fig. 1A). Similar numbers of homologous responders in both groups had multifunctional Gag-specific CD4 responses, though 6/15 HIV-2-infected homologous responders had $CD4^+$ T cells that were positive only for IL-2 (IL-2 single positivity) as their only detectable HIV-specific response, compared to only 1/15 within the HIV-1 cohort (Fig. 1B and D).

Overall, we observed similar frequencies for $CD4^+$ T cells able to recognize homologous Gag peptides in HIV-1 and HIV-2 cohorts matched for CD4 depletion. In agreement with previous reports (2, 6), the cytokine profiles of HIV-2 responders were dominated by IL-2 production alone or in combination with other cytokine-producing subsets, whereas in the HIV-1 cohort, more responders had single IFN-γ-producing specific $CD4^+$ T cells forming part or all of their response.

In contrast, on assessment of heterologous responses, we found that more patients with HIV-2 responded to HIV-1 peptides than did those with HIV-1 to HIV-2 peptides, though this did not reach a level of significance (9/11 [82%] and 5/10 [50%], respectively; $P = 0.1224$ [$\chi^2$ test]). The magnitude of this response was significantly higher in the HIV-2 cohort than in the HIV-1 cohort ($P = 0.0483$) (Fig. 1A). The heterologous response of the HIV-1 cohort was also significantly reduced compared to its homologous response ($P = 0.0122$), which was largely due to the reduced frequency of IL-2 single-positive CD4 ($P = 0.0105$) (Fig. 1A). Of note, none of the HIV-1-infected heterologous responders had detectable IFN-$\gamma^+$ IL-$2^+$ CD4 (Fig. 1A and E). Within the HIV-2 cohort, the functionality of heterologous responses was similar to that observed for homologous responses (Fig. 1B and C). However, HIV-1 heterologous cytokine profiles were almost exclusively dominated by IFN-γ single-positive CD4 (5/5, versus 4/9 in the HIV-2 cohort) (Fig. 1E). The ability of a given individual, independent of the type of HIV infection, to mount a heterologous response was not dependent upon a detectable homologous response and vice versa.

In summary, we detected a clear difference in the magnitudes of $CD4^+$ T-cell response to heterologous peptides in HIV-2-infected and HIV-1-infected individuals. Additionally, in contrast to HIV-2, the frequency and magnitude of heterologous responses in the HIV-1 cohort were significantly reduced compared to those of the homologous responses. It is unlikely that the latter is related to the differing lengths of the HIV-1 (15-mer) and HIV-2 (20-mer) peptides, as both types of peptides have been shown to be equally efficient at revealing HIV-specific CD4 responses (17). Moreover, the functionality of heterologous responses was similar to that observed for homologous responses in both cohorts. Our data revealing responses to heterologous peptides in both HIV cohorts contradict an earlier report (30). However, this probably reflects differences in the populations assessed, CD8-depleted PBMC versus $CD4^+$ T cells in our study, and the readouts, IFN-γ enzyme-linked immunospot assays versus intracellular cytokine staining used here. Moreover, other studies have shown that PBMC isolated from HIV-2-infected individuals respond to simian immunodeficiency virus recombinant proteins and peptide pools, both in terms of proliferation (21, 22) and cytokine production (2). Overall, these data suggest that HIV-2-specific CD4 responses may feature a degree of flexibility similar to that reported for their CD8 counterparts (16).

Next, we assessed the relationships between HIV-specific responses and virological parameters. Plasma viremia was assessed by reverse transcription-PCR (detection limit for HIV-2, 200 RNA copies/ml, as described previously [27]; detection limit for HIV-1, 50 RNA copies/ml; Roche Molecular Systems, Branchburg, NJ), and PBMC-associated proviral load was assessed by quantitative real-time PCR, as we have previously described (25).

Unlike others (11, 19, 20), we found no correlation between the magnitude of HIV-specific CD4 responses and HIV-1 viremia (Table 2), which probably relates to the absence of nonprogressors in our untreated cohort. The aviremic status of the majority (18/19) of HIV-2-infected individuals precluded this analysis. However, in contrast to the HIV-1 cohort, a significant negative correlation between magnitude of homologous IFN-γ-producing CD4 responses and level of proviral DNA was observed (Table 2). Of note, this differs from the current paradigm that polyfunctionality of response, preservation of IL-2 production in particular, is better related to viral control (6). Interestingly, HIV-2-specific cytotoxic T-lymphocyte activ-

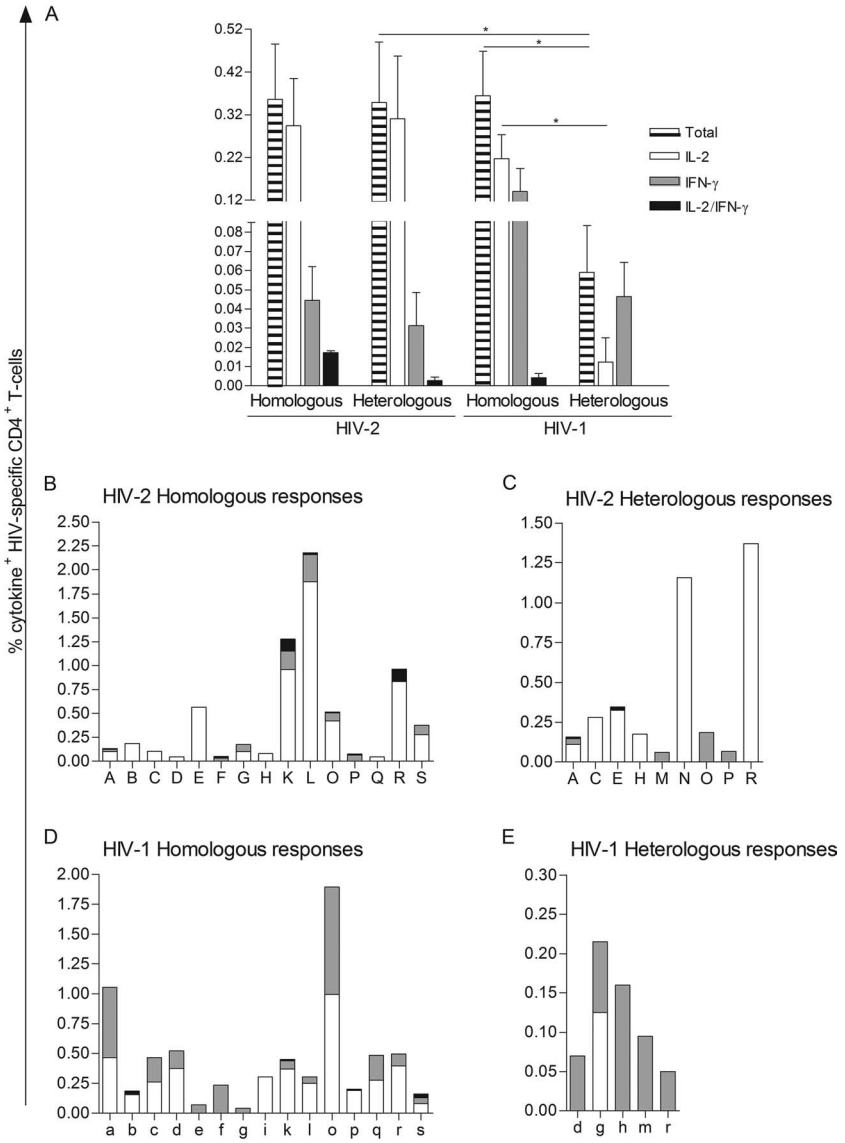


FIG. 1. Homologous and heterologous responses to Gag peptides. HIV-specific CD4 responses were assessed by cytokine production at the single-cell level after short-term culture with overlapping Gag peptides, and results are shown as the proportion of total $CD4^+$ T cells. Responses of HIV-2 individuals against HIV-2 Gag peptides (homologous) and of those against HIV-1 Gag peptides (heterologous) are shown, as well as the corresponding responses of an HIV-1 cohort. The total percentage of HIV-specific $CD4^+$ T cells able to recognize homologous or heterologous peptide sets in each cohort (horizontally hatched bars) is illustrated in panel A. The frequencies of IL-2 single-positive (open bars) and IFN-γ single-positive (gray bars) $CD4^+$ T cells and of $CD4^+$ T cells that were positive for both IL-2 and IFN-γ (black bars) are also illustrated. The bars indicate the means ± the standard errors of the means. Intercohort differences were assessed using unpaired *t* or Mann-Whitney U tests, as appropriate, using GraphPad Prism version 4.00 (GraphPad Software Inc., SD). *, $P < 0.05$. The heterogeneity of the HIV-specific CD4 responses in terms of cytokine production is further defined for each responsive individual with respect to homologous and heterologous responses for the HIV-2 cohort (panels B and C, respectively) and for the HIV-1 cohort (panels D and E, respectively). HIV-2 responders are identified by uppercase letters A to S and HIV-1 responders by lowercase letters a to s. Each bar represents the total response for a given individual, with the relative contributions of cytokine-producing HIV-specific CD4 subsets indicated as above.

ity has also been shown to inversely relate to proviral DNA in a Gambian cohort (3), further supporting the idea that the immune response in general may be more effective in controlling virus replication/dissemination in HIV-2 infection. Although the levels of proviral DNA are similar in HIV-2 and HIV-1 infections, the dynamics of cell-associated proviral load in relation to latency, productive infection, and cell death are likely to be distinct given the disparity in viremia. This may explain the contrasting associations between specific responses and proviral DNA in these two infections.

Finally, we determined whether correlations between markers of CD4 T-cell activation and magnitude of HIV-specific CD4 responses existed for either cohort. The factors driving immune hyperactivation and whether their relative contributions to HIV immunopathogenesis are beneficial or deleterious are still subjects of ongoing debate (10, 26). This is further

TABLE 2. Correlation between frequency of Gag-specific CD4 responses and possible surrogate markers of HIV disease progression[a]

| Response type and cytokine(s) | Value (*P*, r) for cohort infected with: | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | HIV-2 | | | | | HIV-1 | | | | | |
| | $CD4^+$ T cell count | % $CD4^+$ T cells | Viremia | Proviral DNA | % HLA-$DR^+$ within CD4 | % HLA-$DR^+$ $CD38^+$ within CD4 | $CD4^+$ T cell count | % $CD4^+$ T cells | Viremia | Proviral DNA | % HLA-$DR^+$ within CD4 | % HLA-$DR^+$ $CD38^+$ within CD4 |
| Homologous response | | | | | | | | | | | | |
| Total | 0.849, −0.047 | 0.663, −0.107 | NA | 0.067, −0.469 | **0.048, 0.519** | **0.023, 0.58** | 0.715, −0.092 | 0.45, −0.19 | 0.591, 0.145 | 0.832, −0.06 | 0.793, −0.077 | 0.718, −0.219 |
| IL-2 | 0.905, −0.029 | 0.605, −0.127 | NA | 0.082, −0.448 | 0.064, 0.499 | **0.036, 0.544** | 0.366, −0.227 | 0.353, −0.233 | 0.676, 0.113 | 0.508, −0.186 | 0.315, 0.29 | 0.245, 0.333 |
| IFN-γ SP | 0.31, −0.246 | 0.451, −0.184 | NA | **0.008, −0.636** | 0.177, 0.368 | 0.157, 0.385 | 0.983, −0.005 | 0.494, −0.172 | 0.104, 0.421 | 0.413, 0.228 | 0.413, −0.238 | 0.453, −0.031 |
| IL-$2^+$ IFN-$\gamma^+$ | 0.389, −0.21 | 0.301, −0.251 | NA | 0.335, −0.258 | **0.033, 0.551** | **0.049, 0.516** | 0.59, −0.136 | 0.471, −0.182 | 0.977, −0.008 | 0.807, 0.069 | 0.811, −0.071 | 0.917, 0.116 |
| Heterologous response | | | | | | | | | | | | |
| Total | 0.615, −0.173 | 0.577, −0.189 | NA | 0.291, −0.393 | 0.678, 0.159 | 0.81, 0.1 | 0.387, 0.301 | 0.682, 0.153 | 0.25, −0.424 | 0.121, −0.569 | 0.123, 0.522 | 0.155, 0.485 |
| IL-2 SP | 0.946, 0.029 | 0.86, 0.057 | NA | 0.25, −0.424 | 0.493, 0.254 | 0.613, 0.203 | ND | ND | ND | ND | ND | ND |
| IFN-γ SP | 0.342, −0.316 | 0.299, −0.187 | NA | ND | ND | ND | 0.759, 0.106 | 0.946, −0.031 | 0.521, −0.339 | 0.359, −0.244 | **0.039, 0.675** | 0.067, 0.607 |

[a] Relationships were tested for significance with Spearman rank correlation using GraphPad Prism. *P* values of $<0.05$ were considered significant and are in boldface type. SP, single positive; NA, not applicable; ND, not done. Correlations were not assessed for cytokine-producing HIV-specific CD4 T-cell subsets and immuno/virologic parameters when these values were available for ≤3 individuals.

illustrated by the distinct rates of disease progression that distinguish HIV-2- and HIV-1-infected patients despite exhibiting the same levels of immune activation at a given degree of CD4 depletion (28). Freshly isolated PBMC were surface stained and analyzed as previously described (28). Despite a lack of significant correlations between absolute numbers or frequency of circulating $CD4^+$ T cells and magnitude of the specific $CD4^+$ T-cell response in either cohort, the HIV-2-infected individuals exhibited significant positive correlations between IL-2-producing homologous Gag responses and CD4 T-cell activation (Table 2). Although the possibility of a deleterious role for HIV-2-specific responses in driving immune activation could be considered, their direct correlation with CD4 T-cell activation uniquely observed in our HIV-2 cohort suggests a possible counterbalance of the deleterious effects of immune activation through an expansion of the virus-specific responses. Moreover, this subset is characterized by IL-2-producing cells and, therefore, likely to be enriched in central-memory cells with preserved proliferative capacity (20), capable of generating more-differentiated, IFN-γ-producing progeny, and better able to target virally infected cells.

In conclusion, despite the similar frequencies and magnitudes of their homologous responses, HIV-2-infected individuals had stronger heterologous responses than their HIV-1 counterparts. More importantly, we demonstrated that HIV-2 homologous responses alone correlated positively with CD4 activation and negatively with proviral load. Although the correlative nature of this study precludes causal connections, it is reasonable to speculate that the closer link between immune activation and HIV-specific responses is related to the better control of viral replication that characterizes HIV-2 disease progression.

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