Early Gag Immunodominance of the HIV-Specific T-Cell Response during Acute/Early Infection Is Associated with Higher CD8+ T-Cell Antiviral Activity and Correlates with Preservation of the CD4+ T-Cell Compartment

Gabriela Turk, Yanina Ghiglione, Juliana Falivene, María Eugenia Socias, Natalia Laufer, Romina Soledad Coloccini, Ana María Rodriguez, María Julia Ruiz, María Ángeles Pando, Luis David Giavedoni, Pedro Cahn, Omar Sued, Horacio Salomon and Maria Magdalena Gherardi

Early Gag Immunodominance of the HIV-Specific T-Cell Response during Acute/Early Infection Is Associated with Higher CD8+ T-Cell Antiviral Activity and Correlates with Preservation of the CD4+ T-Cell Compartment

Gabriela Turk, Yanina Ghiglione, Juliana Falivene, María Eugenia Socías, Natalia Laufer, Romina Soledad Coloccini, Ana María Rodríguez, María Julia Ruiz, María Ángeles Pando, Luis David Giavedoni, Pedro Cahn, Omar Sued, Horacio Salomon, María Magdalena Gherradi

The important role of the CD8+ T-cell response on HIV control is well established. Moreover, the acute phase of infection represents a proper scenario to delineate the antiviral cellular functions that best correlate with control. Here, multiple functional aspects (specificity, ex vivo viral inhibitory activity [VIA] and polyfunctionality) of the HIV-specific CD8+ T-cell subset arising early after infection, and their association with disease progression markers, were examined. Blood samples from 44 subjects recruited within 6 months from infection (primary HIV infection [PHI] group), 16 chronically infected subjects, 11 elite controllers (EC), and 10 healthy donors were obtained. Results indicated that, although Nef dominated the anti-HIV response during acute/early infection, a higher proportion of early anti-Gag T cells correlated with delayed progression. Polyfunctional HIV-specific CD8+ T cells were detected at early time points but did not associate with virus control. Conversely, higher CD4+ T-cell set points were observed in PHI subjects with higher HIV-specific CD8+ T-cell VIA at baseline. Importantly, VIA levels correlated with the magnitude of the anti-Gag cellular response. The advantage of Gag-specific cells may result from their enhanced ability to mediate lysis of infected cells (evidenced by a higher capacity to degranulate and to mediate VIA) and to simultaneously produce IFN-γ. Finally, Gag immunodominance was associated with elevated plasma levels of interleukin 2 (IL-2) and macrophage inflammatory protein 1β (MIP-1β). All together, this study underscores the importance of CD8+ T-cell specificity in the improved control of disease progression, which was related to the capacity of Gag-specific cells to mediate both lytic and nonlytic antiviral mechanisms at early time points postinfection.

H uman immunodeficiency virus (HIV) still represents a major public health concern. Although the instauration of highly active antiretroviral treatment (HAART) had a tremendous impact on the epidemic dynamics, the development of an effective prophylactic vaccine is still a main objective in the HIV-related research field. As HIV is highly diverse among different isolates, evolves continuously under selective pressure, infects immune cells, and encodes proteins with the capacity to modulate immune cell functions, it imposes definite challenges that should be overcome in the race of getting a successful vaccine. However, the description of (i) infected subjects able to control HIV replication over long periods of time to very low levels without therapy (known as long-term nonprogressors [LTNP] and elite controllers [EC]); (ii) uninfected subjects who, despite being highly exposed to the virus, remain seronegative (exposed seronegatives [ESN]); and (iii) the results from the Thai vaccine trial RV-144, which showed 30% efficacy (1), suggests that the objective is reachable. In this line, much of the research work conducted over the past few years was aimed to define the immune correlates of protection, i.e., desirable characteristics that the vaccine-elicted immune response should have in order to contain viral challenge. Within this field, special emphasis has been focused on the HIV-specific CD8+ cytotoxic T lymphocytes (CTLs), which are thought to play a key role in reducing viral replication (2, 3).

The first evidence that specific CD8+ T cells were involved in the control of viral replication was reported in studies conducted in humans and nonhuman primates during the acute phase of infection. After infection, emergence of specific CD8+ T cells correlates with the decline of peak viremia toward set point establishment, which varies from person to person and is a strong predictor of disease progression (4). Also, CTL escape mutants have been described (5, 6), and superior viral control has been attributed to specific human leukocyte antigen (HLA) class I alleles (7, 8). Moreover, recent proof-of-concept vaccine studies in nonhuman primates indicate that vaccine-elicted CD8+ T-cell responses are associated with partial protection from infection and with en-
TABLE 1 Summary of clinical data corresponding to enrolled HIV+ subjects per study group

<table>
<thead>
<tr>
<th>Group (no. of subjects)</th>
<th>Viral load&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean log&lt;sub&gt;10&lt;/sub&gt; ± SD</th>
<th>Viral set point&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CD4&lt;sup&gt;c&lt;/sup&gt; T-cell count&lt;sup&gt;c&lt;/sup&gt;,&lt;sup&gt;d&lt;/sup&gt;</th>
<th>CD4 set point&lt;sup&gt;d&lt;/sup&gt;,&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHI (n = 44)</td>
<td>98,684 (13,161–477,708)</td>
<td>4.6 ± 1.2</td>
<td>4.5 ± 0.8</td>
<td>525 (320–678)</td>
<td>526 (357–656)</td>
</tr>
<tr>
<td>PHI &gt; 350 (n = 23)</td>
<td>32,473 (9,532–116,129)</td>
<td>4.2 ± 1.2</td>
<td>4.3 ± 0.6</td>
<td>633 (587–786)</td>
<td>586 (491–680)</td>
</tr>
<tr>
<td>PHI &lt; 350 (n = 18)</td>
<td>269,532 (101,394–500,000)</td>
<td>5.2 ± 1.0</td>
<td>5.0 ± 0.8</td>
<td>292 (244–375)</td>
<td>281 (234–327)</td>
</tr>
<tr>
<td>Chronic (n = 16)</td>
<td>16,682 (2,136–41,677)</td>
<td>4.1 ± 0.7</td>
<td>4.5 (164–577)</td>
<td>455 (164–577)</td>
<td></td>
</tr>
<tr>
<td>Chronic excluding viremic controllers (n = 12)</td>
<td>22,267 (13,296–44,758)</td>
<td>4.4 ± 0.5</td>
<td>287 (140–544)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC (n = 11)</td>
<td>&lt;50</td>
<td>&lt;1.7</td>
<td>595 (562–817)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Versant HIV-1 RNA 3.0 assay, Siemens. Lower and upper detection limits are 50 and 500,000 RNA copies/ml, respectively (1.7 and 5.7 log<sub>10</sub>).  
<sup>b</sup> Flow cytometry double platform, FACSCanto, BD Biosciences.  
<sup>c</sup> For PHI subjects, data correspond to baseline samples. For chronic and elite controller subjects, data correspond to samples obtained at enrollment.  
<sup>d</sup> Set points were not calculated for subjects that initiated HAART during the first year postinfection.

MATERIALS AND METHODS

Study subjects. A total of 81 subjects participated in this study; 10 healthy HIV-seronegative donors (HD) and 71 HIV-infected patients, of whom 44 were enrolled during acute/early primary HIV infection (PHI), 16 were chronically infected patients (chronics), and 11 were elite controllers (EC) (Table 1; see also Table S1 in the supplemental material). PHI subjects were enrolled by the Grupo Argentino de Seroconversión Study Group under the following inclusion criteria (26): (i) detection of HIV RNA or p24 antigen with a simultaneous negative or indeterminate Western blot assay or (ii) positive Western blot assay with a negative test within the previous 6 months. Chronically infected patients were defined as subjects with established HIV infection older than 3 years, with detectable viral load (VL; >50 HIV RNA copies/ml plasma), and who are HAART naive, while EC were defined as subjects infected for more than 5 years with undetectable VL (<50 HIV RNA copies/ml plasma) who are HAART naive and have no record of opportunistic infections and/or AIDS-related diseases. The study was reviewed and approved by two institutional review boards (IRB): Comité de Ética Humana, Facultad de Medicina, Universidad de Buenos Aires, and Comité de Bioética, Fundación Huésped (Buenos Aires, Argentina). Both HIV-infected participants and healthy donors provided written informed consent accepting to participate in this study.

Samples. Blood samples were collected from study participants at enrollment and centrifuged to separate plasma, which was stored at −80°C until use. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation (Amersham, Sweden) and cryopreserved for subsequent functional assays. For PHI subjects, additional samples were obtained at 3, 6, 9, and 12 months postinfection. In the case of chronic, EC, and PHI subjects, plasma VL (branched-DNA, Versant HIV-1 RNA 3.0 assay; Siemens Healthcare) and CD4<sup>T</sup> T-cell count (flow cytometry double platform, BD FACSCanto; BD Biosciences) were determined.

HLA typing. Human leukocyte antigen (HLA) class I typing was performed using an in-house protocol consisting of PCR amplification, nucleotide sequencing with nested primers, and Web-based sequence interpretation (R. S. Coloccini and D. C. Monaco, unpublished data). HLA-A
exons 2 and 3 were amplified in one amplicon. HLA-B exons 2 and 3 were amplified separately. The amplification of exon 3 was performed by heminested PCR, using 2 different reverse primers. Similarly, HLA-C exons 2 and 3 were amplified separately using a heminested PCR strategy (2 different forward primers) in the case of exon 2. Amplicons were directly sequenced using the BigDye Terminator sequencing kit (Amersham, Sweden) on an automatic sequencer (Applied Biosystems DNA Sequencer 3100). Nucleotide sequences were analyzed and manually adjusted using Sequencher 4.10.1 software (Gene Codes Co.). Sequence interpretation was performed using the NCBI SBT Interpretation software, available online (http://www.ncbi.nlm.nih.gov/gv/mhc/sbt.cgi?cmd=main).

Peptides. Potential T-cell epitope (PTE) peptide panels corresponding to Nef, Gag, and Env proteins and the cytomegalovirus (CMV), Epstein-Barr virus, and influenza virus (CEF) peptide pool were obtained from the NIH AIDS Reagent Program (27, 28). Lympholized peptides were dissolved in dimethyl sulfoxide (DMSO) at 40 μg/μl and stored at −20°C. The PTE peptides are 15 amino acids (aa) in length and contain naturally occurring 9-aa sequences that are potential T-cell determinants embedded in the sequences of circulating HIV-1 strains worldwide. Here, the PTE peptides were grouped in 9 pools: 1× Nef (n = 127 peptides), 3× Gag (corresponding to p17 [n = 97], p24 [n = 128], and p2p7p1p6 [denoted as RG; n = 95]), and 5× Env (Gp120A [n = 73], spans HXB2 Env aa positions 1 to 154, Gp120A2 [n = 73, aa 157 to 284], Gp120B [n = 105, aa 287 to 511], Gp41A [n = 114, aa 513 to 689], Gp41B [n = 115, aa 689 to 842]).

ELISPOT assay. Gamma interferon (IFN-γ)-secreting cells were evaluated using enzyme-linked immunospot (ELISPOT) assays as described previously (25). Briefly, cryopreserved PBMCs were thawed in complete RPMI medium (RPMIc; RPMI 1640 [Gibco BRL], 10% fetal bovine serum [FBS; Gibco BRL], 2 mM l-glutamine [Gibco BRL], 100 U/ml penicillin [Gibco BRL], 100 μg/ml streptomycin [Gibco BRL], 10 mM HEPES [Gibco BRL]) supplemented with 50 U/ml DNase I (Benzonase nuclease; Roche Diagnostics) and 10% FBS. Cell viability was checked before and after overnight rest by trypan blue exclusion (only samples with ≥95% viability after the overnight rest were used for the assays). Costimulatory antibodies (anti-CD28 and anti-CD49d antibodies, 1 μg/ml; BD Biosciences), monensin (Golgistop, 0.7 μl/ml; BD Biosciences), brefeldin A (10 μg/ml; BD Biosciences), and the corresponding peptide pool (2 μg/μl) were added. An unstimulated (peptide-free medium plus 0.5% DMSO and costimulatory antibodies) and two positive controls (2 μg/ml CEF peptide pool and PMA-ionomycin) were included in each assay. A mixture of anti-CD107A-fluorescein isothiocyanate (FITC) and anti-CD107B-FITC antibodies (BD Biosciences) was added to one of the replicates. Cells were incubated for 6 h at 37°C, washed, and stained for 30 min at 4°C with LIVE/DEAD Fixable near-IR (Invitrogen), in order to exclude dead cells, and with anti-CD3-PerCP and anti-CD8-APC surface antibodies (BD Biosciences). Then, cells were permeabilized and fixed using the Cytofix/Cytoperm kit (BD Biosciences). After the permeabilization/fixation step, one of the replicates was stained using anti-IL-2–phycoerythrin (PE), anti-tumor necrosis factor alpha (TNF-α)-FITC, and anti-IFN-γ–PE/Cy7 antibodies (BD Biosciences), while replicates already containing the anti-CD107A/B mix were stained with anti-IL-2–PE and anti-IFN-γ–PE/Cy7. Cells were then washed and stored until data acquisition in a 2-laser, 6-color BD FACSVerse flow cytometer. Data acquisition and analysis were performed using the BD FACSDiva software. Instrument settings and fluorescence compensation were performed for each day of testing using unstained and single-stained samples. Isotype controls (consisting of stimulated cells stained with conjugated antibodies to surface molecules and isotype controls corresponding to intracellular markers) were performed for each patient in order to accurately set negative populations. First, a plot of forward scatter area (FSC-A) versus height (FSC-H) was constructed to remove doublets. Then, gating was performed on small lymphocytes in a plot of FSC versus side scatter (SSC). At least 80,000 events were acquired in the lymphocyte gate. Dead cells were then excluded on the bases of LIVE/DEAD fluorescence. Subsequently, CD3+ CD8+ cells were gated in a CD3-versus-CD8 dot plot. Following identification of these cells, a gate was made for each function studied (see Fig. 5A). To study 2-function and 3-function positive populations, “derived gate tools” available in the FACSDiv software was used. For this purpose, intersections of two and three gates were created, respectively. Once the percentages of events were determined for each derived gate, the value of triple-positive events was subtracted from those of double-positive events and, in turn, double- and triple-positive events were subtracted from the total events positive for a given function to calculate monofunctional cells. Samples with a nonspecific background higher than 0.05% for any function were retested using a new vial of frozen cells. Data presented corre-
spond to background-subtracted results using the CD28/CD49d-only stimulation. This was performed on a cytokine-subset-by-cytokine-subset basis, i.e., subtracting the result from the CD28/CD49d-only condition for a given cytokine subset to the same subset of a peptide-stimulated condition. Two standard deviations (SDs) above background was set as the threshold for determining positive responses. Values below this threshold were set at 0.

For certain cell functions, relative mean fluorescence intensity (rMFI) was calculated as the ratio between MFI corresponding to specific versus total CD8+ T-cells for a given channel.

Quantification of plasma soluble factors. Simultaneous determination of the following 39 cytokines and chemokines was performed in plasma samples from a subset of 28 PHI subjects (at baseline time point only) using Luminex technology (MILLIPLEX MAP Human Cytokine/Chemokine; Millipore): epidermal growth factor (EGF), eotaxin, FGF-2, Flt-3 ligand, fractalkine, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), GRO, IFN-α-2, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, monococyte chemotactic protein 1 (MCP-1), MCP-3, MDC (CCL22), MIP-1α, MIP-1β, SCF, sIL-2Rα, transforming growth factor alpha (TGF-α), TNF-α, TNF-β, VEGF. Samples were processed and analyzed as described by Gaviedona (30).

Data analysis. For PHI subjects, viral and CD4 set points were calculated as the geometric mean of the determinations obtained between 6 and 12 months after the presumed date of infection. Set points were not calculated for those subjects who started HAART during the first 12 months after infection or if no stable set point was reached during that period.

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). All data except log_{10} VL and breadth of response were analyzed using nonparametric statistics. Two-tailed Wilcoxon and Mann-Whitney tests were used to compare intra- and intergroup variables, respectively. Correlations were determined using Spearman’s rank test. All tests were considered significant if the P value obtained was less than 0.05. For correlations between plasma cytokine levels and immune parameters, P values were adjusted for multiple comparisons using a false discovery rate (FDR) procedure, according to the Benjamini & Hochberg method, with R Project software version 2.10.0. Adjusted P values were considered significant when less than 0.1.

RESULTS

Study subject descriptions. Three groups of HIV-infected subjects were enrolled as follows in order to attempt to dissect immune mechanisms associated with control over disease progression during acute/early HIV infection: 44 subjects recruited during HIV seroconversion and/or within 6 months from presumed date of infection (PHI group), 16 chronically infected subjects (chronics), and 11 subjects defined as elite controllers (EC) according to the criteria defined in Materials and Methods. Details of descriptions of the HIV-infected participants are shown in Table S1 in the supplemental material. Baseline samples for most PHI subjects were obtained during Fiebig stages V and VI (31). Within this group, median baseline VL was 98,684 RNA copies/ml (25 to 75% interquartile range [IQ], 13,161 to 477,708 copies/ml). As expected, it was statistically higher than chronics’ VL (median, 16,682 RNA copies/ml [IQ, 2,136 to 41,677], P = 0.012) and, of course, EC’s VL (Table 1 and Fig. 1). Median baseline CD4+ T-cell count for PHI (525 cells/μl [IQ, 320 to 678]) did not differ significantly from the median CD4+ T-cell count of chronics (455 cells/μl [IQ, 164 to 577]) or EC (595 cells/μl [IQ, 562 to 817]), but a statistically significant difference was found between chronics and EC (P = 0.018) (Fig. 1). For those PHI subjects who remained HAART naive during the first year postinfection, viral and CD4+ T-cell set points were calculated (Table 1 and Fig. 1; see Table S1 in the supplemental material). These parameters did not differ significantly from chronics’ VL and CD4+ T-cell count. Of note, the group of chronically infected subjects enrolled for this work is very heterogeneous and includes four subjects that could be classified as what some authors refer to as viremic controllers, i.e., individuals able to spontaneously control viral load below 2,000 RNA copies/ml (32) (C03, C05, C07, C08; Fig. 1; see also Table S1 in the supplemental material). After removing these subjects from the analysis, median VL for the chronic group was 22,267 RNA copies/ml (IQ, 13,296 to 44,758), and median CD4+ T-cell count was 287 cells/μl (IQ, 140 to 544) (Table 1).

PHI subjects were further divided into two subgroups whether their CD4+ T-cell count dropped below 350 cells/μl at any time during the first year postinfection or not (PHI < 350 and PHI > 350). By doing this, we were aimed to differentiate subjects with more rapid or aggressive progression of early infection (PHI < 350 group) and to investigate the association of this pattern with the immune parameters analyzed in this work. The 350-cells/μl endpoint was chosen based on the national and international recommendations for initiation of HAART current by the year 2010,
when most of these individuals were already enrolled (26). The PHI < 350 and PHI > 350 groups differed significantly in both baseline and set point VLS and CD4 T-cell counts (Table 1, Fig. 1).

Distribution of HLA alleles in enrolled subjects as a whole reflected the frequency distribution described for Argentina’s urban population (www.allelefrequencies.net). To exclude the possibility of enrichment of particular HLA alleles associated with faster (A01, A68, B35) or slower (B27, B51, B57) HIV disease course, the frequency of these alleles was studied per study group. No significant differences in the frequency of A01, A68, or B35 alleles were found among groups, even for the EC group, where these alleles were found in 4 out of 11 subjects (36% versus 41% of chronics and 47% of PHI subjects) (see Table S1 in the supplemental material). On the other hand, “protective alleles” (i.e., HLA-B27, B51, or B57) were present in one subject from the PHI < 350 group, three subjects from the PHI > 350 group, one PHI subject with undetermined progression status, three chronically infected subjects, and only three EC. So, the different disease progression rates among groups cannot be explained merely by differences in the genetic background, at least based on the HLA-I locus level.

Screening of HIV-specific T-cell responses by ELISPOT revealed differences between progressive and nonprogressive infection regarding magnitude, preferred targets of specific response, and quality. To evaluate how the central targets, magnitude, and quality (in terms of breadth and capacity to respond upon stimulation) of the HIV-specific cellular immune response during the acute/early phase of infection impact early disease progression, HIV-specific IFN-γ-secreting cells were screened by ELISPOT in PHI subjects using samples obtained at baseline (defined as the first sample obtained after enrollment according to the criteria defined in Materials and Methods). PTE peptide pools spanning the viral proteins Nef (1 pool), Gag (3 pools), and Env (5 pools) were used as stimuli. Also, these HIV-specific immune responses were screened in chronics and EC for comparison purposes.

(i) Magnitude and targets of HIV-specific cellular immune response in PHI, chronics, and EC. The median magnitude of the specific immune response (expressed as the number of SFU/10^6 PBMCs) was higher in EC for all three antigens evaluated than in chronic and PHI subjects. This difference was particularly important in terms of Gag antigen: EC showed a >5-fold-higher median Gag-specific magnitude than chronic and PHI subjects (median Gag-specific magnitude = 3,570, 692, and 145 SFU/10^6 PBMCs for EC, chronics, and PHI subjects, respectively) (EC versus chronics, P = 0.025; EC versus PHI subjects, P = 0.0003; Fig. 2A).

Intragroup analysis showed that anti-Gag magnitude was significantly higher (≈6-fold) in EC than Nef (median, 656 SFU/10^6 PBMCs; P = 0.005) and Env (median, 590 SFU/10^6 PBMCs; P = 0.007), while the anti-Nef magnitude in the PHI group (median, 308 SFU/10^6 PBMCs) tended to be higher than anti-Gag magnitude (median, 145 SFU/10^6 PBMCs; P = 0.06). Also in the PHI group, both anti-Nef and anti-Gag magnitude were significantly higher than anti-Env magnitude (median, 35 SFU/10^6 PBMCs; P = 0.015 and P = 0.014, respectively) (Fig. 2A).

The relative contribution of each antigen to the total response, i.e., the proportion of cells specific for each protein relative to the total anti-HIV response, is an important factor to be taken into account when evaluating the association of HIV-specific immune response with viral control (11). Thus, the proportion of specific cells against each HIV antigen in relation to the total response (sum of the T-cell responses against the individual antigens) was analyzed (Fig. 2B). Again, the contribution of the different targets differed among the different study groups: PHI subjects showed a statistically higher proportion of Nef-specific cells (median, 55%) than chronics (median, 17%; P = 0.005) and EC (median, 20%; P = 0.007) and lower proportion of anti-Gag-specific cells (PHI, 32% versus EC, 64% [P = 0.004]; PHI versus chronics, 64% [P > 0.05]). In the same line, intragroup analysis revealed that anti-Gag response (median, 64%) clearly predominated over Nef (20%) and Env (7.4%)-specific cells in EC (Gag versus Nef, P = 0.005; Gag versus Env, P = 0.007), whereas anti-Nef cells predominated in PHI (median, 55%) over Gag (32%, P = 0.001) and over Env (6%, P < 0.001). The chronic group showed the same immunodominance pattern (Gag > Nef > Env) than EC, although the response dispersion among chronics was much higher, which reflects the heterogeneity of this group. When analyzing separately the chronic subjects referred to as viremic controllers, it could be observed that the median percentage of Gag immunodominance in this chronic subgroup was 86%, considerably higher than that of regular chronics (43%).

When a similar analysis was performed within PHI subgroups, it could be observed that the scenario is clearly different between PHI > 350 and PHI < 350 groups: while no statistically significant difference could be observed between anti-Nef and anti-Gag magnitude or proportion of total response in the PHI > 350 group, anti-Nef cells clearly dominated the anti-HIV response in the PHI < 350 group (median magnitude, 500 SFU/10^6 PBMCs; median proportion of total response, 62%) over Gag and Env both in terms of magnitude (80 SFU/10^6 PBMCs [P = 0.0125] and 77 SFU/10^6 PBMCs [P = 0.001], respectively) and proportion (32% [P = 0.01] and 7% [P = 0.003], respectively) (Fig. 2A and B).

Given the significant role that anti-Nef and anti-Gag responses seem to play in defining the different rates of progression among groups, the contribution of anti-Nef and anti-Gag T-cell responses (relative to the sum of the magnitude obtained for both antigens) was analyzed per individuals included in the different groups (Fig. 2C). Anti-Gag responses clearly dominated over Nef in all EC (P = 0.002). On the opposite sense, anti-Nef responses dominated the anti-HIV response in the group of rapid progressors, PHI < 350 (P = 0.008). In chronics and the PHI > 350 group, an intermediate situation was observed: Gag dominated over Nef only in a subset of individuals, while the rest showed either Nef immunodominance or a balanced situation between both antigens. Among the chronic group, those with clear Gag immunodominance included those previously referred to as “viremic controllers” (C03, C05, C07, and C08).

It must be noted that the immunodominance hierarchy obtained with baseline samples was maintained when using samples obtained 1 year postinfection (data not shown), thus similar tendencies were observed when analysis was performed with the 12-month samples.

Then, a more detailed analysis of the Gag polypeptide subunits targeted by cells from the recruited individuals was performed. In all groups (chronic, EC, and PHI subjects as a whole), the immunodominance pattern (according to medians) was as follows: p24 > p17 > RG (Fig. 2D). Only in EC, the anti-Gag response was focused into p24 protein in a significantly higher proportion over both p17 and RG (P = 0.047 and 0.032, respectively). In chronics and PHI subjects, the proportion of anti-p24 response was significantly higher than the anti-RG response (P = 0.0012 and P =
0.0021, respectively) and also tended to be higher than the anti-p17 response, although the difference was not statistically significant in either group. When PHI subjects were split into PHI > 350 and PHI ≤ 350, it was observed that the PHI > 350 group maintained the immunodominance pattern observed before, whereas a shift to p17 > p24 > RG was observed in the PHI ≤ 350 group.

(ii) Quality (in terms of breadth and intensity of IFN-γ production upon stimulation) of the HIV-specific cell immune response in PHI, chronic, and EC groups. Afterward, the breadth of the response was calculated for each individual as the number of peptide pools recognized (out of the 9 pools used to screen HIV-specific response) (Fig. 2E). This analysis revealed that EC were able to recognize a higher number of pools than chronic and PHI (P = 0.05 and 0.002, respectively). Even more, EC were able to recognize more Gag-specific pools (n = 3 pools) than chronics and the PHI group (not shown). No statistically significant difference was observed between the PHI ≤ 350 and PHI > 350 groups.

Finally, the mean spot size obtained for both CEF and HIV peptide pools was recorded as the measure of the amount of IFN-γ produced by the individual specific T cells (which in turn correlated with the T-cell functional avidity) (33) (Fig. 2F). No intra-subject difference was observed among the different HIV-specific pools (Nef, Gag, and Env) (data not shown). Hence, for the purpose of this analysis, the mean spot size for all HIV antigens was averaged, taking into account only those pools for which a positive response was obtained in the ELISPOT assay on a subject-by-subject basis. Regarding the control CEF-specific spots, PHI subjects showed the smallest size being statistically different from EC (EC CEF-specific spots were 1.5 times larger than in the PHI group, P = 0.029), which in turn showed the largest CEF-specific spots, even slightly larger than healthy donors (P > 0.05). This difference in the PHI group was driven by PHI ≤ 350 subjects who even differed substantially from their counter group, PHI > 350 (spots from the PHI > 350 group doubled, on average, those of the PHI < 350 group). A similar scenario was observed for HIV-specific spots but, in this case, differences among groups were stronger. EC showed significantly larger spots than the chronic and PHI groups (1.5× [P = 0.004] and 1.7× [P = 0.002], respectively). Within the PHI group, a significant difference could be observed between the PHI > 350 and PHI ≤ 350 groups, showing the former group had larger HIV-specific spots (1.87×, P = 0.022). Interestingly, when CEF- versus HIV-specific spot size was analyzed intragroup, the HIV spots were smaller than CEF spots. In fact, the spot size difference between the two antigens reached statistical significance in chronic (P = 0.036), PHI as a whole (P = 0.011), and the PHI ≤ 350 group (P = 0.018). Although the trend was also observed in EC and the PHI > 350 group, the breach between both antigens was less important, especially in EC.

(iii) In acute/early infected subjects (PHI group), baseline relative immunodominance of anti-Gag cellular response correlates with both baseline and set point CD4+ T-cell counts. In Fig. 2C, it can be observed that the EC and PHI < 350 group (opposed groups in terms of disease progression) showed exactly opposite immunodominance patterns regarding Nef and Gag proteins. Based on this observation, it was hypothesized that in the much heterogenous chronic and PHI > 350 groups, Gag immunodominance correlates with slower disease progression. To test this hypothesis, correlation analyses were performed between the percentages of anti-Gag responses and clinical data of enrolled subjects. It was found that baseline relative Gag immunodominance
Gag immunodominance correlated with higher CD4+ T-cell counts, both at baseline and set point, further denoting it represents a key immune factor involved in delayed progression.

All together, data collected so far demonstrate that although Nef dominates the anti-HIV response in acute/early infection, a higher proportion of early T-cell responses targeting preferentially Gag (in particular p24) and larger spot size (indicative of improved capacity to secrete IFN-γ and higher avidity) were associated with delayed progression during the first year of infection. Also, these same factors were observed in chronically infected elite controllers (EC), indicating that these T-cell features may play an important role in protection from progression. In particular, early Gag immunodominance correlated with higher CD4+ T-cell counts, both at baseline and set point, further denoting it represents a key immune factor involved in delayed progression.

HIV-specific CD8+ T cells elicited during PHI were capable of inhibiting heterologous viral replication in vitro, and the magnitude of this activity was related to Gag immunodominance. We next aimed to investigate whether HIV-specific CD8+ T cells arising during acute/early HIV infection could suppress the replication of lab-adapted X4 and R5 HIV strains and to which extent, in comparison with chronic infection. It was also our aim to establish a possible relationship between this viral inhibitory activity (VIA) and the viral proteins targeted by the immune response during the early stage of infection.

In PHI subjects, VIA was shown to be higher than that of chronic infection. EC equally inhibited both R5 and X4 viruses, indicating that these individuals had a broad VIA. It is worth noting here that the use of heterologous lab-adapted viral strains might lead to a VIA underestimation, and stronger differences among groups may be masked due to this reason (16). As for the PHI group, most subjects (70%) demonstrated to have CD8+ T cells able to mediate viral inhibitory activity (VIA) and the viral proteins targeted by the immune response at baseline sample in the ELISPOT assay; (C) correlation between the magnitude of Gag-specific response in PHI subjects at baseline sample and VIA; (D) PHI subjects with substantial capacity to mediate VIA (>25%) also showed higher CD4+ T-cell set points. In panels A, B, and D, symbols show the values for each individual subject. Within the chronic group (A), viremic controllers are denoted with black dots. Horizontal lines within boxes represent the median values. Intergroup and intragroup (PHI baseline versus year) differences were analyzed using Mann-Whitney and Wilcoxon tests, respectively; *, P < 0.05.
VIA arise early during infection. Longitudinal analysis using samples obtained at 12 months postinfection indicated that this activity persists over time even beyond set point establishment (Fig. 4A).

When the PHI group was split into the PHI > 350 and PHI < 350 groups, no significant differences were observed in VIA either at baseline or 12-month samples. Given our observation obtained from the ELISPOT analysis, where Gag immunodominance during PHI was associated with preservation of the CD4+ T-cell count over the first year postinfection, we decided to reassign PHI individuals into Gag responders or Nef responders according to preferred (>50% of total response) viral protein target. This analysis revealed that CD8+ cells from PHI individuals in which Gag-specific cells dominated the early HIV-specific T-cell response had a stronger capacity to mediate VIA against the R5 virus than Nef responders, both at baseline (P = 0.043) and at the 12-month samples (P = 0.011) (Fig. 4B). This result agrees with the fact that EC consistently show the strongest VIA compared to other HIV+ groups (in this and other studies) and concomitantly show a robust anti-Gag immunodominance of the HIV-specific cellular immune response. In the same line, a statistically significant correlation was found within the PHI group between VIA and the magnitude of Gag-specific SFU (Spearman’s r = 0.5885, P = 0.0343; Fig. 4C). Overall, these results suggest that the ability of HIV-specific CD8+ T cells arising early during infection to suppress viral replication in autologous CD4+ T cells might be related, among other factors, to the relative Gag immunodominance out of the total HIV-specific CD8+ T-cell response as well as the absolute number of Gag-specific CD8+ T cells. Also, it was observed that EC and PHI Gag responders more frequently showed VIA against both R5 and X4 viruses, while chronic and PHI Nef responders recognized one, other, or no viruses, suggesting that the former groups have broader activity (data not shown).

Given the consistent association of higher VIA with EC status observed in this and other studies (16, 19, 20, 34, 35), we decided to seek for any association of stronger VIA with clinical parameters related to acute/early infection. No association was observed between VIA magnitude and baseline viral load, viral set point, or baseline CD4+ T-cell count. However, it could be observed that those subjects showing substantial capacity to mediate VIA (>25%) had higher CD4+ T-cell set points (P = 0.02, Fig. 4D), suggesting that the association of Gag immunodominance with delayed progression in terms of CD4+ T-cell count preservation described earlier in the manuscript (Fig. 3) would be related to a higher capacity of Gag-specific cells to mediate VIA.

Low frequency of polyfunctional HIV-specific CD8+ T cells was detected during PHI; it slightly increased over time during the first year postinfection but did not associate with viral or CD4+ T-cell set points. In order to further characterize HIV-specific CD8+ T cells arising during early infection, the functionality of these cells, in terms of their ability to degranulate (evidenced by CD107A/B mobilization) and to produce IFN-γ, TNF-α, and/or IL-2 upon peptide stimulation, was studied by flow cytometry. For these assays, data obtained in the ELISPOT screening was used as a starting point to define which peptide pools were used as stimuli. So for each subject, only those peptide pools for which positive responses were found by ELISPOT were used as stimuli in the ICS assay. The gating strategy used is illustrated in Fig. 5A. The proportion of cells expressing each of these functions alone or in combination was analyzed, not only in PHI but also in chronic and EC subjects, in order to help distinguish any association with disease progression.

As previously reported by our group (25), HIV-specific CD8+ T cells expressing all the functions analyzed (either alone or in combination) could be measured very early during infection. Among PHI baseline samples, mean ± SD percentages of HIV-specific CD3+ CD8+ cells for each single function were 0.16 ± 0.28 for IFN-γ, 1.13 ± 5.23 for TNF-α, 0.06 ± 0.14 for IL-2, and 0.21 ± 0.4 for CD107. Figure 5B depicts the relative contribution to the total specific CD8+ response made by each function or function combination. No particular function or function combination could be distinguished that differed substantially among PHI, EC, or chronic subjects, except for bifunctional CD107A/B+ IFN-γ− CD8+ T cells. All EC individuals had detectable bifunctional CD107A/B+ IFN-γ− CD8+ T cells (mean, 30% ± 11% out of the specific CD8+ T cells), differing, in this sense, significantly from chronic (13% ± 23%; P = 0.007) and PHI individuals (9% ± 14%; P = 0.0004), indicating that this sub-subset of specific CD8+ T cells could be relevant to virus control. Then, the proportion of cells expressing one, two, or three functions was studied among groups independently of any particular function. As expected, the proportion of polyfunctional cells was greater in EC than in chronic and PHI subjects (Fig. 5C). In particular, EC had a significantly higher proportion of trifunctional cells (mean, 8.1% ± 7%) and significantly lower proportion of monofunctional cells (mean, 46% ± 19%) than chronic subjects (mean, 2.7% ± 9% [P = 0.013] and 68% ± 27% [P = 0.033], respectively). As mentioned above, bifunctional and trifunctional cells could be measured very early in PHI, together accounting for 25.5% of the HIV-specific CD8+ pool in these subjects. Moreover, the proportion of these cells seemed to slightly increase over time to the 12-month sample (32.3%), although the difference between the baseline and year samples did not differ significantly. Within the PHI group, no significant differences were observed in any particular function or function combination when individuals were segregated into PHI > 350 and PHI < 350 groups. Similarly, no difference was observed between both groups in the proportion of mono-, bi-, or trifunctional cells. Also, no significant correlation was observed for any function, combination, or proportion either with baseline or set point VL or CD4+ T-cell count (data not shown).

It was then hypothesized that the stronger CD8+ T-cell VIA found in PHI subjects in whom Gag dominated the cellular immune response, as well as the slower progression observed within this subgroup, could be related to higher polyfunctionality of Gag-specific cells. Consequently, the proportion of Gag- and Nef-specific CD8+ T cells expressing one, two, or three functions was compared within the PHI group at baseline and 12-month samples (Fig. 5D). No differences were observed for both antigens, indicating that polyfunctionality was not related to specificity. Moreover, both Gag- and Nef-specific responses showed a nonstatistically significant increase in polyfunctionality over time.

Overall, these results suggest that although a higher proportion of polyfunctional CD8+ T cells is associated to EC status and even when these cells could be measured very early in HIV infection, they would not be associated with better or worse resolution of acute phase. Also, the benefits of Gag-specific cells would not be related to polyfunctionality in the terms analyzed here.
FIG 5 (A) Gating strategy used for the identification of degranulating and cytokine-secreting HIV-specific T cells. Illustration data were derived from one representative subject, stimulated with an HIV peptide pool. Initial gating was performed on a plot of forward scatter area (FSC-A) versus height (FSC-H) to remove doublets. Then, gating was performed on small lymphocytes in a plot of forward scatter (FSC) versus side scatter (SSC). Dead cells were then excluded.
A higher proportion of HIV-specific CD8+ T cells able to degranulate and secrete IFN-γ was associated with improved capacity to suppress viral replication during PHI. As mentioned before, out of the multiple CD8+ T-cell effector functions studied by flow cytometry, the combined expression of CD107A/B and IFN-γ was significantly associated with EC status (Fig. 5B). Given this, it was reasoned that the particular combination of these two functions (instead of polyfunctionality as a whole) would be associated with improved resolution of acute infection. Thus, associations among percentages of specific CD107A/B+ IFN-γ+ CD8+ T cells and virological and immunological parameters (all corresponding to baseline samples) were analyzed. It was found that, within PHI subjects, those showing substantial CD8+ T-cell VIA (>25%) had higher numbers of CD107A/B+ IFN-γ+ CD8+ T cells than those having weak (<25%) VIA (P = 0.025). Even more, the percentage of CD107A/B+ IFN-γ+ CD8+ T cells in the PHI VIA > 25% group was comparable to that of EC (Fig. 6A and B). Moreover, a significant correlation between the percentage of specific CD107A/B+ IFN-γ+ CD8+ T cells and the CD8 T-cell VIA was found for PHI subjects (Spearman’s r = 0.6383, P = 0.01; Fig. 6C).

These observations indicate that the capacity to degranulate and express IFN-γ might play a relevant role in the ability of specific cells to mediate VIA (i.e., to suppress viral replication in vitro and the frequency of HIV-specific T cells able to degranulate and secrete IFN-γ upon stimulation. (A) Frequency of HIV-specific CD107A/B+ IFN-γ+ CD8+ T cells in EC, chronic, and PHI subjects (segmented according to CD8+ T-cell VIA magnitude). *, P < 0.05 Mann-Whitney test. (B) Representative IFN-γ+ versus CD107A/B- dot plots (gated on CD3+ CD8+ events) obtained for subjects of each group. (C) Correlation between the frequency of HIV-specific CD107A/B+ IFN-γ+ CD8+ T cells and VIA in PHI subjects at baseline sample. r and P values correspond to Spearman’s test.

FIG 6 Association between the CD8+ T-cell capacity to suppress HIV replication in vitro and the frequency of HIV-specific T cells able to degranulate and secrete IFN-γ upon stimulation. (A) Frequency of HIV-specific CD107A/B+ IFN-γ+ CD8+ T cells in EC, chronic, and PHI subjects (segmented according to CD8+ T-cell VIA magnitude). *, P < 0.05 Mann-Whitney test. (B) Representative IFN-γ+ versus CD107A/B- dot plots (gated on CD3+ CD8+ events) obtained for subjects of each group. (C) Correlation between the frequency of HIV-specific CD107A/B+ IFN-γ+ CD8+ T cells and VIA in PHI subjects at baseline sample. r and P values correspond to Spearman’s test.
vitro), which in turn associated both with the preservation of the CD4+ T-cell subset during acute/early infection and the status of EC. On these bases, the subpopulation of specific CD107A/B+ CD8+ T cells was subjected to a deeper analysis. This analysis revealed that, out of the total CD107A/B+ HIV-specific CD8+ cells, most of them coexpressed IFN-γ and/or IL-2 in the EC cohort, i.e., most degranulating HIV-specific CD8+ T cells from EC were bi- or trifunctional. Of note, all EC had measurable HIV-specific CD107A/B+ IFN-γ+ CD8+ T cells, and around 60% of subjects had measurable HIV-specific CD107A/B+ IL-2+ CD8+ T cells. On the contrary, only a minor portion of chronic and PHI subjects exhibited polyfunctional HIV-specific CD107A/B+ CD8+ T cells. When a similar analysis was performed but considering response specificity, it could be observed that Gag-specific CD107A/B+ CD8+ T cells showed higher levels of polyfunctionality than Nef-specific cells in chronic, even comparable to Gag-specific cells from EC. However, no difference was observed between Nef- and Gag-specific CD8+ T cells in terms of polyfunctionality associated to degranulation capacity in the PHI group, either at baseline or 12-month samples (not shown).

Finally, the relative mean fluorescence intensity (rMFI) of IFN-γ and CD107A/B was studied in specific CD8+ T cells as an indicator of cell function strength (Fig. 7). IFN-γ rMFI analysis (Fig. 7A) produced results that paralleled those obtained after the examination of spot sizes in the ELISPOT assays. CEF-specific cells showed 1.7-fold-higher (1.7×) IFN-γ rMFI than HIV-specific cells in the chronic (P = 0.03) and EC (P > 0.05) groups but not in the PHI group. As for CD107 expression, no difference was observed among groups regarding CEF-specific cells (Fig. 7B). When CD107A/B rMFI was compared intragroup, it was found that Gag-specific cells showed higher CD107A/B rMFI than Nef-specific cells both in chronic (1.7×, P > 0.05) and PHI subjects (1.4×, P = 0.033). Even more, CD107A/B rMFI for Gag-specific cells was comparable to that of CEF-specific cells in all three groups of subjects analyzed. When the same analysis was performed but gated on double-positive CD107A/B+ IFN-γ+ CD8+ T cells, it could be observed that rMFI was higher for each function, as already reported for polyfunctional cells (36), and the same trends shown in Fig. 7 were maintained. Even more, the differences on CD107A/B expression between Nef- versus Gag-specific cells became more pronounced. However, this observation was derived from only a minor subset of patients and a small number of events (especially for chronic and PHI subjects) in order to draw definite statements.

In summary, EC showed a higher number of degranulating HIV-specific CD8+ cells than chronic and PHI subjects. Even more, in these subjects, this activity was more frequently accompanied by the capacity to secrete cytokines (IFN-γ and/or IL-2), while in the chronic and PHI groups, these cells were mostly monofunctional. Within PHI, a direct correlation between the percentage of HIV-specific CD107A/B+ IFN-γ+ CD8+ T cells and the magnitude of CD8+ T-cell VIA was found. Finally, Gag specificity was associated with a stronger ability to degranulate (evidenced by higher CD107 rMFI), which seems consistent with these cells having a stronger capacity to mediate viral inhibition.

**Plasma levels of MIP-1β and IL-2 are associated with Gag immunodominance.** Cytokines play a key role in many infectious diseases, both shaping the immune response mounted against pathogens and contributing to pathogenesis. Several reports have established that plasma cytokine levels measured during acute HIV infection may predict subsequent disease progression (37–39). In this scenario, we sought to analyze the relationship between plasma cytokine levels during acute/early HIV infection and the Gag or Nef immunodominance hierarchy obtained also at PHI baseline samples. This was performed with the aim of determining if there existed an association between both soluble and cellular immune signatures associated with protection from disease progression and following the hypothesis that if two immune parameters are truly determinants of protective immunity, then a direct association between them should be observed. It was found that the percentage of Gag-specific cells out of the total HIV-specific cells (baseline Gag%) significantly correlated in a direct fashion with plasma levels of MIP-1β and IL-2, among PHI subjects at baseline samples (Fig. 8A and B). Although the correlation between plasma MIP-1β and baseline Gag% was not significant after the adjusted analysis, the clear trend indicating a direct association between both parameters is still worth noting. Conversely, the proportion of Nef-specific cells inversely correlated with plasma MIP-1β (Fig. 8C). These findings are of particular importance, since these molecules are produced by CD8+ T cells upon stimulation and the percentage of cells expressing IL-2 and MIP-1β either alone or in combination with other functions were previ-
progression. It is also local, viremic chronics and elite controllers. Epidemiological, characterizations of the T-cell responses in a cohort of acute/early infection in a subset of subjects from a well-characterized cohort of Argentinean seroconverters, in comparison to that found in, also local, viremic chronics and elite controllers. Epidemiological, clinical, immunological, and virological characteristics of the patients enrolled in the cohort are described elsewhere (26). Aiming at delineating CD8+ T-cell features that best associate with disease progression in our population, it was found that (i) there exist early differences regarding the immunodominant viral targets of the specific T-cell response between subjects with different rates of progression to disease (in terms of CD4+ T cell loss) during the first year of infection (Fig. 2 and 3), (ii) polyfunctional HIV-specific CD8+ T cells could be detected during PHI but their frequency was not associated with virus control or protection from progression (Fig. 5), (iii) CD8+ T cells capable of mediating viral inhibitory activity (VIA) in vitro could also be detected during PHI (Fig. 4), and (iv) the improved capacity to mediate VIA during PHI was associated with a higher CD4+ T-cell set point and was related to Gag immunodominance and a higher proportion of HIV-specific CD8+ T cells able to degranulate and secrete IFN-γ (Fig. 4 and 6). The main contribution of this study relies on the correlation between the HIV-specific CD8+ T-cell functional properties during acute/early infection and the clinical outcomes during the first year postinfection. On the other hand, to our knowledge, this is the first report to perform an immunological characterization of the T-cell responses in a cohort of acute/early HIV-infected subjects from South America.

FIG 8 Correlation between anti-Gag and anti-Nef cellular immune response immunodominance hierarchy and plasma cytokine/chemokine levels in PHI subjects at baseline samples. Significant correlations were found between the relative immunodominance of Gag (A and B) and Nef (C) with plasma IL-2 (A) and MIP-1β (B and C) levels. r and P values correspond to Spearman’s test. * P values adjusted by the Benjamini and Hochberg method for false discovery rate (FDR) procedure.

Clear differences in the viral proteins that are targeted by the HIV-specific cellular response have been described between the acute/early and chronic phases of infection: while Nef-specific cells dominate the early antiviral response (42), this response afterward broadens toward epitopes contained within other viral proteins, such as Gag, Env, and Vpr (11–15). Many of these studies have also established that Gag-specific CD8+ T-cell responses are associated with low viremia in chronic infection. Other reports indicate that Gag is the immunodominant target in elite controllers (43), both in blood and mucosal tissues (44), providing further support to the important role of Gag-specific cells in restricting viral replication. Possible mechanisms to explain this better ability to control viral replication include ability to kill very recently infected cells (even before viral genome integration to host genome) (45, 46), Gag fitness constraints to escape immune pressure (47, 48), and higher capacity to mediate antiviral activity, which will be discussed in the following paragraphs. In line with these findings, we found that Gag-specific cells (particularly p24-specific cells) dominated the HIV-specific response in EC, both in terms of magnitude and hierarchy (Fig. 2A and B). Also, Gag immunodominance was observed in a subgroup of chronically infected subjects referred to as “viremic controllers.” On the contrary, Nef-specific cells dominated the response in PHI subjects. However, when this group was split into PHI > 350 and PHI < 350 groups, it was observed that in the group with more rapid
progression (PHI < 350), Nef clearly dominated the anti-HIV response (Fig. 2A to C). More importantly, significant correlations between baseline Gag immunodominance and both baseline and set point CD4+ T-cell counts were observed in the PHI group (Fig. 3). In line with this result, other authors described a direct correlation between the magnitude of the anti-Nef CD8+ T-cell response and viral load in a cohort of HIV subtype C acutely infected subjects (49). Overall, these results argue in favor of that an earlier and higher contribution of Gag-specific cells to the hierarchy of the total anti-HIV response at very early time points postinfection contributes to a slower disease progression.

The ability of CD8+ T cells to kill virus-infected cells may rely in their specificity, phenotype, and/or functionality and could even be mediated by distinct mechanisms (mediated by soluble factors or cell-to-cell contact). The evaluation of the ex vivo virus inhibitory activity (VIA) results in an overall measurement of the total CD8+ T-cell antiviral potency. Previous reports have used this assay to evaluate VIA mediated by CD8+ T cells obtained from elite controllers, treated and untreated chronically infected individuals (16, 19, 20, 34, 35), as well as human and simian vaccinees (20, 50). In these studies, it was demonstrated that VIA correlates with the magnitude of the Gag-specific CD8+ T-cell response (16, 35), that the expression of “protective” HLA-I alleles is not a required condition (20, 35), and that it is associated with higher frequencies of degranulating (as measured by CD107A/B expression) specific CD8+ T cells, usually accompanied by other functions, such as secretion of IFN-γ and MIP-1β (20, 35, 50). In agreement with this background, we found that CD8+ T cells from EC mediated stronger and broader VIA than those cells from untreated chronic subjects. Additionally, we also demonstrate in this work that VIA can be measured very early after infection in most acutely infected subjects (although to a lower magnitude than EC) and that this activity persists over time (Fig. 4). To our knowledge, there exists only one very recent report studying VIA in acute HIV infection (41). In that work, Freel and collaborators also demonstrate (i) that VIA magnitude correlates with the percentage of HIV-specific CD8+ T cells expressing CD107A, MIP-1β, and IFN-γ and with the secretion of MIP-1α, MIP-1β, IFN-γ, IL-10, and IL-1α after cell stimulation with peptide antigens and (ii) that early escape from CD8+ T-cell-mediated VIA occurs during PHI. In our work, further insights are provided into the role of CD8+ T-cell-mediated VIA during PHI. Aiming at delineating the specificity and functionality of VIA-mediating cells during PHI, it could be established that CD8+ cells from PHI individuals in whom Gag-specific cells dominated the early HIV-specific T-cell response had stronger capacity to mediate VIA than Nef responders, and a statistically significant direct correlation was found within the PHI group between VIA and the magnitude of Gag-specific cellular immune response (Fig. 4). This is in line with results obtained for EC and chronic subjects with broad anti-Gag responses (16, 35) and could provide an additional mechanistic explanation for the pivotal role of Gag-specific cells in delayed disease progression. Although Freel et al. (41) found that Nef-specific cells could mediate VIA to the same extent as Gag-specific cells in acutely infected subjects, no association between these activities and clinical parameters was studied by these authors, as it is the case of the present work. Also, it is important to denote that both studies differ in definition of the specificity of VIA-mediating cells, which may account for the different results obtained. Regarding the functionality of VIA-mediating cells, we found that, within PHI subjects, those showing substantial CD8+ T-cell VIA (≥25%) had a higher frequency of CD107A/B+ IFN-γ+ CD8+ T cells (even comparable to EC) than those having weaker VIA. Moreover, a significant correlation between the percentage of specific CD107A/B+ IFN-γ+ CD8+ T cells and the CD8+ T-cell VIA was found for PHI subjects (Fig. 6). In regard to the expression of CD107A/B, this result is in line with previous data obtained for EC, chronic, acute/early infected subjects and simian vaccinees (35, 41, 50). However, in our study, the simultaneous coexpression of both markers (CD107A/B and IFN-γ) is necessary to maintain the association. Other groups reported that MIP-1β secretion is also associated with VIA both in chronic and acute infection (20, 41). Because of technical constrains, we could not include the evaluation of this cytokine in our multicolor flow cytometry assay. However, it is presumable that a similar association would have been found due to the high contribution of MIP-1β to the total response during acute infection and its high rate of coexpression with CD107A/B and IFN-γ (6, 25). What we did observe was a nonsignificant yet border correlation between plasma MIP-1B (see further discussion below) and the proportion of bi-functional degranulating cells, i.e., the proportion of CD107+ IFN-γ+ plus CD107+ IL-2+ HIV-specific CD8+ T cells out of the total CD107+ HIV-specific CD8+ T cells (Spearman’s r = 0.4368, P = 0.0699; data not shown), suggesting an association between VIA, degranulation, and expression of MIP-1B. Finally, as it has been documented for chronic infection (35), no association was found between VIA magnitude and viral load. However, we could observe an association between VIA and the CD4+ T-cell set point (Fig. 4D). This result, together with the higher VIA observed in EC (in our and other studies mentioned above) and an observation made in vaccinated rhesus monkeys where strong VIA was related to enhanced virus control in breakthrough infections (50), clearly indicates that CD8+ T-cell-mediated VIA is a desirable feature to be elicited by prophylactic or therapeutic interventions aimed at delaying disease progression.

Early reports comparing the capacity of CD8+ T cells to degranulate and secrete multiple soluble mediators upon stimulation, between individuals with progressive versus long-term non-progressive HIV infection, have suggested that CD8+ T-cell polyfunctionality would be a functional correlate of virus control (17, 18). However, these studies also raised the question of whether polyfunctionality was not the cause but the consequence of virus control. Results presented here argued in favor of the latter hypothesis: in our PHI cohort, polyfunctional HIV-specific CD8+ T cells could be measured very early during infection (in consonance with our previous observation [25]), and the proportion of these cells seemed to slightly increase over time to the 12-month sample (Fig. 5), as also reported by Ferrari et al. (6). Although significant differences in the proportion of polyfunctional cells were observed in EC versus viremic chronic subjects (as expected, based on previous reports [17, 51]), no significant differences were observed when PHI individuals were segregated as rapid (PHI < 350) or regular (PHI > 350) progressors. Moreover, polyfunctional profiles were similar in PHI individuals with different immunodominant targets, indicating that polyfunctionality was not related to specificity (Fig. 5). These findings shed more light into the notion that, instead of being a marker of antiviral function, T-cell polyfunctionality would be directly affected by viral persistence. In other words, lack of T-cell polyfunctionality would be the consequence of constant antigen stimulation dur-
ing viremic chronic infection, which ultimately may lead to cell exhaustion and functional impairment, as postulated previously (6, 21). In this sense, CD8$^+$ T-cell phenotypic characterization in terms of exhaustion and memory markers in our cohort is being currently performed. On the other hand, the demonstration that, out of the many CD8$^+$ T-cell function combinations studied, only the frequency of specific bifunctional CD107A/B$^+$ IFN-γ$^+$ CD8$^+$ T cells correlated with the magnitude of CD8$^+$ T-cell VIA (Fig. 6) would indicate that not all the functions exerted by a polyfunctional cell would be equally relevant in mediating antiviral T-cell effectors.

T-cell functional outcomes upon antigen engagement depend on the strength of the stimulus, which can be directly affected by the level of antigen sensitivity or cell avidity (52, 53). These parameters, in turn, can be indirectly inferred by computing the mean spot size in the IFN-γ ELISPOT assay (33) or the rMFI of a given function evaluated by flow cytometry (52). Here, we found that the analysis of IFN-γ mean spot size and IFN-γ rMFI gave comparable results, together indicating that HIV-specific cells showed less quality in all groups analyzed compared to CEF-specific cells (in EC the difference was not significant) (Fig. 2F and 7). CEF-specific cells were analyzed for comparison since they represent cells that target pathogens that were either cleared (influenza) or controlled (CMV and Epstein-Bar viruses), thus their analysis could provide putative signatures associated with virus control. This result suggests again that constant HIV antigen stimulation may be driving cell exhaustion. Moreover, and regardless of the specificity, cells from PHI subjects showed the lowest quality among all the groups analyzed, suggesting that, during acute/early HIV infection, there exists an overall deterioration of the CD8$^+$ T-cell compartment. In regard to CD107A/B mobilization, it was found that rMFI for Gag-specific cells was higher than for Nef-specific cells and comparable to that of CEF-specific cells in all three groups of subjects analyzed (Fig. 7). A relation among the magnitude of CD107A/B mobilization (evaluated by flow cytometry as in this work), antigen sensitivity, and antiviral activity has previously been established in another setting (52), supporting our association between the stronger ability of Gag-specific cells to degranulate and their stronger capacity to mediate viral inhibition.

A large amount of data indicate that the expression of certain HLA-I molecules is related to HIV control. Part of the evidence came from cohort studies where some alleles were overrepresented in EC and long-term nonprogressors (7, 8), while other studies provided insights into functional performance of the “protective alleles” regarding specificity, proliferation, polyfunctionality, antigen presentation, immune pressure, among other factors (8, 54–61). However, it has also been demonstrated that these protective alleles perform qualitatively different in controllers compared to progressors (60), indicating that a combination of T-cell function and phenotypes should coexist in order to achieve virus control. Conversely, some individuals reach EC status in the absence of protective alleles (as it can be observed in our cohort), indicating that they are not a requirement for virus control. For instance, CD8$^+$ T-cell VIA can be observed both in the presence and absence of protective HLA-A1 alleles (20, 35). Although in this work a simultaneous evaluation of multiple CD8$^+$ T-cell functions (in different terms such as magnitude, breadth, immunodominant targets, polyfunctionality, and VIA) was performed in HLA-typed individuals with known disease status, both the cohort size and the heterogeneity of alleles and responses preclude us from unequivocally establishing the relationship between CD8$^+$ T-cell function and phenotype and disease outcome. Further studies aimed at developing a model involving these three parameters will be needed.

The cytokine network elicited after HIV/simian immunodeficiency virus (SIV) infection has been studied in order to shed light on the early pathogenic events of the infection and to understand how these events trigger the future course of the disease. Moreover, the level of different plasma cytokines and/or chemokines during acute infection has been proposed as a potential biomarker to predict the rate of disease progression (37–39, 62). However, to our knowledge, no report has established so far the relationship between plasma molecules and the HIV-specific cellular immune response. Here, a correlation between the relative immunodominance of the anti-Gag response and the levels of IL-2 and MIP-1β was found (Fig. 8). As mentioned before, these molecules are produced by CD8$^+$ T cells upon stimulation, and the percentage of cells expressing IL-2 and MIP-1β either alone or in combination with other functions were previously associated with virus control (4, 11, 14, 43, 49). IL-2 is an important molecule for T-cell homeostasis and immune system activation (63). Although different levels of plasma IL-2 during PHI infection have not been previously associated with HIV/AIDS disease outcome, decreased levels of other molecules also involved in T-cell homeostasis and T-cell phenotype determination, such as IL-15 and IL-12, respectively, have been associated with progressive infection in a cohort of chronically infected African-American women (38). On the other hand, it was determined that MIP-1β-producing CD8$^+$ T cells represent an elevated proportion within the HIV-specific CD8$^+$ T-cell response during acute infection and that they exert considerable immune pressure over viral replication (6). At the functional level, it was suggested that the production of MIP-1β is rapidly elicited upon stimulation on the specific cells (52), which may account for an enhanced activity of the MIP-1β-producing CD8$^+$ T cells over cells exerting other functions. Moreover, other authors have found an association between MIP-1β plasma levels and lower VL and higher CD4$^+$ T-cell counts during chronic infection (38). Taken together, all this evidence provides a rationale for the association between the protective role of immunodominant Gag-specific cellular response and plasma levels of IL-2 and MIP-1β. As already mentioned, and although further investigations will be needed to determine the nature of this relationship, these results are in line with the evidence indicating that early relative Gag immunodominance is somehow related to the generation of an effective antiviral immune response leading to slower disease progression.

In summary, multiple aspects of the HIV-specific CD8$^+$ T-cell subset (specificity, ex vivo viral inhibitory capacity, and polyfunctionality) arising early after infection were characterized in a cohort of acute/early infected subjects, in comparison with that found in viremic chronics and elite controllers. Importantly, the association of these functions with disease progression was studied. In the first place, it was found that specificity of cells arising early after the transmission event is critical for immune control: early relative immunodominance of Gag-specific cells was associated with delayed disease progression, in terms of CD4$^+$ T-cell count preservation, during the first year postinfection, in concordance with Gag immunodominance in EC and viremic controllers. Further insights into the functionality of CD8$^+$ T cells from
enrolled subjects allowed us to establish that the advantage of Gag-specific CD8+ T cells would rely on their ability to mediate both lysis of infected cells (evidenced by higher capacity to degranulate and to mediate viral inhibition activity in vitro) and soluble inhibition of viral replication by the simultaneous secretion of IFN-γ. Also, higher relative contribution of anti-Gag-specific cells to the total anti-HIV cellular immune response correlated with plasma levels of IL-2 and MIP-1β, molecules that further contribute to viral inhibition. The identification of phenotypic and functional properties of the CD8+ T-cell subsets associated with viral control is urgently needed to aid in the design and performance evaluation of an effective HIV vaccine. In this scenario, data presented in the manuscript underscore the importance of considering both cell specificity and quality, in terms of both cytotoxic and noncytotoxic mechanisms, to boost preventive and therapeutic anti-HIV immune-based strategies.

ACKNOWLEDGMENTS

This research was funded by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT; grant numbers 2008/0549 and 2011/1658) to M.M.G. and a grant from Universidad de Buenos Aires (UBACYT 2011-2014 2002010002155) to G.T.

We thank Maria Florencia Quiroga for helpful discussions during experiment design and results interpretation and Sergio Mazzini for assistance during manuscript preparation. We are indebted to all the physicans belonging to the Grupo Argentino de Seroconversion Study Group and their patients for their central contributions to this work.

We have no conflict of interests to declare.

Grupo Argentino de Seroconversion Study Group: Lorena Abusamra, Marcela Acosta, Carolina Acupiú, Viviana Alonso, Liliana Amante, Graziela Ben, M. Belén Bouzas, Ariel Braverman, Mercedes Cabrini, Pedro Cahn, Osvaldo Cando, Cecilia Cánepa, Daniel Cangelosi, Juan Castelli, Carolina Cesar, Gustavo Echenique, María I. Figueroa, Valeria Fink, Claudia Galasso, Palmira Garda, Manuel Gómez Carrillo, Ana Gun, Alejandro Kroelewicie, Natalia Lafruy, María E. Lázaro, Alberto Leoni, Eliana Loiza, Patricia Maldonado, Horacio Mingrone, Marcela Ortiz, Patricia Patterson, Héctor Pérez, Norma Porteiro, Daniel Pryluka, Carlos Remondegui, Raul Román, Horacio Salomón, M Eugenia Socías, Omar Sued, J. Gonzalo Tomás, Gabriela Turk, Javier Yave, Carlos Zala, Inés Zapiola.

REFERENCES


