Conserved HIV-1 Gag p24 Epitopes Elicit Cellular Immune Responses That Impact Disease Outcome

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Abstract

Although the breadth of the human immunodeficiency virus type 1 (HIV-1)-specific cellular immune response and its impact on the control of viral replication have already been addressed, reported data have proven controversial. We hypothesize that the nature of targeted epitopes, rather than the simple breadth or magnitude of responses, correlates with disease outcome. In this study, we explore the occurrence of patterns of Gag p24 recognition among untreated HIV-1-infected patients by identifying the epitopes that compose such patterns and how they distinctly associate with disease progression. Utilizing enzyme-linked immunospot (ELISPOT) interferon gamma (IFN- γ), we screened cellular responses of 27 HIV-1-infected subjects against 15-mer peptides encompassing the whole Gag p24 protein. Obtained data were used to develop a clustering analysis that allowed definition of two groups of individuals with totally distinct patterns of recognition. Although targeted Gag p24 peptides were completely different between the two groups, the breadth and magnitude of the responses were not. Interestingly, viral control and preservation of CD4⁺ T cells were increased in one group. In addition, we compared genetic conservation of amino acid sequences of the recognized peptides, as well as of the human leucocyte antigen class I (HLA-I)-restricted epitopes within them. Subjects presenting higher control of HIV-1 replication targeted more conserved epitopes, and higher genetic variation was present mainly in anchor residues for HLA-I molecules. We strengthen the existing evidence from cases of HIV-1 infection in humans that, cellular immune responses targeting conserved epitopes, rather than the magnitude and breadth of responses, associate with a better control of viral replication and maintenance of peripheral CD4⁺ T cell counts.

Keywords: antigen processing and presentation, Gag, immune response, T cells

Introduction

THE IDEA THAT BROAD T CELL responses to certain human immunodeficiency virus type 1 (HIV-1) proteins impact the control of viral replication has been proposed before.^{1–3} The specificity of the cellular response to HIV-1 is thought to be relevant to both control of infection and maintenance of CD4⁺ T cell populations. This relationship is supported by published data that found a correlation between these parameters and the immune response to viral Gag and Env proteins.^{1,4–8} While the breadth of vaccine-elicited immune responses is likely to be essential for protection,⁹ a low quality of CD8⁺ T cell response may not be adequate.¹⁰ The actual impact of the breadth of virus-specific responses is not fully understood. Some studies have shown no correlation between vaccine efficacy or disease outcome and broad cellular response to Gag and other HIV-1 proteins.^{10–15}

Although the correlation between magnitude and breadth of HIV-1-specific responses and disease outcome is still a controversial topic,^{4,5,11,15–18} it has been recently shown that the presence of CD8⁺ T cells of certain peptide specificities positively correlates with CD4⁺ T cell count and negatively with viral load, while cells of other peptide specificities may not present the same correlation.^{18–21} Also, a vaccine designed to elicit effective cellular responses is expected to act against conserved epitopes of HIV-1.^{20,22} However, empirical evidence of a beneficial effect of targeting conserved epitopes on disease outcome is scarce, being mostly extrapolated conclusions from comparisons of conservation between targets for protective versus nonprotective human leucocyte antigen (HLA) molecules.^{11,13}

To address this, we assessed sequence conservation of regions of HIV-1 Gag p24 targeted by cellular responses in a group of HIV-1-infected individuals and explored the

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association with individuals' clinical progression. Gag protein epitopes are highly expressed during infection and are able to induce host cytotoxic T lymphocytes with the ability to recognize and kill infected cells at early postinfection time points.¹ Gag-specific cells are strongly associated with slow disease progression, compared to Nef², and are dominant among responses in elite controllers.³ In addition, the Gag protein is highly conserved among viral isolates, leading to severe fitness reductions in escape variants.^{7,8} Nevertheless, previous vaccination attempts that focused on inducing Gag-specific CD8⁺ T cells failed,⁴ suggesting that a careful selection of conserved elements able to elicit effective cellular responses is a logical step.^{5,6}

In this study, by screening Gag p24-specific cellular responses in HIV-1-infected patients, we demonstrate that despite 17 out of 27 patients having Gag p24-specific interferon gamma (IFN- γ) responses, the presence of these responses did not predict control of viral load or maintenance of CD4⁺ T cell counts. To address this, we performed a novel clustering approach to interpret enzyme-linked immunospot (ELISPOT)-IFN- γ results and determined two different patterns of cellular immune responses against the Gag p24 protein among the patients. Importantly, not only were the Gag p24 peptides to which patients responded different between the groups but CD4⁺ T cell counts and plasma viral load also differed. Our analyses revealed that responses against more conserved peptides were predominantly found in the group that presented attenuated disease outcome.

Although HIV and SIV vaccinologists have long agreed that immune responses against conserved epitopes are important for viral control and preservation of CD4⁺ T cells counts, our study complements the evidence with cases of HIV-1-infected individuals. Our results suggest that the effect of specific cellular immune responses on disease outcome may be more linked to epitope characteristics, such as genetic conservation, than to characteristics of the response, such as its breadth, magnitude, and HLA-molecule mediation.

Material and Methods

Study cohort

Blood samples were collected from 27 recently HIV-1-infected patients. Following the Serologic Testing Algorithm for Recent HIV Seroconversion (STAHRS), patients were considered to be recently HIV-1-infected when they had at least one positive HIV-1 enzyme-linked immunosorbent assay (ELISA) test confirmed with a Western Blot assay, and a negative result using the less sensitive HIV-1 ELISA test Vironostika HIV-1 micro-ELISA system (BioMérieux, Durham, NC). Sample collections were done between 2004 and 2009 and all subjects were naive for treatment with antiretrovirals at the time of collection of blood for laboratory assays. We obtained informed written consent from all the patients and the study was approved by the Ethics Committees of the Federal University of Sao Paulo, University of Sao Paulo, and by the *Comissão Nacional de Ética em Pesquisa* (CONEP).

HLA class I typing

Subjects were typed with intermediate resolution for major histocompatibility complex (MHC) class I antigen expres-

sion using sequence-specific primer polymerase chain reaction kits (Pel-Freez SSP UniTray; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

IFN- γ ELISPOT assay

Fifteen-mer peptides overlapped by 11 amino acids corresponding to HIV-1 consensus B Gag p24 protein (NIH AIDS Research and Reagent Program, Rockville, MD) were used at a final concentration of 10 μ g/ml. Ninety-six-well flat-bottomed nitrocellulose plates (Multiscreen; Millipore, Bedford, MA) were coated with 0.5 μ g of anti-IFN- γ monoclonal antibody (mAb; Mabtech, Nacka, Sweden) for a 1-h incubation at 4°C. Plates were washed with 1 $\times$ phosphate-buffered saline (PBS) and then 1.0 $\times$ 10⁵ peripheral blood mononuclear cell (PBMC) suspended in RPMI 1640 supplemented with penicillin, streptomycin, and 10% fetal bovine serum (R10) were added to the wells. The R10 also contained each Gag p24 peptide, Concanavalin-A (positive control), or 2.0 μ l of 100% dimethyl sulfoxide (DMSO) with no peptide (negative control). Plates were incubated for 16 h at 37°C in 5% CO₂, after which the cells were discarded. After washing the plates with PBST (1 $\times$ PBS containing 0.05% Tween 20), 0.05 μ g of biotinylated anti-IFN- γ mAb (Mabtech) was added and the plates were incubated for further 2 h at room temperature. Following additional washes with PBST, 0.07 μ g per well of alkaline phosphatase (Vector Laboratories, Inc., Burlingame, CA) in 50 μ l of PBS was added for 1 h at room temperature. Following additional washes with PBST, 50 μ l of BCIP/NBT (5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium) substrate solution (Sigma-Aldrich, St Louis, MO) per well was added and the plates were developed for $\sim$ 30 min. Spots were counted using AID reader (AID), which reported HIV-1-specific responses as the number of spot-forming units (SFU)/1.0 $\times$ 10⁶ cells after subtraction of the background IFN- γ secretion. A response was considered positive when the number of SFU exceeded 50 SFU/1 $\times$ 10⁶ cells and was at least four times the level of the wells with no peptide.

Clustering analysis

To perform a clustering analysis of our ELISPOT-IFN- γ data, we considered them in a binary manner, where "0" meant the absence of a positive response and "1" the presence of a response to a given peptide. This dataset was used to accomplish pairwise comparison according to the Link's coefficient of diversity (CD) following the equation below:

$$CD_{xy} = (N_x + N_y) / (N_x + N_y + N_{xy})$$

where N_x is the number of positive responses against a given peptide x and not against another given peptide y , N_y is the number of positive responses against peptide y and not against peptide x , and N_{xy} is the number of positive responses against peptides x and y . A CD between two patients may vary from 0% to 100%, as the paired responses ranged from completely different Gag p24 peptides to an exactly equal pattern. The coefficients of diversity of responses presented by each patient were then compared for all possible pairs of patients to generate a matrix of diversity. Those data were then used as an input for a clustering analysis made by the

method UPGMA (Unweighted Pair Group Method with Arithmetic Mean), which employs a sequential clustering algorithm able to identify local topological relationships in order of similarity and build a dendrogram tree in a stepwise manner. Thus, the tree topology clusters the patients based on the similarity of the patterns of Gag p24-directed responses. The software Treason for Windows was used for making all the cited calculations and also inferring the tree topology. Likewise, we used the same approach to cluster the peptides in clades based on the sharing of patients responding to them. As a result, a bidimensional clustered view of the data was generated.

Amino acid sequence alignments and identification of HLA-I-restricted epitopes

We used QuickAlign, an online tool from the Los Alamos HIV Molecular Database, to produce alignments of the sequences of the Gag p24 peptides with the 968 HIV-1 subtype B Gag amino acid sequences available in this database. We also aligned sequences of HLA-I-restricted epitopes within the 15-mer peptides with the same 968 Gag sequences. Number of different variant sequences, that is, homolog sequences for the same region, but differing in one or more amino acids, was recorded.

We used ELF (Epitope Location Finder), an online tool from the Los Alamos HIV Molecular Database, to inspect the sequences of recognized Gag p24 15-mer peptides for occurrence of HLA-I-restricted epitopes. The ELF bioinformatics tool finds known and predicted epitopes in a given

amino acid sequence by (1) comparing the input sequence with the HIV CTL (cytotoxic T lymphocyte) and Helper Epitope databases and (2) searching the sequence for anchor motifs of an informed HLA type. Sequences of peptides recognized by a given patient were screened against the HLA-I loci A and B alleles of the same patient. By aligning sequences of the identified HLA-I-restricted epitopes with the 968 reference HIV-1 subtype B sequences with QuickAlign, we identified the position of mutated amino acids in the variant sequences. Number of different variant sequences with mutations in C-terminal and internal HLA anchor residues and number of different variant sequences with mutations in other amino acid residues were recorded.

Results

Association of breadth and magnitude of responses against Gag p24 peptides and CD4⁺ T cell counts and RNA-HIV load

We have screened cellular immune responses by ELISPOT-IFN- γ assays in 27 chronic phase HIV-1-infected individuals (Table 1) enrolled in our cohort of early HIV-1 infection.²³ Patients were selected based on their HLA class I loci A and B alleles to obtain a heterogeneous group for epitope recognition. Seventeen out of the 27 individuals (67%) presented responses against the Gag p24 peptides (1–8 positive peptides per patient). The magnitude of the responses also widely varied, ranging from 80 to 1,390 SFU/10⁶ cells. Interestingly, we did not detect a correlation between either the breadth or magnitude of the immune

TABLE 1. HLA-I ALLELES AND CLINICAL CHARACTERISTICS OF THE STUDIED PATIENTS

ID	Gender	Age (years)	HLA-I alleles				Follow-up (days)	Plasma viral load (copies/ml)	CD4 ⁺ T cell count (cells/μl)
			locus A		locus B				
1018	M	23	A*23	A*24	B*35	B*58	2,636	36,064	864
1019	M	27	A*02	A*03	B*39	B*35	2,622	27,098	642
1021	M	29	A*24	A*68	B*35	B*58	809	141,755	338
1025	M	25	A*11	A*74	B*35	B*40	2,491	16,096	740
1026	M	35	A*02	A*02	B*15	B*51	2,491	18,004	563
1030	F	28	A*02	A*30	B*15	B*51	2,134	20,579	360
1032	M	30	A*02	A*66	B*35	B*58	2,505	17,192	466
1035	M	42	A*01	A*03	B*08	B*40	688	18,021	381
1048	M	34	A*02	A*32	B*14	B*44	2,193	2,630	737
1054	M	42	A*01	A*68	B*08	B*51	658	97,843	378
1056	M	23	A*01	A*66	B*08	B*39	2,359	3,801	653
1057	M	31	A*02	A*29	B*07	B*49	1,540	81,118	581
1064	M	34	A*02	A*32	B*49	B*58	699	125,763	543
1079	M	24	A*30	A*33	B*14	B*14	1,505	10,180	677
1104	M	32	A*01	A*02	B*49	B*57	1,603	64,803	388
1112	M	36	A*11	A*23	B*15	B*35	735	58,363	528
1124	M	25	A*26	A*30	B*13	B*39	1,358	10,130	539
1154	M	35	A*03	A*34	B*15	B*44	961	147,251	395
1165	M	27	A*26	A*33	B*07	B*14	933	406	417
2004	M	36	A*23	A*23	B*45	B*49	930	39,125	495
2011	M	34	A*01	A*24	B*18	B*49	433	46,384	350
2013	M	28	A*01	A*33	B*14	B*37	2,486	12,218	478
2019	F	31	A*02	A*33	B*51	B*58	968	9,854	441
2027	M	35	A*23	A*24	B*08	B*51	255	12,533	146
2032	M	27	A*01	A*24	B*08	B*35	659	13,539	500
2039	M	42	A*32	A*33	B*45	B*49	520	15,041	321
2041	M	24	A*02	A*02	B*35	B*50	1,539	28,236	444

response and the predictive marker of disease progression, peripheral CD4⁺ T cell counts. For plasma viral load, breadth of the immune response was also not correlated, but the magnitude of such responses was marginally negatively correlated ($r = -0.39$, $p = .04$; Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/aid).

Clustering analyses reveal the presence of distinct patterns of responses targeting different Gag p24 peptides

As the breadth and magnitude of the Gag p24-specific cellular responses did not seem to play a role in disease outcome when we studied our 27 patients as a single homogenous group, we attempted to investigate the existence of possible patterns of Gag p24 peptide recognition among the screened subjects. We analyzed our ELISPOT-IFN- γ data in a binary manner, where “0” meant absence of positive response and “1” meant presence of response to a given peptide. The resultant data set was used to perform pairwise comparisons of (1) subjects in terms of the peptides they responded to and (2) peptides in terms of the patients that

recognized them. Then, Link’s CD for each possible pair of individuals and each possible pair of peptides generate a matrix of diversity. A CD between two patients may vary from 0% to 100%, as their responses range from completely different Gag p24 peptides to an exactly equal pattern of peptides recognized and peptides not recognized. Data were then used as input for clustering analysis using UPGMA, with inference of a dendrogram tree whose topology clustered patients based on the similarity of Gag p24-specific responses. Likewise, we used the same approach to cluster Gag p24 peptides based on patients responding to them. As a result, a bidimensional clustered view of the data was generated (Fig. 1).

According to the dendrogram topology, we clearly identified two groups of patients (called hereafter Groups A and B) as they showed responses against two different sets of peptides. One additional group of patients did not present responses to any Gag p24 peptide (Group C). Nine individuals were clustered in Group A, 8 in Group B, and 10 in Group C (Fig. 1). Three patients (1025, 1104, and 2004) showed responses shared by Groups A and B. As our goal was to discover groups of HIV-1-infected individuals bearing immune responses targeted to distinct peptides, we removed

FIG. 1. Clustering analysis of the p24-specific cellular immune responses presented by 27 HIV-1-infected patients screened with ELISPOT-IFN- γ assays. Dendrogram clustering trees were built by the UPGMA method. *Upper left tree* clusters 27 HIV-1-infected patients based on the reactivity to p24 peptides presented in the ELISPOT-IFN- γ assays. *Lower tree* shows the p24 15-mer peptides clustered based on the responses. Having the patients’ tree as axis y and peptides’ tree as axis x, a bidimensional view of three distinct patterns of virus-specific immune responses among those 27 patients is seen and three groups (A, B, and C) were defined by the UPGMA method. ELISPOT, enzyme-linked immunospot; IFN- γ , interferon gamma; UPGMA, Unweighted Pair Group Method with Arithmetic Mean.

these three patients from the data set to define three groups of individuals with totally distinct patterns of Gag p24 recognition.

Groups of patients defined by the specificity of responses to Gag p24 present different disease outcomes

Although the sets of recognized Gag p24 peptides were different between the two groups with positive Gag p24-specific responses (Groups A and B), neither breadth (A: 3.2 ± 2.0 vs. B: 2.3 ± 1.2 positive peptides; $p = .15$) nor magnitude (A: 443 ± 353 vs. B: 222 ± 183 SFU/ 10^6 cells; $p = .23$) of their immune responses was different. In addition, we compared demographic aspects, frequency of protective HLA-I alleles and CCR5 $\Delta 32$ polymorphism, and coinfections with GB virus-C, HSV-2, and HTLV between the groups and found no difference for any of the investigated variables (Supplementary Table S1). Consequently, we became especially interested in comparing disease outcomes between the groups.

Interestingly, Group A presented higher CD4⁺ T cell counts than Group B (A: 615 ± 213 vs. B: 355 ± 328 cells/ml; $p < .05$) and even higher than the CD4 counts found in Group C ($p < .01$). Of note, CD4⁺ T cell counts of individuals in Group B were no different than those of Group C's patients, even though Group B had HIV-specific responses and Group C did not. Nadir CD4⁺ T cell values presented by the individuals during their clinical follow-up since early infection were also higher in Group A (A: 390 ± 109 vs. B: 229 ± 186 , $p = .03$; A vs. C: 256 ± 95 cells/ml, $p = .01$) and were not different between Groups B and C (Fig. 2A).

Plasma HIV-1 RNA load was also compared among the groups. Similarly, compared to Group B, Group A's individuals showed lower values for viral load at the assay visit (A: $14,162 \pm 10,141$ vs. B: $23,283 \pm 7,829$ copies/ml; $p < .05$) and for the peak viral load (A: $49,089 \pm 42,675$ vs. B: $171,116 \pm 137,697$ copies/ml; $p = .01$). Lower HIV RNA copies/ml values were found in Group A compared to Group C, and Group B did not differ from Group C (Fig. 2B).

Although we find a weak correlation between the magnitude of the immune responses and viral load for the whole group of 27 patients (Supplementary Fig. S1), when we made the same comparisons within each group, we observed a strong correlation ($r = -0.73$; $p = .01$) between the magnitude

of Gag p24 responses and viral load for the Group A's patients. The same finding was not seen among the individuals in Group B (Fig. 2C). We also investigated whether patients in the three groups progressed differently to the point of needing to start antiretroviral therapy (ART). In Group A, there were fewer patients who had started ART than in Group B (11% vs. 60%; $p = .04$; Fig. 2D). By Kaplan–Meier survival analysis, time to starting ART in Group A was clearly longer than in Group B and Group C ($p = .03$; Fig. 2E).

Gag p24 peptides targeted by patients with better disease outcome present higher genetic conservation

The relevance of the specificity of the anti-HIV-1 cellular responses to viral control and CD4⁺ T cell count maintenance has been already suggested and exemplified by different correlations found between these clinical parameters and immune responses directed against different viral proteins, notably Gag versus Env.^{1,4–8} Also, it has been recently addressed that different responses targeting different epitopes within a single HIV-1 protein are distinctly associated with clinical progression.^{18,20,21} Following this observation, we investigated aspects of the nature of the Gag p24 peptides to which responses in the two groups of patients were distinctly directed. Specifically, we characterized amino acid conservation of the 15-mer peptides' sequences and of the sequences of HLA-I-restricted epitopes within them. Using the ELF tool (Los Alamos HIV molecular database), we searched the amino acid sequences of the Gag p24 peptides to which Group A's and Group B's patients responded for the occurrence of epitopes predicted to bind the patients' HLA-I loci A and B molecules (found epitopes are listed in the Supplementary Table S2). We then used the QuickAlign tool (Los Alamos HIV molecular database) to align the sequences of Gag p24 peptides and their epitopes with 968 HIV-1 subtype B Gag amino acid sequences (available in Los Alamos HIV molecular database).

Two variables were employed to infer about "conservation of the peptide and epitope sequences." First is the number of sequence variants described for a given Gag peptide or epitope region, among 968 Los Alamos Gag B sequences. Second variable is the percentage of described sequences (among those 968, again) that correspond to the variant with no mutations. Amino acid sequences of the peptides recognized by individuals in Group A, and also the HLA-I epitope

FIG. 2. Predictive markers of disease outcome among the groups A, B, and C of patients defined by the specificity of cellular immune responses presented against the p24 peptides. Peripheral blood CD4⁺ T cell counts were used as a predictive marker for disease outcome and the values for analyses were established as the one presented by the time of the blood draw used for the ELISPOT assays, the mean value of all counts during the patients' follow-up periods, and the lowest count (nadir) presented by the patients. Data from all the 27 patients together (gray bars) are shown and also data from patients clustered in the 3 groups (Group A in green, Group B in red, and Group C in black bars) defined by the p24-specific responses (A). Data from all individuals together and also separately from the three clustered groups are shown for HIV RNA copies/ml by the time of the blood draw used for the ELISPOT assays, the mean value for all quantifications during the patients' follow-up periods, and the highest viral load presented by the patients (B). Spearman's rank-order correlations between breadth and magnitude of responses with the average of plasma viral loads during patients' follow-up periods are plotted for all patients together (gray points), and separately for patients from Group A (green) and Group B (red). Group C has no data for such analysis (C). Percent of patients who started antiretroviral therapy (ART) during the clinical follow-up considering either the total 27 patients or only patients from each group (D). Kaplan–Meier survival analyses to time to ART inception based either on the breadth (0 or 1 peptide vs. 2 or more) or magnitude (mean SFU/ 10^6 cells <250 vs. >250 SFU/ 10^6 cells) of the responses for the whole group of 27 patients. Survival analysis comparing time to start ART among the three groups is also shown (E). SFU, spot-forming units. *, $p < 0.05$; **, $p < 0.01$.

sequences within these 15-mer peptides, were more conserved than peptide and epitope sequences from Group B. Peptide and epitope sequences from Group A showed a much lower frequency of variants than Group B's sequences, when aligned to correspondent 968 HIV-1 Gag sequences from Los Alamos database (A: 64 ± 27 vs. B: 116 ± 54 , $p = .008$; and A: 30 ± 13 vs. B: 39 ± 10 variants, $p = .005$, respectively, for peptide and epitope sequences; Fig. 3A). The percentage of Los Alamos sequences presenting mutations within the regions correspondent to our peptide sequences was also compared between groups A and B. Peptide sequences recognized by Group A's patients showed to be more conserved, as the percentage of sequences with no mutation was higher in this group compared to Group B (A: $74\% \pm 21$ vs. B: $54\% \pm 15$ of sequences with no mutation, $p = .03$). Comparing Los Alamos sequences correspondent to the epitopes found within the peptides, 82% (± 15) of Group A's sequences presented mutations, while only 77% (± 10) of Group B's epitope sequences presented mutations ($p = .15$, data not shown).

Next, we examined the HLA-I molecules possibly mediating the Gag p24 responses seen in the patients from both groups. No HLA-I allele was overrepresented in any group of patients (Table 1). Surprisingly, we found that only one out of 14 HLA-I-restricted epitopes from Group B was restricted to a locus A molecule. All the others were locus B restricted. Inversely, within the Gag p24 peptides' sequences from

Group A, we found a nondiscrepant number of locus A and locus B epitopes (51 vs. 28 epitopes, respectively). This could suggest an enhanced ability of Group A individuals to recognize more epitopes presented by a wider set of HLA-I molecules in a given viral region than the individuals from Group B.

It is broadly known that, at a molecular level, the interaction of a peptide with an HLA-I molecule is crucial for stability of the resultant HLA-peptide complex and the consecutive interaction of it with a T cell receptor, and further development of a CD8 T cell response. Usually, there are two major sites for interaction of a peptide with its respective HLA-I molecule, the internal site and the C-terminal one. C-terminal binding residue is essential to the HLA-peptide stability and directly impacts the T cell recognition.²⁴ So, we also investigated the conservation of the anchor residues for HLA binding in the epitope sequences from our two groups of patients. When aligned to the 968 Gag sequences from Los Alamos, we found a high frequency of mutations in anchor residue positions in Group B's epitope sequences, 3.3 times higher than the frequency of mutations in the HLA-I anchor residues found within the sequences from Group A ($p = .004$).

We further deconvoluted these data to explore whether these mutations were equally distributed in the internal and C-terminal anchor residues. We observed that mutations were most commonly found in the C-terminal residues in

FIG. 3. Distinct characteristics among the peptides eliciting cellular immune responses in our groups of patients. The amino acid sequences of the peptides and HLA-I-epitopes within them were compared between Group A and Group B of patients. The number of sequence variants among the 968 Gag B sequences available in Los Alamos HIV Molecular database is lower for the regions encompassing Group A-recognized peptides, when compared to Group B-recognized peptides. Also, the epitope sequences from Group A, when aligned to Los Alamos HIV-1 sequences, showed lower number of variants compared to Group B's epitope sequences (A). It also compared the percent of Los Alamos HIV-1 sequences showing mutations in anchor residues for the patients' HLA-I molecules. Epitopes from the peptides recognized by Group A's patients presented lower mutation frequency in internal and C-terminal anchor residues than the epitopes in the Group B's peptide sequences (B). **, $p < 0.05$; ***, $p < 0.001$.

Group B's epitope sequences, but not in Group A's epitope sequences. We found four times more sequences with C-terminal mutations from Group B compared to Group A ($p = .004$; Fig. 3B). As Group B's patients may have mounted their responses almost only upon HLA-I locus B presentation, we repeated all these comparisons considering only the HLA-I locus B-restricted epitopes in Group A and B. Consistently, similar or greater differences were found. For instance, considering only the HLA-B epitopes, the frequency of variant sequences of epitopes with C-terminal mutations was nine times higher in the Group B's epitopes than in the Group A's epitopes ($p < .001$; data not shown).

Of note, intracellular protein degradation is also an important mechanism by which short peptides are produced and made available for presentation to CD8⁺ T cells by HLA-I molecules.²⁵ Thus, the ability of the most crucial cytosolic protease, the 20S proteasome, to generate peptide fragments for binding HLA-I molecules, *in vivo*, could also contribute to the explanation of why Group B's patients present worse disease outcome than Group A's patients, despite showing no difference in either breadth or magnitude of Gag p24-specific responses. To address this issue, we predicted 20S proteasome cleavage sites within the HLA-I-restricted epitope sequences from the peptides recognized by the individuals in both groups using NetChop 3.1 Server (C-term 3.0 network). We found that Group B's epitopes presented a higher number of sites for proteasome cleavage than the epitopes from Group A (A: 0.8 ± 0.7 vs. B: 1.8 ± 0.7 , $p = .0001$; data not shown).

Discussion

Cellular immune responses targeting the Gag p24 HIV-1 protein are reported to play an important role in the control of viremia and preservation of CD4⁺ T cell count. However, other studies have found contradictory results when the same correlation was investigated. In this study, we screened Gag p24-specific cellular responses by ELISPOT-IFN- γ assays in 27 patients with chronic HIV-1 infection. As in some previous studies, we did not find strong correlations between either the breadth or magnitude of such responses with plasma viral load and CD4⁺ T cell count. Although the breadth and magnitude of immune responses have not been associated with disease progression by some studies and also by our data when we analyzed our 27 subjects together, some immunologic features of T cell-mediated responses have been more clearly demonstrated to impact disease outcome.²⁶ To illustrate, high proliferative capacity of HIV-1-specific CD8⁺ T cells^{27,28} and the ability of T cells to perform multiple functions upon antigen encounter, commonly referred to as polyfunctionality,^{2,13} have been associated with enhanced immune control of HIV-1 infection. However, it is still unknown to what extent proliferative and polyfunctional cells are a cause or an effect of viral containment and a direct link between anti-HIV-1 efficiency, and any particular cellular function is yet to be fully clarified.

We hypothesized that, along with successful effector functions of responsive cells, the nature of the T cell epitopes eliciting cellular immune responses could help explain different disease outcomes. According to that, it has been shown that control of viral replication in HIV-infected individuals is affected by the viral proteins that are targeted by the immune system.¹ Gag-directed cellular responses, for

instance, have been repeatedly associated with lower viral loads,^{1,4,5,8,18,29,30} whereas T cell responses against Env have actually been linked to elevated viremia.^{1,29,31} This might reflect that some epitopes are able to elicit successful antiviral cellular responses, while others, although they are also recognized by the T cell repertoire, do not show the same ability. In addition, some recent data have shown that targeting Gag conservative epitopes is important for controlling HIV replication *in vivo*.^{18–21} Identifying target-related characteristics critical to the efficacy of the immune responses could have an important role in future selection of candidate anti-HIV-1 immunogens. To reinforce this concept in HIV-1 *in vivo* infection, we attempted to investigate possible patterns of Gag p24 recognition among 27 screened patients. By considering our ELISPOT data in a binary manner, we clustered our patients in three different groups based on their distinct specificity of cellular immune responses against Gag p24 peptides. While one group consisted of patients with no response to any Gag p24 peptide, two other groups were derived and showed responses to different viral antigens. Although the breadth and magnitude of responses between these two groups were not different, Group A patients undoubtedly presented better clinical scenarios for CD4⁺ T cell count and viral load.

As the circulating HIV-1 population is tremendously diverse, as is the viral population found in a single infected individual, one possible approach for an effective AIDS vaccine would be to elicit immunity against the diverse population of viruses.³² Indeed, HIV-1 sequence diversity is considered one of the major hurdles for the design of an effective and broadly applicable HIV vaccine.³³ However, if potent cellular responses could be generated against highly conserved domains on the viral proteome, efficacy could also be theoretically achieved.^{34,35} Experimental data from Wang *et al.*³⁵ reinforce this hypothesis as the study describes that escape mutations to acute-phase CD8⁺ T cell responses impairing viral control were preferentially located in more conserved viral residues of epitopes for protective HLA molecules. Responses restricted by hazardous HLA alleles did not present preferential placement of escape mutations. Moreover, it has also been shown that reversion of escape polymorphisms to more common consensus residues upon viral transmission occurs mainly and more rapidly at conserved residues.^{36,37} This lends support to the notion that structurally conserved regions of the virus particularly refractory to sequence changes do exist.

Contributing to that, our analyses enabled us to identify specific targets in the Gag p24 protein that were more associated with reduced viral load and preserved CD4⁺ T cell count. Of note, these targets were found to be different from ones not associated with such better clinical outcome, mainly based on their higher genetic conservation. Peptides in regions of Gag protein that are more prone to show mutations were more frequently targeted by individuals in the group with worse disease outcome (Group B). Mutations concentrated in amino acid residues that work as anchor sites for HLA binding were also more frequently found in this group of patients. Once stability of peptide-HLA-I complex is influenced by perfect interaction between the peptide anchor residues and their respective binding sites in the HLA molecule, our results could suggest that Group B's patients are mounting responses against viral peptides that are more prone to present mutations in these residues and then evade from

host immunity, compared to peptides recognized by Group A's patients, helping to explain the worse disease outcome seen in Group B. These data imply that control of HIV-1 is likely due to the targeting of highly conserved regions of the virus particularly difficult to evade through sequence evolution.

Also, Group B of HIV-1-infected subjects, those with worse disease outcome, presented response against epitopes that bear twice more proteasome cleavage sites than the epitopes targeted by responses of Group A's patients. Proteasomes are key proteases involved in a variety of cellular processes, including processing viral proteins and further presentation of their antigens to CD8⁺ T cells. Number and nature of cleavage sites within a target protein and also how fast these proteins are degraded have impact on immune system function.²⁵ So, along with the different level of conservation between the targets of immune response of groups A and B, the cellular processes for processing and presentation of peptides in a given viral protein could be different between individuals with better and worse disease outcomes.

Finally, a possible limitation of our conclusions might be attributed to a small number of subjects investigated (i.e., $n=27$). By comparing our work to other screening studies of cellular immune response with larger sample sizes, we could suggest that our statistical power to detect an effect might be affected by studying only 27 individuals, but not the effect detected itself. For instance, Kiepiela *et al.*¹ have screened CD8⁺ T cell responses against Gag (and other proteins) in 578 untreated HIV-infected individuals from KwaZulu-Natal, South Africa. Their results also indicated a weak negative correlation ($r=-0.25$) between breadth of Gag responses and plasma HIV-1 load, similar to our results ($r=-0.22$). Notwithstanding, this correlation is very trustful ($p<.0001$) for their study and not for ours ($p=.27$). So, even if narrowly enough, screening 27 individuals in this study allowed the identification of at least 2 groups of individuals, based on specificities of immune responses and, consequently, the assessment of differences in clinical progression between the groups, which were our main objectives. Then, our observations support the hypothesis stated by recently published data that, it is the specificity rather than total breadth or magnitude of responses that would be important for viral control and so even a narrow, but well-focused response could suppress viral replication.^{18–21}

In conclusion, our study offers empirical evidence for a common belief among HIV vaccinologists that, eliciting cellular immune responses against conserved HIV epitopes might play an important role in vaccination-induced protection or viral control seen in HIV-1-infected subjects. Our data support suggestions that eliciting cellular immune responses targeting highly conserved regions of the HIV-1 proteome may be important for developing an effective vaccine against HIV. We suggest that along with being necessary to sensitively identify successful cellular immune responses against the HIV-1, it is also important to determine which among them are the critical ones acting on the viral control.

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