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Higher cross-subtype IFN-γ ELISpot responses to Gag and Nef peptides in Brazilian HIV-1 subtype B- and F1- than in C-infected subjects

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ABSTRACT

HIV-1 diversity has been considered a huge challenge for the HIV-1 vaccine development. To overcome it, immunogens based on centralized sequences, as consensus, have been tested. In Brazil, the co-circulation of three subtypes offers a suitable scenario to test T cell cross-subtype responses to consensus sequences. Furthermore, we included peptides based on closest viral isolates (CVI) from each subtype analyzed to compare with T cell responses detected against the consensus sequences. The study included 32 subjects infected with HIV-1 subtype B ($n=13$),C ($n=11$), and F1 ($n=8$). Gag and Nef-specific T cell responses were evaluated by IFN-γ-ELISpot assay. Peptides based on CVI sequences were similar to consensus in both reducing genetic distance and detecting T cell responses. A high cross-subtype response between B and F1 in both regions was observed in HIV-1 subtype B and F1-infected subjects. We also found no significant difference in responses to subtype B and C consensus peptides among subtype B-infected subjects. In contrast, the magnitude of T cell responses to consensus C peptides in the Gag region was higher than to consensus B peptides among HIV-1 subtype C-infected subjects. Regarding Nef, subtype C-infected subjects showed higher values to consensus C than to consensus F1 peptides. Moreover, subtype F1-infected subjects presented lower responses to subtype C peptides than to subtype F1 and B. A similar level of responses was detected with group M based peptides in subtype B and F1 infected subjects. However, among subtype C infected subjects, this set of peptides detected lower levels of response than consensus C. Overall, the level of cross-subtype response between subtypes B and F1 was higher than between subtype C and B or C and F1. Our data suggests that the barrier of genetic diversity in HIV-1 group M for vaccine design may be dependent on the subtypes involved.

1. Introduction

The development of safe and effective HIV vaccines offers the best hope for the prevention of new infections. However, HIV-1 vaccine efforts have not yet proven successful [1]. Among the four vaccine efficacy trials in human volunteers, only the recent RV144, conducted in Thailand, showed statistically significant rate of protection, despite the fact that vaccine efficacy was 31.2% [2].

The high mutation rate of HIV-1 allows the virus to rapidly evade immune responses [3]. In fact, the genetic variability of HIV-1 is considered one of the major challenges for the design of effective vaccines that could protect from heterologous viral infection [4] and for the development of reagents to evaluate vaccine immunogenicity. Even within the same HIV-1 subtype the amino acid sequence can diverge by >15%, whereas genetic distances between isolates of distinct subtypes can exceed 30% depending on the genomic region analyzed [5].

In areas where different HIV-1 subtypes co-circulate, this diversity is reflected in the emergence of unique recombinant forms and CRFs. In Brazil, the subtype B is prevalent in most geographic regions, followed by BF1 recombinants, and subtype F1 [6–9]. This scenario is different in the Southern Brazilian region, where subtype C and BC recombinants are highly prevalent [10]. The co-circulation of subtype B and BF1 recombinants is also found in other South American countries, such as Argentina, Chile, Uruguay and Paraguay [11–13].

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A globally effective vaccine should protect against a variety of HIV-1 genetic forms. To overcome this diversity, several strategies of immunogen design have been proposed, as inclusion of distinct HIV subtype target antigens [2] engineered antigens, such as polyvalent mosaic [14] and centralized [15–18] sequences. The goal of these strategies is to reduce the amino acid sequence distance between immunogens and circulating viruses. Since HIV-1 phylogeny present a star-like configuration, the use of a "central sequence" should diminish the amino acid difference between immunogens and circulating virus [16]. Among centralized sequences, consensus sequences have been commonly used to evaluate immune responses in HIV-1-infected subjects [19–22]. Vaccines based on consensus sequences stimulated cross-subtype responses in animal models [23–25], and cross-reactivity was detected using peptides based on the consensus of target regions in cohorts from different ethnicities infected with diverse subtypes [26–28].

Evaluation of cross-reactivity responses among HIV-1 subtypes prevalent in South America are scarce, and the high miscegenation of the Brazilian population offers a heterogeneous HLA background to study T cell responses in HIV-1 infected subjects [29]. The objective of this study was to evaluate the potential usage of consensus sequences for the definition of viral immunogens, and to analyze the cross-subtype responses in a cohort of HIV-1 subtype B-, C-, and F1-infected Brazilian subjects. An additional strategy of immunogen design, here called closest viral isolate (CVI), which was based on the identification of the circulating virus with the lowest genetic distance to the consensus in a given population, was also evaluated in this study.

2. Materials and methods

2.1. Study population

The study included 32 Brazilian subjects infected with HIV-1 subtypes B ($n = 13$), C ($n = 11$), and F1 ($n = 8$), followed at Hospital Evandro Chagas (Rio de Janeiro, RJ), Hospital Geral de Nova Iguaçu (Nova Iguaçu, RJ), and Hospital Homero de Miranda Gomes (São José, SC). All subjects have detectable viral load and lymphocytes TCD4$^+$ counts > 200 cells/μL. The study was approved by the respective Institutional Review Committees and all participants gave written informed consent.

2.2. Cells

Blood was collected by sterile venipuncture, and samples were processed on the day of collection. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Histopaque 1077 (Sigma–Aldrich, USA), and cryopreserved in 90% fetal bovine serum (FBS-Gibco, Invitrogen, USA), and 10% dimethyl sulfoxide (Sigma–Aldrich, USA).

2.3. Lymphocytes T CD4$^+$ counts and viral load

Absolute TCD4$^+$ cells counts were measured using the MultiTest TruCount-kit and the MultiSet software on a FACSCalibur (BD Biosciences, USA). Plasma HIV-1 viral loads were measured using the Versant HIV-1 3.0 RNA assay (bDNA, Siemens Healthcare Diagnostics, USA).

2.4. Extraction, PCR amplification, and sequencing of HIV-1 DNA

DNA samples were extracted from whole blood using QIAamp viral DNA Kit (QIAgen Inc., USA), according to the manufacture's protocol, and PCR-amplified using nested primers. Amplification of *gag* region was performed using SCAOSD [30] and G17 [31] as outer primers, and SCANSD [30] and p24-1 [32] as inner primers. Amplification of the *nef* fragment was carried out using NEF-1 [33] and SCDOAD [30] as outer primers, and NEF-3 [34] and SCDNAD [30] as inner primers. PCR conditions were performed as described elsewhere [6].

PCR products were purified with Illustra GFX PCR DNA Kit (GE Healthcare, USA), and sequenced using the Big Dye Terminator v3.1 Kit (Applied Biosystem, USA). Sequencing reactions were analyzed with an ABI 3100 automated sequencer. Sequences were edited with Seqman v7.0 program (DNASTAR; Lasergene, USA).

2.5. Consensus and closest viral isolate (CVI) sequences

A total of 220 Brazilian sequences were retrieved from the Los Alamos HIV database (http://www.hiv.lanl.gov), and from the sequence database from our laboratory (subtype B [*gag* = 21, *nef* = 51], subtype C [*gag* = 34, *nef* = 45], and subtype F1 [*gag* = 31, *nef* = 38]). They were used to infer the subtype B, C, and F1 Brazilian consensus sequences of *gag* and *nef* regions employing the software Dambe v.5.0.10. Group M-consensus sequence was retrieved from the Los Alamos HIV database. The subtype B, C, and F1 Brazilian sequences, with the lowest genetic distance to the corresponding subtype consensus in both *gag* and *nef* regions, were defined as the closest viral isolates (CVI). Nucleotide distance between sequences and subtype consensus sequences were calculated using the Tamura–Nei model as implemented in MEGA v40 [35]. Amino acid sequences were obtained by translating consensus and CVI nucleotide sequences.

2.6. Synthetic peptides

Sets of peptides (15-mers with 11-aa overlaps) based on the Brazilian consensus and CVI subtype B, C, and F1 covering Gag (amino acids 17–43, and 64–103 related to the HXB2 Gag protein), and Nef (amino acids 68–160 related to the HXB2 Nef protein) fragments were produced by polypeptide (USA). These regions were chosen based on a previous study from our group [36], that showed high frequencies of responses to these positions in Gag p17 and the Nef central region. Overlapping peptides were divided into two pools for Gag p17 and four pools for Nef, according to the position in protein. The final concentration of each peptide within a peptide pool was 4 μg/mL.

2.7. IFN-γ ELISpot

The ELISPOT IFN-γ assay was performed as described previously [37]. Briefly, 96-well plates (Millipore, USA) were coated with anti-human IFN-γ mAb (Diaclone, France). HIV-1 peptide pools were diluted in complete culture medium [RPMI 1640 (Sigma, USA) supplemented with 10% of FBS, Penicillin–Streptomycin (10,000 U–10,000 μg/mL), L-glutamine 200 mM, non-essential amino acids 10 mM, and sodium pyruvate 100 mM (all purchased from invitrogen, USA)]. PBMCs were added at an input cell number of 1×10^5 cells/well. Phytohemagglutinin-5 μg/ml (Sigma, USA) was used as a positive control, and cells suspended only in culture medium served as a negative control. The spots were counted using an automated ELISPOT reader (CTL Analyzers LLC, Cellular Technology, USA). The results were expressed as spot-forming cells (SFC)/million PBMCs. The response was considered positive if ≥50 SFC/10^6 PBMCs were detected.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad 5.0 (Prism Software, USA). The Wilcoxon test was used to compare genetic distance between infecting viral sequences and consensus or CVI

Table 1
Summary of viral load and CD4+ T cell counts according to the HIV-1 subtype infection.

Laboratory data, median (IQRs)	HIV-1 subtypes (number of participants)			
	Subtype B (13)	Subtype C (11)	Subtype F1 (8)	Overall (32)
Plasma HIV RNA level, copies/mL*	3,483 (375–12,521)	12,663 (920–16,200)	12,915 (3,738–32,396)	8,543 (911–15,711)
$CD4^+$ T cell count cells/μL*	736 (565–1,014)	519 (431–654)	648 (353–842)	617 (485–790)

* No significantly differences were observed among the $CD4^+$ T cell count and viral load. All p values > 0.05.

sequences, and the magnitude of responses against consensus and CVI peptides. The Friedman test was performed to compare genetic distances and the magnitude of HIV-1 cross-subtype responses, followed by a Dunn's post-test for multiple comparisons. Correlations were determined using Spearman's rank test. All tests were considered significant if the p value was below 0.05.

3. Results

3.1. Study population

The summary of laboratory data for subjects included in this study, distributed according to the subtype of the HIV-1-infecting virus, is presented in Table 1. Overall, the median of absolute lymphocyte T $CD4^+$ cell count was 617 cells/μL (IQR = 485–790), and the median of plasma RNA viral load (VL) was 8,543 copies/mL (IQR = 911–15,711). No statistically significant differences were found for VL and CD4T cell counts among the groups.

3.2. Genetic distance to consensus and CVI of HIV-1 subtypes B, C, and F1

The mean genetic distances between the Brazilian sequences, used to derive consensus, and the corresponding subtype consensus for *gag* and *nef* regions were: 3.9% and 5.3% for subtype B; 3.0% and 3.2% for subtype F1; and 3.7% and 4.1% for subtype C, respectively. The genetic distances between the CVI sequences and the corresponding subtype set of sequences were: 5.8% (*gag*) and 6.8% (*nef*) for subtype B; 4.9% (*gag*) and 5.2% (*nef*) for subtype F1; and 5.8% (*gag*) and 4.2% (*nef*) for subtype C. Phylogenetic trees of *gag* and *nef* genes showing the positions of both consensus and CVI sequences are presented in Supplemental File (Fig. S1).

Next, we determined the mean genetic distance between consensus and CVI overlapping peptide sequences for each target used for the ELISpot IFN-γ assays and the viral amino acid sequence of each study subject. The mean genetic distances to consensus and CVI were similar for all subtypes on both regions analyzed, with exception of subtype B infected subjects that displayed significantly ($p = 0.0015$) lower mean genetic distance to consensus (8.6%) than to CVI (13.8%) in the Gag region (Fig. 1A and B). These results suggest that the capacity of both consensus and CVI sequences to minimize the genetic distance to Brazilian circulating viruses was roughly similar, although they might vary according to the genetic region and subtype considered.

3.3. Immune response to consensus and CVI of HIV-1 subtypes B, C, and F1

To compare the capacity of peptide pools corresponding to CVI and consensus sequences of HIV-1 subtypes B, C, and F1 to elicit T cell responses, the median of the magnitude of responses was calculated based on the sum of the number of IFN-γ-secreting cells/10^6 PBMCs for each Gag or Nef peptide set. Consensus and CVI peptides showed similar median of magnitude of responses, independently of subtype evaluated (Fig. 1C and D) for both regions. The overall magnitude of response to Nef was higher than to Gag peptide pools. It is important to indicate that F1 subtype-infected subjects showed higher responses to Gag peptides than those infected with subtypes B or C.

3.4. Cross-reactivity responses among subtype B, C, F1 and group M

Intra and inter pairwise distances were calculated based on the amino acid sequence of each sample against consensus peptides used as ELISpot reagent (Fig. 2A and B). The mean intra-subtype genetic distances were significantly lower than the mean inter-subtype, except for the subtype F1-infected patients that displayed a mean genetic distance to consensus F1 (8%) similar to consensus B (7.2%) in Nef region. Mean genetic distances to M consensus ranged from 14 to 24% for Gag and 10–13% for Nef.

The degree of cross-reactivity to each subtype consensus and group M peptides was evaluated in Gag and Nef. Positive response to at least one consensus peptide set was detected for 93% of the patients. In contrast with what was observed in genetic distances, the mean magnitude of responses, expressed as SFC/10^6 PBMCs, in subtype B-infected individuals, showed no significantly differences against the four peptides sets tested in both Gag and Nef. In subtype C-infected subjects, the mean magnitude of responses to consensus C (Gag = 190; Nef = 628) were significantly higher than to consensus B (Gag = 24; Nef = 463), consensus M (Gag = 35; Nef = 421), and consensus F1 (Nef = 368). In subtype F1-infected subjects, similar to observed in subtype B-infected subjects, a high cross-reactivity was demonstrated to consensus B and M. However, the magnitude of responses to consensus C, in Gag, was significantly lower (Fig. 2C and D).

3.5. Correlation between genetic distance and magnitude of T cell responses to Gag and Nef peptides

The genetic distance between the viral infecting sequences from the three groups and consensus B, C, F1, and group M peptides had a significant impact on the anti-Gag T cell responses, demonstrated by a negative correlation (Spearman's $r = -0.2227$; $p = 0.0145$) (Fig. 3A), while no significant correlation was observed for Nef (Fig. 3B).

4. Discussion

The genetic diversity of HIV-1 is considered one of the major challenges for the design of effective vaccines. One of the most popular strategies of immunogen design proposed to overcome this problem is the use of centralized sequences, such as consensus, that are also frequently used to derive reagents to assess immune responses in HIV-infected subjects. In the present study, we compared the capacity of consensus sequences, and primary isolate sequences that most closely resemble consensus (CVI), to both reduce the genetic distance and maximize the immune response

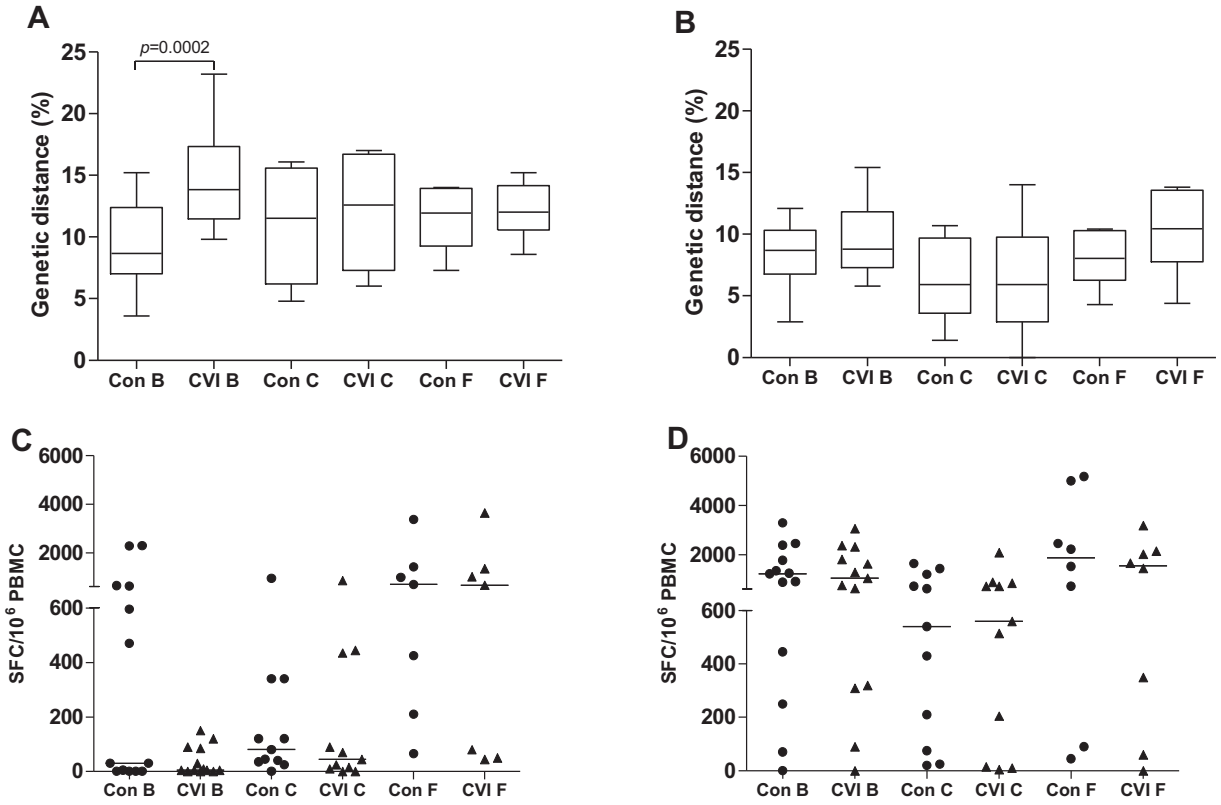

Fig. 1. Comparison between consensus and CVI peptides. Amino acid distances between infecting viral sequences and peptides derived from consensus and CVI sequences from subtype B, C, and F1 in Gag (A) and Nef (B). Magnitude of T cell responses from HIV-1 subtype B-, C-, and F1-infected subjects, stimulated with Gag (C) or Nef (D) peptides pools based on consensus and CVI sequences from subtype B, C, and F1. Responses ≥ 50 SFC/10^6 PBMCs were considered positive. *P* values were calculated using the Wilcoxon test.

against the three prevalent HIV-1 subtypes circulating in Brazil: B, C, and F1.

Previous studies demonstrated a high similarity between consensus and isolate sequences most similar to consensus (>80% in Gag and >70% in Nef), and also a high percentage of positive responses in IFN-γ ELISpot assays against peptides derived from isolate sequences [38–40]. However, no comparison has been performed between consensus and isolate sequences in context of T cell immune response. Here we demonstrate that the CVI sequence is similarly powerful for both reducing the genetic distance to circulating viruses, and for the detection of HIV-specific T cell responses to Gag and Nef when compared to consensus for all three subtypes analyzed.

For vaccine design, it is of paramount importance the identification of cross-reactive HIV antigens to cover the high HIV diversity. Several studies indicate that Gag and Nef highly conserved epitope regions are commonly recognized, and give rise to high inter-subtype cross-reactive T-cell responses [26,28,41–43]; although the overall frequency and magnitude of inter-subtype T-cell responses is typically lower than that of the intra-subtype [26,28,44]. We found a high cross-subtype response between subtype B and F1 for both Gag and Nef regions, comparable to the frequency and/or magnitude of intra-subtype T-cell responses. This data is in agreement with a previous study that also described a high T-cell cross-reactivity between subtypes B and F1 among HIV positive individuals from Argentina using Nef peptides [45]. High cross-reactivity between subtypes B and F1 have also been observed for neutralizing antibodies [46]. These results open a good perspective for vaccine design for countries where these two subtypes are predominating, as in South America.

No significant difference in the magnitude of the responses to subtype B and C Gag and Nef consensus peptides was found among subtype B-infected subjects, consistent with other studies demonstrating a similar magnitude or breadth of T cell responses to subtype B and C peptides in populations infected mainly with subtype B [38,43]. In contrast, the magnitude of T cell responses to consensus C peptides in the Gag region was significantly higher than to consensus B peptides among HIV-1 subtype C-infected subjects. Previous studies with subtype C-infected subjects also demonstrated a lower magnitude of response to subtype B consensus when compared with intra-subtype responses [26,28]. Significant differences in the level of cross-subtype responses were also observed between subtype C and F1. Among subtype C-infected subjects, the median magnitude of responses to consensus C was higher than to consensus F1 peptides in Nef, whereas among subtype F1-infected subjects the median magnitude of responses to consensus F1 peptides in Gag was higher than to consensus C peptides.

Consensus group M based immunogens have been proposed as an alternative to vaccine design in regions where different subtypes co-circulate [27,47]. Peptide pools based on group M consensus sequences detected responses of similar breadth and magnitude as did consensus B or consensus C peptides in subtype B- and C-infected patients, respectively [27]. Gag and Nef peptides derived from HIV-1 consensus group M also detected responses in subtype A1 and D infected subjects [47] demonstrating that this strategy is useful for different subtypes. Our results confirm that group M consensus peptides were as efficient as subtype-specific consensus peptides for subtype B- and F1-infected subjects, but not for subtype C-infected patients. Among subtype C-infected patients, group M consensus peptides elicited a significant lower magnitude

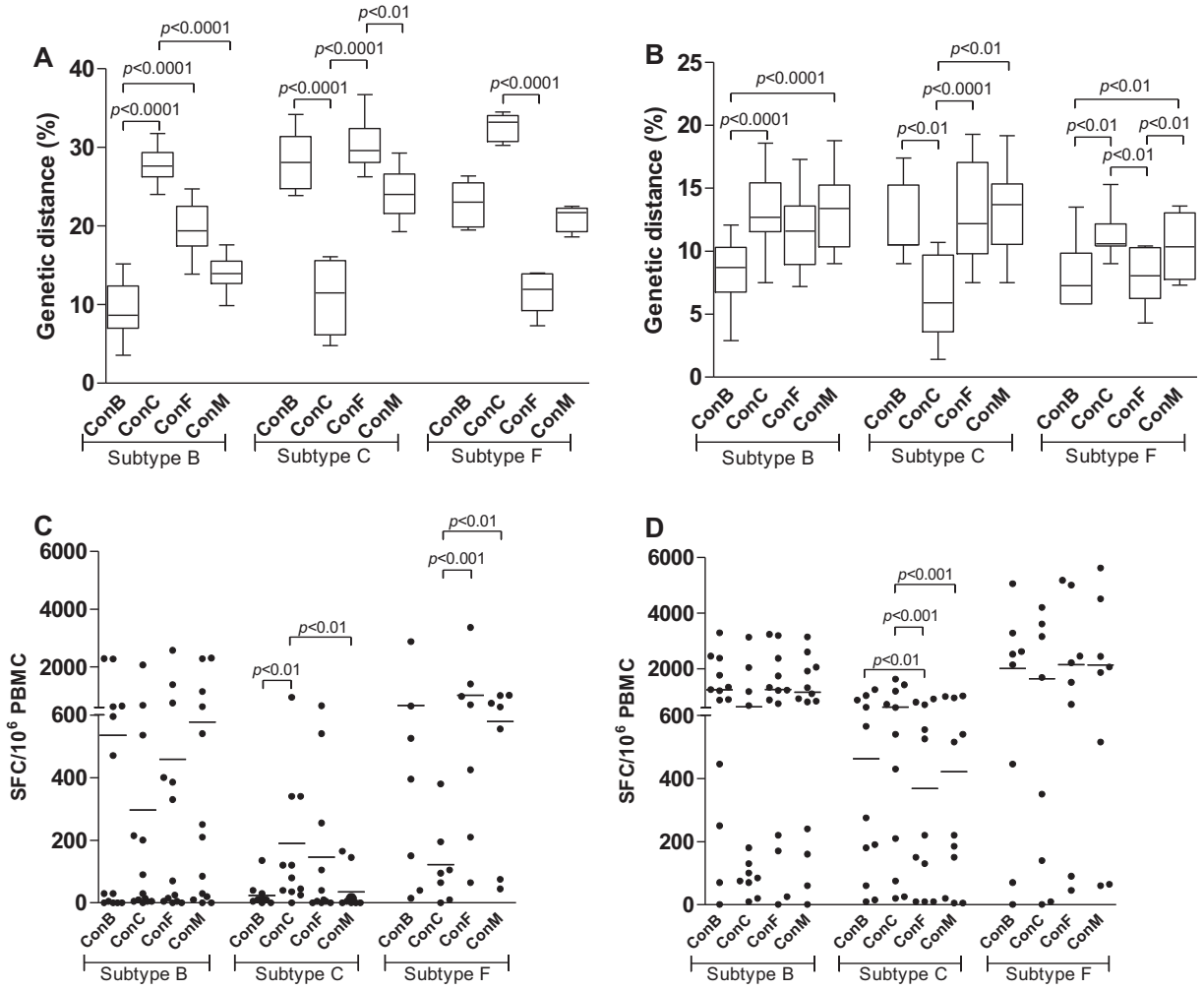

Fig. 2. Evaluation of cross-reactivity. Amino acid distances between infecting viral sequences and peptides derived from subtype B, C, F1, and group M consensus sequences in Gag (A) and Nef (B). Magnitude of T cell responses from HIV-1 subtype B-, C-, and F1-infected subjects, stimulated with Gag (C) and Nef (D) peptides pools based on subtype B, C, F1, and group M consensus sequences. Responses ≥ 50 SFC/10^6 PBMCs were considered positive. *P* values were calculated using Friedman's test followed by a Dunn's post-test for multiple comparisons.

of response than subtype C consensus peptides. This difference in T cell response to peptides derived from group M and subtype C consensus was not observed in a study of Bansal et al. [27], that found a similar level of responses against group M and subtype C consensus in Zambians subtype C HIV-1 infected subjects.

The extensive genetic diversity of the HIV-1 group M isolates and its implications for vaccine design have long been debated. While some studies point to an influence of genetic distance on T cell responses, others were unable to detect a correlation between these variables [18,26,43,48]. We found a significant negative correlation between amino acid genetic distance and ELISpot IFN-γ responses in Gag, but no correlation was demonstrated in Nef. Although the genetic distances to the homologous consensus peptides in Nef were significantly lower than to heterologous consensus ones, the

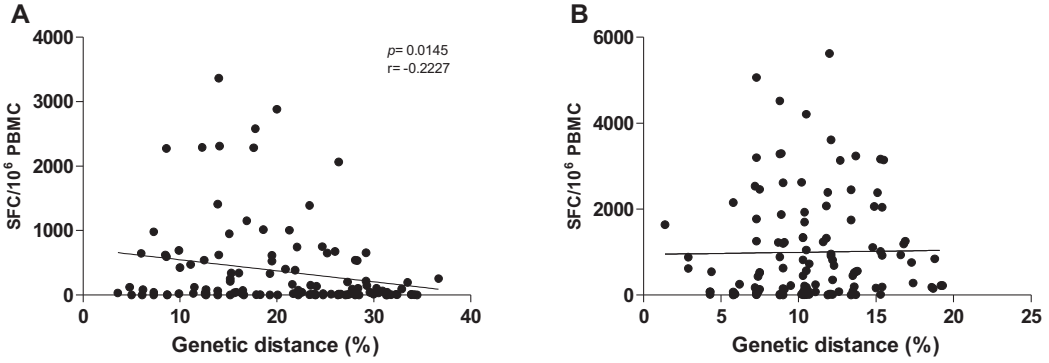

Fig. 3. Correlation between genetic distance and T cell response. Association between the magnitude of IFN-γ ELISpot response to Gag (A) and Nef (B) and the amino acid distance between autologous viral sequences and consensus sequence. *P* values were determined by the Spearman rank test.

magnitude of intra-subtype and inter-subtype responses was quite homogenous for most comparisons in this region. Finally, although the mean genetic distance among prevalent Brazilian subtypes was roughly similar, we observed that the level of cross-subtype response between subtypes B and F1 was higher than between subtype C and B or between subtype C and F1. These data demonstrate that associations between T cell responses and phylogenetic proximity are complex.

Our data demonstrate that peptide pools based on natural CVI strains are able to minimize the genetic distance to circulating viruses and to detect responses of similar breadth and magnitude as peptides based on artificial consensus sequences. They also indicate that the significance of the HIV-1 group M genetic diversity for vaccine design may be dependent of the subtypes involved and the genomic region considered. We also point out that the negative impact of genetic distance on T cell recognition could be more important for Gag than Nef. Overall, these results emphasize that it is probably necessary to use a multi-subtype immunogen to match the predominant HIV-1 subtypes that circulate in the Brazilian population, especially if peptides based on Gag are included in the vaccine formulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.12.023.

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