MAJOR ARTICLE

Cross-Reactivity of Anti–HIV-1 T Cell Immune Responses among the Major HIV-1 Clades in HIV-1–Positive Individuals from 4 Continents

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Background. The genetic diversity of human immunodeficiency virus type 1 (HIV-1) raises the question of whether vaccines that include a component to elicit antiviral T cell immunity based on a single viral genetic clade could provide cellular immune protection against divergent HIV-1 clades. Therefore, we quantified the cross-clade reactivity, among unvaccinated individuals, of anti–HIV-1 T cell responses to the infecting HIV-1 clade relative to other major circulating clades.

Methods. Cellular immune responses to HIV-1 clades A, B, and C were compared by standardized interferon-γ enzyme-linked immunospot assays among 250 unvaccinated individuals, infected with diverse HIV-1 clades, from Brazil, Malawi, South Africa, Thailand, and the United States. Cross-clade reactivity was evaluated by use of the ratio of responses to heterologous versus homologous (infecting) clades of HIV-1.

Results. Cellular immune responses were predominantly focused on viral Gag and Nef proteins. Cross-clade reactivity of cellular immune responses to HIV-1 clade A, B, and C proteins was substantial for Nef proteins (ratio, 0.97 [95% confidence interval, 0.89–1.05]) and lower for Gag proteins (ratio, 0.67 [95% confidence interval, 0.62–0.73]). The difference in cross-clade reactivity to Nef and Gag proteins was significant ($P < .0001$).

Conclusions. Cross-clade reactivity of cellular immune responses can be substantial but varies by viral protein.

With an estimated 2.9 million deaths from AIDS and 4.8 million new HIV-1 infections per year worldwide [1], the development of vaccines against this virus is an urgent priority. Virus-specific cell-mediated immune (CMI) responses may not be sufficient to protect against HIV-1 infection but have been implicated in the control of HIV-1 replication [2] and will probably play an important role in any HIV-1 vaccine paradigm [2–5]. Vaccination of monkeys to elicit CMI responses, followed by infection with simian immunodeficiency virus or simian-human immunodeficiency virus, results in a more benign clinical course, compared with that in unvaccinated controls, including longer survival, lower viral loads, and better-preserved $CD4^+$ T cell popula-

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[b] We note with sadness that one of the coauthors, Dr. Scott Thaler, recently passed away suddenly. Scott was kind, compassionate, a talented and outstanding researcher, and a pleasure to work with. We sorely miss him.

tions. Generally, the extent of clinical protection in monkeys has been shown to correlate with the magnitude of the vaccine-elicited CMI response at the time of virus challenge [2]. As a result, a substantial vaccine development effort has been focused on inducing anti–HIV-1–specific CMI responses, particularly that of $CD8^+$ cytotoxic T lymphocytes (CTLs).

An important consideration in the development of CMI-based vaccines is the diversity of HIV-1 worldwide [3, 4]. HIV-1 is classified into 3 groups (M, O, and N) [6]. The majority of the HIV-1 circulating globally is type M, which has 9 defined subtypes, or clades [7]. Of these, clades A, B, and C represent the large majority (~86%) of circulating HIV-1 variants [8]. Clade A is prevalent in East Africa; a recombinant variant of clade A (CRF01_AE) with *gag* and *nef* derived from clade A and *env* derived from clade E is predominant in Thailand [9]. Clade B is prevalent in Europe, Brazil, the United States, Canada, Australia, and Haiti, as well as in South African men who have sex with men [10, 11], whereas clade C is predominant in the southern African subcontinent, Ethiopia, India, and China [12–14]. It is unclear whether this observed viral diversity limits broadly reactive CMI responses. This is an important concern for developing a globally effective vaccine against HIV-1/AIDS [15], since inadequate cross-reactive CMI responses may lead to the need to construct several clade-specific vaccines.

It has been shown that clade B–based HIV-1 vaccines can elicit cross-clade CMI reactivity [16] and that CMI responses from individuals infected with one clade can lyse target cells infected with a different clade [17–19]. However, an informative quantification of cross-clade reactivity, by a consistent method in geographic areas where diverse clades predominate and among people with diverse HLA types, has not, to our knowledge, been performed. Therefore, in the present study, we assessed the CMI responses, among humans infected with diverse HIV-1 clades, against a series of HIV-1 proteins (Gag, Pol, Nef, Env, Rev, and Tat) based on near-consensus clade B sequences. We then selected the 2 proteins, Gag and Nef, that yielded the highest responses overall and, within each country, assessed cross-reactivity among clades A, B, and C.

SUBJECTS AND METHODS

Study design. Each study participant signed an informed-consent form before study enrollment. The informed-consent form described the nature and possible consequences of the study. The study protocol was approved by the independent ethical review committee of the universities at each site where the study was conducted and by the Institutional Review Boards of Harvard School of Public Health and the Johns Hopkins School of Public Health, as well as by national authorities, as appropriate. After informed consent was obtained, HIV-1–positive participants 18–55 years of age were enrolled at clinic sites in Brazil (Rio de Janeiro and São Paulo), Malawi (Thyolo tea estate), South Africa (Soweto), Thailand (Bangkok), and the United States (10 sites). Inclusion criteria were a hematocrit $\geq$28% obtained before blood was drawn and a $CD4^+$ T cell count $\geq$300 cells/μL. Subsequent to enrollment, 42 participants were found to have $CD4^+$ T cell counts <300 cells/μL and were included in the analysis. Exclusion criteria included positive urine pregnancy test results, breast-feeding, overt evidence of immune compromise, or documented HIV-1 infection $\geq$5 years from the time of screening. Blood samples were processed, within 12 h of drawing, into peripheral blood mononuclear cell (PBMC) samples that were frozen using a standard procedure and shipped in dry ice to a central US laboratory.

Three gene regions were sequenced to determine viral clade by polymerase chain reaction amplification: *gag, nef,* and the V3 loop of *env.* Since clade determination studies in South Africa and Malawi have reported that $\geq$90% of HIV-1 isolates are clade C [12, 13], only a subset of South African and Malawian specimens were assayed to confirm the predominant clade C infection. US participants were men who have sex with men, among whom clade B infection predominates [20, 21], and were presumed to be infected with clade B virus. Clade assignments were made using the National Center for Biotechnology Information tool (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi). A homologous clade was defined as the viral clade type responsible for infection, and heterologous clades were defined as clades other than the one responsible for the participant's infection.

Selection of HIV-1 antigen sequences for study. The T cell response to each viral antigen was estimated by characterizing the reactivity of T cells, in PBMCs isolated from each participant, to peptide pools derived from near-consensus HIV-1 Gag, Nef, Pol, Rev, and Tat proteins. Peptide pools (synthesized by the SynPep Corporation, Dublin, CA) contained peptides 20 aa in length, overlapping by 10 aa, that spanned the entire viral protein. The Pol pools used in some assays contained 15-aa peptides overlapping by 11 aa. Because of the size of the Pol protein, Pol peptides were divided into 2 pools, Pol-1 and Pol-2, each representing half of the protein. The Gag and Nef amino acid sequences of clades A, B, and C were obtained from primary virus isolate sequences in the Los Alamos HIV-1 database that were as close as possible to the consensus sequence in that clade type [22]. Percentage homology in the identity of amino acid sequences was determined for Gag and Nef proteins from pairwise alignments by use of BioEdit [23] (table 1). Comparisons of these prototypical amino acid sequences with each other and with consensus sequences are shown in the Appendix, which appears only in the electronic edition of the *Journal.*

Measurement of T cell responses. Anti–HIV-1–specific T cell responses were quantified using an enzyme-linked immunospot (ELISPOT) assay of interferon (IFN)–γ secretion that has been shown to have high correlations with other methods, such as intracellular cytokine staining, tetramer staining, and the classical

Table 1. Percentage amino acid identity matrices for clade A, B, and C Gag and Nef proteins.

Protein, clade	Amino acid identity, %, with clade					
	A[a]	B[b]	C[c]	Consensus A	Consensus B	Consensus C
Gag						
A	100	83.6 (83.9)	81.3 (83.7)	92.5 (92.5)	84.4 (84.7)	84.1 (85.5)
B		100	82.2 (84.5)	85.8 (86.5)	98.6 (98.6)	85.6 (86.9)
C			100	83.7 (86.1)	83.0 (85.3)	92.5 (93.6)
Consensus A				100	86.4 (87.1)	87.3 (88.7)
Consensus B					100	86.2 (87.6)
Consensus C						100
Nef						
A (SE8891)	100	73.6 (77.9)	76.5 (78.8)	93.6 (93.6)	80.5 (81.3)	81.1 (82.3)
B (JRFL)		100	71.4 (77.8)	75.4 (79.9)	87.9 (92.2)	79.7 (83.9)
C (IN21068)			100	79.0 (81.4)	74.8 (77.8)	83.6 (87.4)
Consensus A				100	83.4 (84.3)	85.0 (86.2)
Consensus B					100	86.9 (87.3)
Consensus C						100

NOTE. Percentage amino acid sequence identities in Gag *(top)* and Nef *(bottom)* proteins were determined from pairwise alignments, by use of BioEdit [23]. The first values given were calculated with alignment gaps considered as mismatches, and the second values (in parentheses) were calculated without assessing gap penalties.

[a] Gag peptides were based on the sequences of HIV 90CF4071 (GenBank accession no. AF197341), and Nef peptides were based on the sequences of HIV SE8891 (GenBank accession no. AF069373).

[b] The HIV CAM-1 sequence (GenBank accession no. D10112) was used for Gag, and the HIV JR-FL sequence (GenBank accession no. U63632) was used for Nef.

[c] Virus HIV 96ZM751 (GenBank accession no. AF286225) was used for Gag, and virus HIV IN21068 (GenBank accession no. AF067155) was used for Nef.

chromium-51 release assay. The ELISPOT assay has been used as a reliable tool for quantifying both HIV-1–specific and general CMI responses [24–28]. The assay measures antigen-specific memory T cells that secrete IFN-γ upon exposure to antigenic peptides presented by antigen-presenting cells. PBMC samples were placed in 96-well plates with well membranes of polyvinylidene fluoride (Millipore) coated with anti–human IFN-γ monoclonal antibody (MAbTech). Cells at 2×10^5 cells/well and 1×10^5 cells/well, in duplicate, were incubated overnight in a 37°C, 5% CO_2 incubator with peptide pools diluted to an ~2.5 μg/mL final concentration per peptide. After washing, bound IFN-γ was detected with biotinylated anti–IFN antibody (MAbTech), followed by alkaline phosphatase–conjugated anti-biotin antibody (Vector Laboratories) and visualization using 5-bromo-4-chloro-indolyl-phosphatase/nitroblue tetrazolium (BCIP/NBT) substrate (Pierce). IFN-γ–expressing cell spots were enumerated with a digital imager and automated counting system (AutoImmun Diagnostika). A positive response was identified as a minimum of 55 IFN-γ–expressing cells/million PBMCs with values at least 4-fold higher than in nonantigen control wells. This criterion was previously determined by statistical assay validation to result in a <1.0% false-positive rate (authors' unpublished data). Results were expressed as the number of IFN-γ–expressing cells per million PBMCs.

Statistical analysis. It was anticipated that ELISPOT responses to stimulation by Gag, Nef, and Pol proteins would be the most robust among the various antigens considered [29, 30]. Accordingly, the ELISPOT summaries of responses to Gag, Nef, and Pol were compared with the corresponding summaries of responses to Rev and Tat, by use of 2-tailed paired *t* tests on log-transformed data (for comparing geometric means) and exact McNemar's tests (for comparing response proportions). To reduce the potential for "false discoveries," within each analysis, the 8 *P* values (Gag, Nef, Pol-1, and Pol-2 vs. Rev and Tat) were assessed for statistical significance after a multiplicity adjustment [31]. Multiplicity-adjusted *P* values <.05 were considered statistically significant.

The ELISPOT responses for each individual are the product of lymphocyte stimulation probabilities that depend on the antigen, the infecting clade, and the number of cytotoxic T lymphocytes per million PBMCs. The ELISPOT assays quantified the ability of T cells in HIV-1–infected individuals, upon stimulation with Gag- and Nef-specific peptides, to elicit cellular immune responses from a heterologous clade. For each participant, we calculated the ratio of ELISPOT responses to a heterologous clade versus ELISPOT responses to the homologous (infecting) clade. The geometric mean of these ratios over all individuals in a population provides an estimate of the overall ratio of stimulation probabilities for the population, specific to the gene, the clade of infection, and the stimulating clade. We refer to these probability ratios as "cross-clade reactivity ratios" and calculated them using STATA (version 8; Statacorp). Ratios closer to 1 indicate greater cross-clade reactivity, and ratios closer to 0 indicate less cross-clade reactivity. For

each country, Wilcoxon signed rank tests were used to test for differences between anti-Gag and anti-Nef cross-clade ratios, by use of the aforementioned multiplicity adjustment [32, 33].

RESULTS

Study participants. Two hundred fifty-four HIV-1–positive participants consented and were enrolled in the study. Four participants (1.6%) with mock control ELISPOT values ⩾200 IFN-γ–expressing cells/million PBMCs were excluded. Characteristics of the 250 participants are shown in table 2. ELISPOT results were available for 209 participants for all 5 HIV-1 antigens.

Clade type. All 10 tested Gag and Nef sequences from Malawian participants were identified as clade C. The clade types of the viral isolates obtained from the 19 South African participants were identified as 95% ($n = 18$) clade C and 5% ($n = 1$) clade B, for both Gag and Nef. Clade typing among Brazilian participants was based on 3 gene sequences: *gag* ($n = 19$), *nef* ($n = 29$), and V3 *env* ($n = 55$). Among Brazilian participants, clade types were as follows: 69% clade B, 15% clade C, 8% clade B/D, and 8% clade B/F1 recombinants, on the basis of *gag* sequencing; 83% clade B, 7% clade C, and 3% each of clade F1, A1/B, and B/F2 recombinants, on the basis of *nef* sequencing; and 78% clade B, 15% clade F, and 5% clade C, on the basis of V3 *env* sequencing. Similarly, clade typing among Thai participants was based on 3 gene sequences: *gag* ($n = 10$), *nef* ($n = 22$), and V3 *env* ($n = 50$). Clade types among Thai participants were 100% CRF01_AE, on the basis of *gag* sequencing; 82% CRF01_AE and 18% clade B, on the basis of *nef* sequencing; and 90% CRF01_AE and 10% clade B, on the basis of V3 *env* sequencing, consistent with previous reports [33].

Comparison of cellular immune responses to clade B HIV-1 proteins. Quantitative ELISPOT responses (figure 1) to Gag, Nef, Pol-1, and Pol-2 were higher than those to Rev and Tat, for the overall study population ($P < .0001$) and within each country ($P < .04$). The proportions of participants with positive ELISPOT responses to Gag (95%), Nef (84%), Pol-1 (82%), and Pol-2 (75%) were higher than those for Rev (28%) or Tat (17%), for the overall study ($P < .0001$) and within each country ($P < .0001$), except in the United States ($P < .13$), which contributed only 15 participants to this analysis (data not shown). The proportion of the study population that consisted of ELISPOT responders to clade B Gag or Nef was 96%. In countries with non–clade B founder viruses, the proportion of the study population responding to clade B Gag and/or Nef proteins was 99% in Thailand, 100% in Malawi, and 95% in South Africa (data not shown).

ELISPOT responses to peptide pools based on the clade B 89.6P Env protein were assessed for a subset of participants (data not shown). The anti-Env responses were lower than the anti-Gag ($P < .0001$), anti-Nef ($P < .005$), and anti-Pol ($P < .02$) responses. Additional experiments in a subset of individuals ($n = 44$) were performed to determine the relative contributions of $CD8^+$ versus $CD4^+$ T cell responses against the Gag and Nef antigens. These responses were measured by flow cytometry detection of intracellular IFN-γ production by antigen-stimulating cells, by use of the same peptide pools as those used for the ELISPOT assays. All of the participants exhibited strong $CD8^+$ T cell responses, and the majority did not have detectable $CD4^+$ T cell responses. Among participants with both $CD8^+$ and $CD4^+$ T cell responses, the $CD8^+$ T cell response was always greater than the $CD4^+$ T cell response. No participant had an antigen-specific $CD4^+$ T cell response in the absence of

Table 2. Characteristics of the 250 study participants.

Characteristic	Value in participants from				
	Brazil ($n = 55$)	Malawi ($n = 46$)	South Africa ($n = 38$)	Thailand ($n = 75$)	United States ($n = 37$)
Age, median (95% CI), years	31 (30–33)	34 (29–37)	30 (28–32)	29 (927–31)	44 (41–47)
Sex, % women	49	43	89	64	0
Race, %					
Asian	2	0	0	100	NA
Black	15	100	100	0	NA
White	67	0	0	0	NA
Other	16	0	0	0	NA
$CD4^+$ T cell count, median (95% CI), cells/μL	493 (424–545)	432 (389–457)	611 (509–718)	351 (302–405)	668 (516–908)
Plasma viral load, median (95% CI), 1000s of copies/mL	16 (12–26)	74 (3–118)	8 (4–26)	30 (19–59)	0.9[a] (0.4–3)
Risk behavior during past 10 years, % of participants					
Injection drug use	0	NA	0	6	NA
Heterosexual contact	67	NA	96	81	NA
Men had sex with men	33	NA	4	8	NA
Sex work	0	NA	0	5	NA

NOTE. CI, confidence interval; NA, not available.

[a] Of US participants, 50% were receiving antiretroviral therapy and 15% had received antiretroviral therapy.

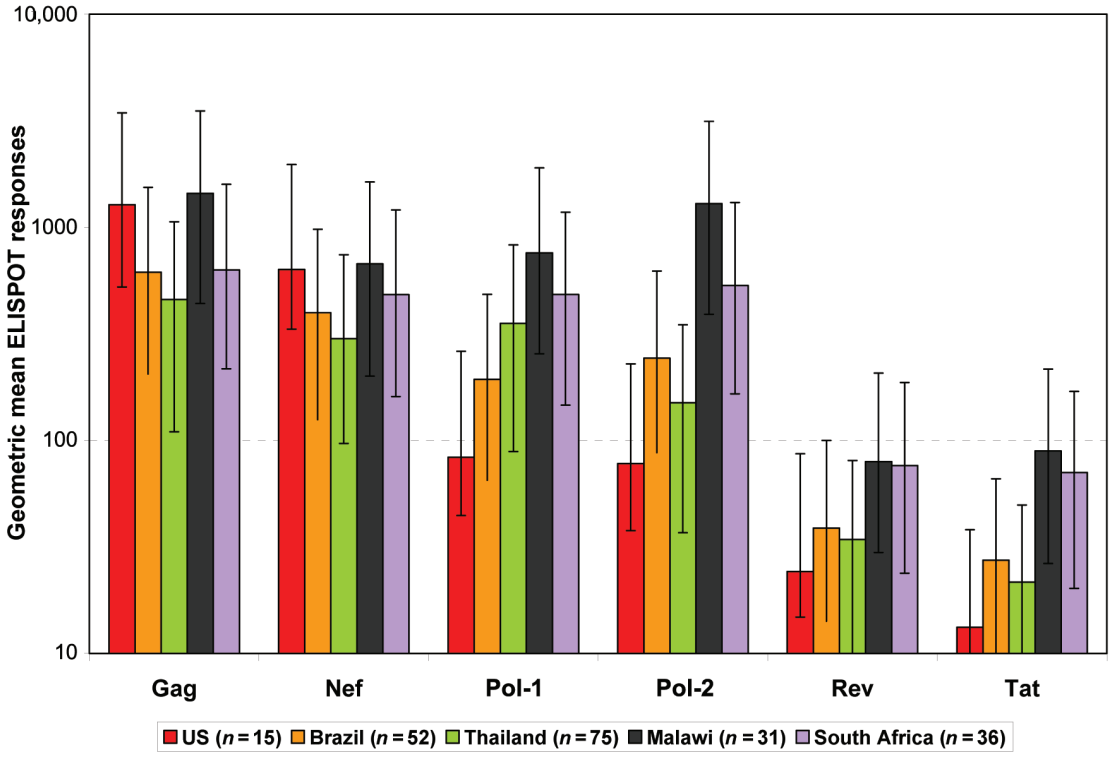

Figure 1. Anti–HIV-1 T cell responses to clade B antigens. Responses are shown as geometric means of interferon (IFN)–γ enzyme-linked immunospot (ELISPOT) assay responses among 209 HIV-1–infected participants, expressed as the no. of IFN-γ–expressing cells per million PBMCs

a $CD8^+$ T cell response. These observations are consistent with reports that chronically HIV-1–infected humans exhibit a predominantly $CD8^+$ T cell response against HIV-1 antigens [34]. In the same subset of 44 participants, the correlation between cellular immune responses measured by IFN-γ ELISPOT and IFN-γ intracytokine staining in the $CD8^+CD3^+$ subpopulation was $\rho = 0.72$ ($P < .0001$).

Evaluation of cross-clade CMI responses. Figure 2 shows $\log_{10}$-transformed ELISPOT responses to HIV-1 Gag and Nef proteins. The 45° line in each graph represents the points for which ELISPOT responses to stimulation by the clade proteins represented on the *X*-axis are quantitatively the same as responses to stimulation by the clade proteins represented on the *Y*-axis. The data points comparing responses to one clade with responses to another clade cluster relatively tightly around the 45° identity line. The slopes of the regression lines for each of the graphs in figure 2 were calculated using the no-constant option, so that the regression line originates at the 0 point on both axes and is analogous to the ratios shown in table 3. The slopes of the regression lines (figure 2) were very close to 1.0, which is the slope of the 45° identity line; therefore, regression lines are not shown.

A quantitative assessment of the ratios of ELISPOT responses to the 3 major HIV-1 clades tested in the present study is presented in table 3. The founder clade in each country was used in the denominator of the ratio, and each of the other 2 clades was used (separately) in the numerator. The geometric mean ratios of cellular immune responses in the entire study population were 0.93 (95% confidence interval [CI], 0.82–1.04) against clade A Gag versus clade B Gag, 0.99 (95% CI, 0.90–1.10) against clade C Gag versus clade B Gag, 0.97 (95% CI, 0.87–1.09) against clade A Nef versus clade B Nef, and 0.98 (95% CI, 0.87–1.10) against clade C Nef versus clade B Nef.

The geometric mean of cross-clade ratios was calculated independently for each country. The data indicate various degrees of lower CMI responses to heterologous clades in comparison with homologous clades, depending on the population and protein evaluated. The lowest cross-clade reactivity was observed for the anti-Gag responses among South African and Malawian participants (clade C founder), but their anti-Nef CMI responses did not show cross-clade attenuation. When Gag and Nef cross-clade reactivity was paired and the higher of the pair selected to represent the cross-clade reactivity of each clade comparison, the lowest cross-clade reactivity was noted among US participants, for the ratio of clade C versus clade B (0.65). The second lowest cross-clade reactivity, 0.73, was observed among Malawians for the ratio of clade A versus clade C and among Thais for the ratio of clade B versus clade A.

The differences between the anti-Gag and anti-Nef cross-clade reactivities shown in table 3 were evaluated by ranking

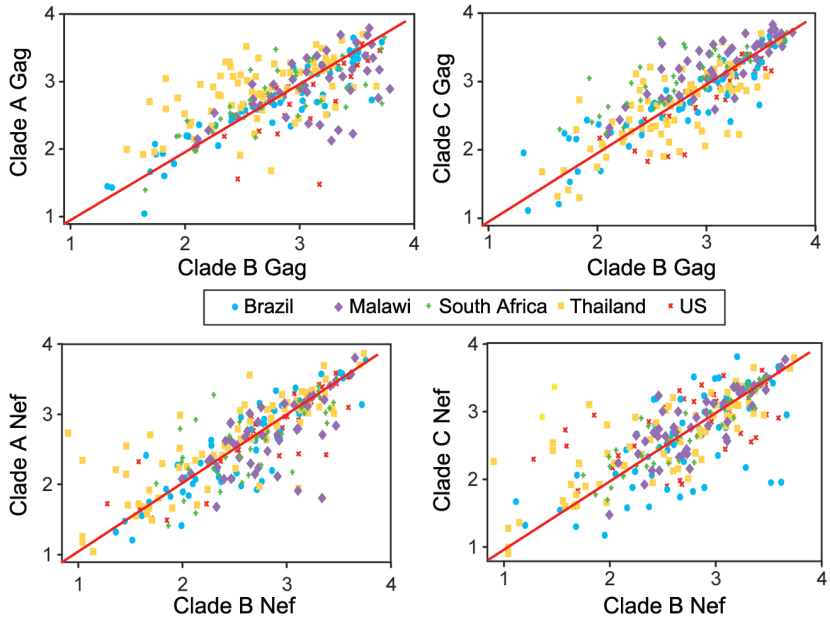

Figure 2. Cross-clade reactivity of anti–HIV-1 T cell immune responses against HIV-1 Gag and Nef (see text). Values on the axes represent the $\log_{10}$ no. of interferon (IFN)–γ–expressing cells per million peripheral blood mononuclear cells. The slopes of the regression lines calculated using the no-constant option, so that the regression line originates at the 0 point on both axes, are as follows: clade C Gag vs. clade B Gag, 0.991 (95% confidence interval [CI], 0.977–1.007); clade A Gag vs. clade B Gag, 0.978 (95% CI, 0.958–0.993); clade C Nef vs. clade B Nef, 0.988 (95% CI, 0.970–1.007); and clade A Nef vs. clade B Nef, 0.984 (95% CI, 0.966–1.001).

the cross-clade reactivity for each study subject, by use of a signed rank test. Anti-Nef cross-clade reactivity was statistically significantly higher than anti-Gag cross-clade reactivity ($P < .0001$). The proportion of participants with positive ELISPOT responses to all 3 clade (A, B, and C) Gag proteins was 90%, to all 3 clade Nef proteins was 72%, and to either all 3 clade Gag or all 3 clade Nef proteins was 94%.

DISCUSSION

Most of the HIV-1 vaccine approaches that are currently being tested in human clinical trials include a component that is focused on eliciting antiviral CMI responses. Although there is a substantial body of evidence suggesting that vaccine-induced CMI responses possibly could control infection and prevent—or significantly delay—progression of AIDS, it is not clear whether vaccine antigens can be defined that are capable of providing broad coverage against the genetically diverse virus variants prevalent throughout the world. Therefore, we have studied this issue by evaluating which HIV-1 antigens appear to be the most broadly recognized by the CMI response in participants infected with such genetically diverse viruses. We then quantified the extent of cross-reactive responses for 2 of these antigens. The results suggest that broadly effective vaccine-induced CMI responses may be feasible and provide guidance for the selection of vaccine antigens.

Some previous studies sharing these objectives have identified Gag, Pol, Nef, and Env proteins as the most common HIV-1 antigen targets for CTL responses, whereas other studies have emphasized the importance of Rev and Tat responses [35]. Investigators have also reported that cross-clade responses are either limited [4] or common [18, 36–39]. Explanations for these apparently conflicting findings include small sample size and a focus on single epitopes; different assay methodologies (e.g., cytokine detection vs. cytotoxic assays) that may measure different response attributes or have different quantitative potential also contribute to the apparent discrepant conclusions. Previously conducted studies were restricted to a maximum of 2 countries, and the selection of HIV-positive participants was inconsistent and often varied by geographic region. All of the studies provided a qualitative assessment of cross-clade reactivity; however, a quantitative assessment of cross-clade reactivity is necessary for designing HIV-1 vaccines. We therefore conducted a more systematic and quantitative assessment of the cross-clade reactivity of CMI responses, by use of a single study protocol with standardized clinical and laboratory procedures at each study site and centralized IFN-γ ELISPOT assays conducted in 1 laboratory to ensure consistency.

Our data show that HIV-1–infected individuals have quantitatively stronger CMI responses to Gag, Nef, and Pol, in comparison with responses to clade B Rev and Tat proteins, within each of the 5 countries studied. The ratio of ELISPOT responses to stimulation by heterologous clade A, B, and C Gag and Nef

Table 3. Ratios of cellular immune responses against HIV-1 Gag and Nef proteins, for heterologous clades versus homologous clades, among 250 HIV-1–infected participants.

Country, clade comparison	Immune response ratio, geometric mean (95% CI)	
	Gag	Nef
Brazil[a] (n = 55)		
A vs. B	0.77 (0.64–0.91)	0.89 (0.74–1.08)
C vs. B	0.82 (0.68–0.98)	0.95 (0.78–1.14)
Malawi[b] (n = 45)		
A vs. C	0.50 (0.39–0.62)	0.73 (0.57–0.94)
B vs. C	0.73 (0.60–0.88)	1.02 (0.83–1.24)
South Africa[b] (n = 38)		
A vs. C	0.47 (0.38–0.57)	0.90 (0.65–1.24)
B vs. C	0.51 (0.40–0.66)	1.04 (0.87–1.24)
Thailand[c] (n = 75)		
B vs. A	0.64 (0.50–0.81)	0.73 (0.58–0.91)
C vs. A	0.53 (0.43–0.65)	0.92 (0.72–1.19)
United States[d] (n = 37)		
A vs. B	0.63 (0.48–0.81)	0.89 (0.70–1.12)
C vs. B	0.65 (0.55–0.77)	0.63 (0.42–0.95)

NOTE. Values in the table represent the geometric means of enzyme-linked immunospot (ELISPOT) response ratios against Gag or Nef for each study population. For each participant, the ratio of ELISPOT responses to one clade vs. another was calculated. The geometric mean of the ratio for the study population was then calculated. For each country, the ratios of heterologous clades relative to the homologous clade that is predominant in that country are shown.

[a] HIV-1 clade frequencies are as follows: clade B, 78%; clade F, 15%; clade C, 5%.

[b] Clade C is the founding HIV-1 clade.

[c] HIV-1 clade frequencies are as follows: CRF01_AE, 90%; clade B, 10%.

[d] Clade B is the founding HIV-1 clade.

antigens versus responses to the infecting virus clade that is predominant in a country indicated substantial cross-reactivities. The lowest level of cross-clade reactivity was apparent among southern African participants, for anti-Gag responses, but this lower cross-clade reactivity was not observed for anti-Nef responses. Indeed, anti-Nef cross-clade reactivity exceeded that of anti-Gag. These results suggest that the anti-Nef response may likely be focused on the internal region that is highly conserved across clades. The cross-clade ratios were not influenced by the $CD4^+$ T cell count, plasma viral load, age, or sex of the participants (data not shown).

The relatively high degree of cross-clade immune responses that we have measured probably occurs in large part as a result of the substantial protein sequence conservation observed for Gag and Nef. Pol is more conserved (>90%) than Gag (>80%) and Nef (>70%) across clades A, B, and C. Cross-clade reactivity to Pol, other than using clade B reference peptides in figure 1, was not further assessed in this study. Its higher degree of conservation would likely mean that anti-Pol cross-clade reactivity would be similar to, or higher than, the observed anti-Gag and anti-Nef cross-clade reactivity. Preliminary epitope mapping for a subset of subjects by use of 9mer and 15mer peptides representing clade B Gag and Nef indicated that there were 2–13 epitopes recognized per participant tested. The recognized epitopes were distributed throughout the length of the Gag and Nef protein and varied from individual to individual. The recognized epitopes appeared to contribute to cross-clade reactivity because (1) peptide sequences were identical between clades, (2) peptide sequences differed by only 1 or 2 conservative amino acid substitutions between clades, or (3) peptide sequences differed by multiple or nonconservative substitutions. The first of these instances, and sometimes the second, would be expected to yield equivalent and biologically significant affinity associations between T cells and target cells, irrespective of clade. By contrast, the last instance could result in lower affinity associations and, therefore, could mediate limited biological activity. Extensions of these observations are an important corollary to the present study and will be pursued for a better understanding of the basis for cross-clade immunity.

Either Gag or Nef cross-clade responses can be somewhat low in some cases, but at least 1 tends to be high in any given circumstance. Therefore, the likelihood of producing cross-clade responses is greater with both antigens than with either alone and would be further enhanced by the inclusion of Pol. It is logical to project from these results that the most effective CTL-based vaccine would be one that drives responses to as many epitopes as possible, as well as to the most-conserved determinants, to increase the probability that vaccinated individuals will have potential matches against epitopes within viruses they may encounter. Such broad responses could also exert multiple immunological pressures against the virus and help minimize the selection of escape mutants. In conclusion, our results show that substantial cross-clade CMI responses exist across the 3 clades that represent most HIV-1 infections worldwide, regardless of infecting virus genotype. This observation suggests that similar CMI responses elicited by a vaccine composed of antigens based on a single clade—especially for Gag, Pol, and Nef—could possibly be broadly effective and warrant further testing in clinical trials.

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