HIV-1 Subtype C Gag-Specific T-Cell Responses in Relation to Human Leukocyte Antigens in a Diverse Population of HIV-Infected Ethiopians

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Summary: Knowledge of the most dominant T-cell epitopes in the context of the local human leukocyte antigen (HLA) background is a prerequisite for the development of an effective HIV vaccine. In 100 Ethiopian subjects, 16 different HLA-A, 23 HLA-B, and 12 HLA-C specificities were observed. Ninety-four percent of the population carried at least 1 of the 5 most common HLA-A and/or HLA-B specificities. HIV-specific T-cell responses were measured in 48 HIV-infected Ethiopian subjects representing a wide range of ethnicities in Ethiopia using the interferon (IFN)-γ enzyme-linked immunospot (Elispot) assay and 49 clade C–specific synthetic Gag peptides. Fifty-eight percent of the HIV-positive study subjects showed T-cell responses directed to 1 or more HIV Gag peptides. Most Gag-specific responses were directed against the subset of peptides spanning Gag p24. The breadth of response ranged from 1 to 9 peptides, with most (78%) individuals showing detectable responses to <3 Gag peptides. The magnitude of HIV-specific T-cell responses was not associated with HIV viral load but correlated positively with $CD4^+$ T-cell counts. The most frequently targeted Gag peptides overlapped with those previously described for HIV-1 subtype C–infected southern Africans, and therefore can be used in a multiethnic vaccine.

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More than 20 years after the first clinical evidence of AIDS,[1] combating HIV/AIDS is still a major global challenge. Ethiopia, like most sub-Saharan African countries, has been experiencing a severe HIV/AIDS epidemic starting in the mid-1980s. The first sera found positive for HIV-1 antibodies date back to 1984,[2] which concurs with the estimated time of introduction of HIV-1 to the country.[3,4] The epidemic has spread rapidly, and the prevalence rate between 1994 and 2001 in the urban population was 14% to 20% among pregnant women;[5,6] 7% in blood donors;[5] 6% to 12% in the general population, including army and police recruits;[3,5] and 47% to 74% among commercial sex workers.[7,8] The number of Ethiopians living with HIV/AIDS in 2001 was estimated at 2.2 million, including 200,000 children (report from Ministry of Health, 2002). Unlike the neighboring East African countries, the HIV epidemic in Ethiopia is predominantly of subtype C[8–13] with a subcluster C'.[2,14] Despite low-background $CD4^+$ T-cell numbers in healthy Ethiopians,[15–17] HIV disease progression in Ethiopia is not faster than in other countries.[18]

Although prevention remains the hallmark of controlling the HIV/AIDS pandemic, a major expectation for curbing the expanding HIV pandemic globally relies on the development of an effective vaccine to complement other preventive strategies, and attention has focused on the use of vaccines that are able to induce cytotoxic T lymphocytes (CTLs).[19] Indeed, several lines of evidence point to a key role of CTLs in the control of HIV infection: HIV-specific CTL responses have been detected in exposed but noninfected subjects;[20–22] protection of severe combined immunodeficiency (SCID) mice against HIV infection has been induced by $CD8^+$ T-cell infusion;[23] $CD8^+$ T-cell depletion in simian immunodeficiency virus (SIV)–infected monkeys has been shown to be associated with increased viral replication, which reverses when T cells repopulate;[24,25] positive correlations have been

found between strong CTL responses and virus clearance in acute infection and/or delaying of overt disease;[26–31] and occurrence of CTL escape mutants, as a result of CTL-mediated selective pressure on the virus,[32] has been found to be associated with disease progression[33] and with anti-AIDS vaccine failure in rhesus monkeys.[34] Several studies further suggest that the ability of $CD8^+$ CTLs to persist and remain functional is highly dependent on the presence of a $CD4^+$ T helper cell response.[35]

Most data on CTL responses in HIV-infected patients are from clade B–infected subjects, although subtype C affects more infected persons worldwide than any other HIV clade.[36] There are several reports on HIV-1 clade C–specific CTL responses in subjects from sub-Saharan Africa,[37–40] of which most have been carried out in South Africa[14,41–44] and only 1 has been conducted among 35 HIV-infected Ethiopian Jews living in Israel.[45] Because Ethiopian Jews constitute only a small proportion of the Ethiopian population, the latter study may not be representative of the total Ethiopian population. The characteristic HIV epitopes that are recognized by CTLs vary between HIV-infected persons of different ethnic origins because of differences in the frequencies of human leukocyte antigen (HLA) class I molecules between populations. Knowledge of the dominant and subdominant epitopes in the context of the local HLA background aids in the design and development of an effective HIV vaccine that would ideally elicit a potent and broad immune response. We therefore studied the HLA background of 50 HIV-infected and 50 HIV-negative Ethiopian subjects from different ethnic groups and performed a cross-sectional study of $CD8^+$ T-cell responses against HIV-1 subtype C synthetic Gag peptides in 48 HIV-infected Ethiopians.

SUBJECTS AND METHODS

Subjects

Study subjects were HIV-infected and noninfected adult factory workers participating in a long-term cohort study on HIV-1 incidence and progression carried out by the Ethiopian-Netherlands AIDS Research Project (ENARP) at the Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa, Ethiopia. A detailed description of the cohort studies has been reported elsewhere.[46,47] In brief, the ENARP established 2 cohort sites: 1 in Akaki, a suburb of Addis Ababa, the capital city, and 1 in Wonji, a sugar estate, 114 km southeast of Addis Ababa. The cohort studies started in February 1997 in Akaki and in October 1997 in Wonji after pilot surveys in 1995 through 1996. All study participants came to the study clinic every 6 months. A total of 1612 subjects were enrolled from 1997 to June 2001 from both sites, of which 9.4% were positive for HIV-1 antibodies. Among the 150 HIV-positive participants, we randomly selected 50 subjects aged 23 to 49 (median = 35) years for HLA typing. Forty-one of the subjects were prevalent cases, whereas 9 were incident cases. Fifty HIV-negative subjects aged 23 to 48 (median = 38) years were included in this study in the same proportion as the HIV-positive individuals from both cohort sites. As shown in Table 1, the study group comprises the 2 major ethnic groups in Ethiopia, Oromo (n = 45) and Amhara (n = 28), which, together, represent more than 60% of the Ethiopian population, and 23 subjects from the southern ethnic groups (Kembata [n = 11], Hadiya [n = 5], Gurage [n = 5], and Wolayta [n = 5]).

TABLE 1. Selected Sociodemographic Characteristics of the HLA Study Subjects

	All (n = 100)	HIV-Negative (n = 50)	HIV-Positive (n = 50)
Age (y)			
Median (range)	36 (23–49)	38 (24–48)	35 (23–49)
Gender			
Male	72	35	37
Female	28	15	13
Ethnicity			
Oromo	45	20	25
Amhara	28	15	13
South (K, H, G, W)	23	13	10
Mixed	2	1	1
Tigray	1	0	1
Others	1	1	0

G indicates Gurage; H, Hadiya; K, Kembata; W, Wolayta.

For all HIV-infected subjects, $CD4^+$ T-cell numbers and viral loads (using the Nuclisens assay; Organon Teknica, Oss, The Netherlands) were determined at each visit. We studied HIV-specific T-cell responses cross sectionally in 48 of the 50 HIV-infected subjects, who were aged 18 to 57 (median = 34) years, at the laboratory of Sanquin Research, Amsterdam, The Netherlands. Characteristics of the study subjects are summarized in Table 2. Because antiretroviral treatment (ART) was not available in Ethiopia during the study period, all subjects were naive to ART.

Peripheral Blood Mononuclear Cell Separation and HIV Peptides

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation and viably frozen using a computerized freezing machine (KRYO 10, Biomedical Series II, Cryotech, Schagen, The Netherlands). Frozen cells were stored in liquid nitrogen until analyzed.

Forty-nine clade C–specific synthetic Gag peptides, whose sequences correspond to the isolate 96 ZM 651.8 (from the National Institutes of Health [NIH]) were used for stimulation. The peptides were 20 amino acids in length, overlapping by 10 amino acids. T-cell responses were determined directly ex vivo after stimulation of PBMCs with the whole pool (49 peptides; total HIV Gag T-cell response) and with 14 subpools, each containing 7 peptides. The final concentration of each peptide was 2 μg/mL. To identify the specific peptide(s) responsible for the observed T-cell responses, we designed a matrix consisting of 7 pools in columns and 7 pools in rows, where each pool in a column contains 1 peptide from each pool in the rows (as described by Mashishi and Gray[48]).

TABLE 2. Characteristics of the Subjects in the CTL Study (n = 48)

	All Subjects (n = 48)
Age (y)	34 [23–49]*
20–29	15% [7/48]
30–45	81% [39/48]
45+	4% [2/48]
Gender	
Male	36
Female	12
Absolute CD4 count (cells/μL)	387 [95–935]*
<200	23% [11/48]
200–500	46% [22/48]
>500	31% [15/48]
Absolute naive CD4 count (cells/μL)	66 [2–366]*
Viral load (log_{10} copies/mL)	3.65 [1.90–5.53]*
$CD4^+$ T cells (%)	
$CD45RO^-CD27^+$ (naive)	21.9
$CD45RO^+CD27^+$ (memory)	43.7
$CD45RO^+CD27^-$ (memory/effector)	26.3
$CD45RO^-CD27^-$ (effector)	1.6
$HLA\text{-}DR^+CD38^+$	6.6
$CD31^+$ within naive	72.4
CD8+ T cells (%)	
$CD45RO^-CD27^+$ (naive)	14.7
$CD45RO^-CD27^+$ (memory)	21.9
$CD45RO^+CD27^-$ (memory/effector)	21.0
$CD45RO^-CD27^-$ (effector)	35.4
$HLA\text{-}DR^+CD38^+$	23.4
$CD57^+$	59.5

*Median [range].

Human Leukocyte Antigen Class I Typing

DNA for typing was isolated from PBMCs using L6 lysis buffer. PBMCs were thawed in 20% fetal calf serum (FCS); after washing with 10% FCS, 1 mL of L6 buffer (containing 0.1 M of Tris-hydrogen chloride [HCl], 1.2 g/mL of guanidine isothiocyanate, ethylenediaminetetraacetic acid [EDTA], and Triton X-100, Roche, Mannheim, Germany) was added and vortexed. The crude lysate was transported to Sanquin, The Netherlands. Two polymerase chain reaction (PCR)–based DNA typing methods, PCR amplification with sequence-specific primers (PCR-SSP) and PCR amplification and subsequent hybridization with sequence-specific oligonucleotide probes (PCR-SSOP), have been evaluated and recommended for better assignment of all HLA class I specificities than the less precise serologic techniques, especially for nonwhite subjects. Therefore, our subjects were tested using the former 2 methods.

Interferon-γ Enzyme-Linked Immunospot Assay to Measure HIV-Specific T-Cell Responses

HIV-specific T-cell responses were measured using the interferon (IFN)-γ enzyme-linked immunospot (Elispot) assay. To this end, multiscreen 96-well membrane-bottomed plates (MAIP N45; Millipore, Billerica, MA) were coated with 50 μL (5 μg/mL in phosphate-buffered saline [PBS]) of antihuman IFNγ monoclonal antibody (mAb 1-D1K; Mabtech, Nacka, Sweden) and kept at room temperature (RT) for 3 hours or at 4°C overnight. Plates were thoroughly washed with PBS containing 0.005% Tween-20 (Sigma GMBH, St. Louis, MO) and blocked with RPMI-1640 medium containing 10% FCS for 1 hour. Frozen PBMCs were thawed and suspended in RPMI-1640 medium containing 10% FCS and penicillin/streptomycin. The viability of the cells was checked using trypan blue staining. Overall viability was >80%. Cells were incubated at a final concentration of 1 to 2 × 10^5 cells/100 μL per well (adjusted by the viability of the cells) in the presence of peptides (whole pool and subpools) for 20 to 24 hours in a humidified 5% carbon dioxide (CO_2) atmosphere at 37°C. After incubation, cells were removed by washing wells 5 times with 0.005% PBS/Tween (PBST). Biotin-conjugated mAb 7-B6-1 for IFNγ (Mabtech) was added at optimal dilution (1 μg/mL in PBS) and incubated at RT for 1 hour. After 5 washes with PBST, 50 μL of 1:6000 diluted streptavidin–horseradish peroxidase (HRP) (Sanquin) was added, and plates were incubated at RT for 1 hour. Unbound conjugate was removed by washing thoroughly with PBST and, finally, 50 μL of tetramethylbenzidine (TMB) substrate solution (Sanquin) was added and incubated until the appearance of spots in the wells (5–15 minutes). Color development was stopped by rinsing in demi H_2O (dH_2O) and, after drying, the number of IFNγ-producing cells was determined using an Elispot reader (A.EL.VIS V3.31B; A.EL.VIS GMBH, Hanover, Germany) and expressed as spot-forming cells (SFCs) per million input PBMCs. Phytohemagglutinin (PHA; Murex, Dartford, England)-stimulated wells were used as a positive control, and medium alone was used as a negative control. All experiments were done in triplicate. The number of IFNγ-producing cells was calculated by subtracting the negative control value. Samples with >100 SFCs per million PBMCs were considered positive.[28,41,49]

Flow Cytometric Analysis of T-Cell Maturation and Activation Markers

To evaluate the maturation state of $CD8^+$ T cells, cryopreserved PBMCs were thawed and incubated with CD8–peridinin–chlorophyll-A protein (PerCP); CD27–fluorescein isothiocyanate (FITC); and unlabeled CD57, a proposed marker for senescence[50] (all from Becton Dickinson Immunocytometry Systems, San Jose, CA) as well as with CD45RO-allophycocyanin (APC; BD Pharmingen, San Jose, CA). CD31 expression was analyzed as a marker for naive T cells in addition to CD27 and CD45RO. After washing with PBS/0.5% bovine serum albumin (BSA) (PBA), cells were incubated with phycoerythrin (PE)-conjugated IgM (SouthernBiotech, Birmingham, AL) to label $CD57^+CD8^+$ T cells. To measure the expression of CD31 on naive $CD4^+$ T cells, cells were stained with CD4-PerCP, CD27-FITC, and CD31-PE (all from Becton Dickinson Immunocytometry Systems) as well as with CD45RO-APC. In addition, expression of activation markers on $CD4^+$ and $CD8^+$ T cells was measured after staining cells with CD4-APC, CD8-PerCP, CD38-FITC, and D-related HLA (HLA-DR) PE (all from Becton Dickinson

Immunocytometry Systems). All incubation steps were performed at 4°C for 20 minutes. Finally, cells in all CD4 and CD8 preparations were washed with PBA, fixed with Cellfix (Becton Dickinson Immunocytometry Systems), and analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) with Cellquest software.

Intracellular Staining for Interferon-γ and Flow Cytometric Analysis

PBMCs at a final concentration of 2×20^6/mL in RF-10 medium were stimulated with the total pool of HIV-1 clade C–specific synthetic Gag peptides for 16 to 18 hours in a humidified 5% CO_2 atmosphere at 37°C. Cells were cultured in flat-bottom 24-well plates (Nunc, Roshilde, Denmark) in a final volume of 1 mL/well. After 2 hours of incubation, the intracellular protein transport inhibitor Brefeldin A (BFA; Becton Dickinson Immunocytometry Systems), was added at a final concentration of 5 μg/mL to inhibit cytokine secretion and to allow intracellular detection. Cells were harvested and washed with PBA. For surface marker staining, the cell pellet was incubated with APC-conjugated CD4, PerCP-conjugated CD3, and PE-conjugated CD8 (Becton Dickinson Immunocytometry Systems). After washing with PBA, cells were fixed and permeabilized by incubating cells with 10% fluorescent-activated cell sorting (FACS) permeabilization buffer and FACS lysing solution (Becton Dickinson Immunocytometry Systems), after which cells were washed and subjected to intracellular staining using saturated concentrations of FITC-conjugated IFNγ mAbs (Becton Dickinson Immunocytometry Systems). All incubation steps were performed at 4°C for 20 minutes; for fixation and permeabilization, samples were kept at RT for 10 minutes. After a final wash, cells were fixed using Cellfix and 100,000 to 200,000 lymphocytes were analyzed on a 4-color FACSCalibur using Cellquest software. Results were expressed as percentages of total $CD4^+$ or $CD8^+$ T-cell counts. Quadrant markers were set based on the no-antigen controls as a reference for each subject, which ranged from 0% to 0.2%. Frequencies of cytokine-producing T cells were calculated after subtraction of frequencies measured in medium controls and considered positive when they attained the previous background levels found in HIV-negative individuals (median = 0.2% of $CD4^+$ T cells), as previously described.[51]

Statistical Analysis

Data were analyzed using STATA statistical software (version 6.0; Stata Corporation, College Station, TX) and SPSS (SPSS, Chicago, IL). Differences between groups were estimated using the χ^2 test. The Fisher exact test was used whenever expected values were lower than 5. Correlations were calculated using Spearman rank correlation coefficients. P values <0.05 were considered significant.

RESULTS

Human Leukocyte Antigen Class I Frequencies in Ethiopians

To determine HLA class I frequencies, we studied a total of 100 HIV-infected and noninfected Ethiopians, 50 in each group, participating in a long-term cohort study on HIV-1 incidence and disease progression. More than 70% of the study subjects are from the 2 major ethnic groups in Ethiopia: Oromo (n = 45) and Amhara (n = 28). As depicted in Table 1, there were no significant differences in age, gender, or ethnic composition between HIV-infected and noninfected subjects.

Sixteen different HLA-A, 23 HLA-B, and 12 HLA-C specificities were observed in our study subjects (Fig. 1). There were no significant differences between the distribution of HLA specificities of the 2 major ethnic groups in Ethiopia, except for higher frequencies of HLA-B13 (14 of 45 vs. 2 of 28, respectively; $P = 0.016$) and Cw06 (19 of 45 vs. 3 of 28, respectively; $P = 0.004$) in the Oromo compared with the Amhara participants.

To estimate the population coverage of an HLA-based vaccine that could be designed for Ethiopians, we determined the percentage of subjects carrying the 10 most frequent HLA-A and HLA-B alleles, which occurred at a frequency of more than 7% among the study subjects. Eighteen percent of the population carried 4, 32% carried 3, and 34% carried 2 of the 10 most common HLA-A and HLA-B alleles. Ninety-four percent of the population carried at least 1 of the common HLA-A and/or HLA-B specificities. Interestingly, HLA-Cw7 ($P = 0.016$) and HLA-A2 ($P = 0.04$) were more prevalent among HIV-positive compared with HIV-negative individuals.

We compared our data with a published study on HLA-A and HLA-B frequencies in 35 Ethiopian Jews who immigrated to Israel from the northern part of Ethiopia.[23] Because the Ethiopian Jews studied were all HIV-positive and HLA frequencies vary between HIV-infected and noninfected subjects, we restricted our comparison to HIV-infected subjects only. In general, differences in the distributions of HLA-A specificities were small between the Ethiopian subjects of the present study and the Ethiopian Jews (see Fig. 1A), except for HLA-A32, which was significantly lower in Ethiopian Jews. More differences were observed at the B locus, however, at which Ethiopian Jews in Israel had higher frequencies of HLA-B49 and HLA-B51 and lower frequencies of HLA-B27 (see Fig. 1B).

HIV-1 Gag-Specific Responses in HIV-Infected Ethiopians

PBMCs from 48 of the 50 HLA-typed HIV 1C–infected Ethiopians were screened for HIV-1 subtype C Gag-specific T-cell responses using 20-mer synthetic peptides overlapping by 10 amino acids in the Elispot assay. Using a peptide matrix-based approach,[6] 58% (28 of 48) of the study subjects showed demonstrable T-cell responses directed to 1 or more HIV Gag-peptides (responders, >100 CFSs/10^6 PBMCs), whereas the remaining 42% (20 of 48) lacked detectable HIV Gag-specific responses despite a good response to mitogens (PHA; nonresponders; Fig 2). The latter suggests that the cells had good viability, and trypan blue staining confirmed >80% viability (data not shown). The breadth of response ranged between 1 and 9 peptides in the responders; most of them (78%) showed detectable responses to <3 peptides. As depicted in Figure 2, 39% of the responders (11 of 28) showed a T-cell response directed to 1 peptide, 39% (11 of 28) showed a T-cell response directed to 2 peptides, and the remaining 22%

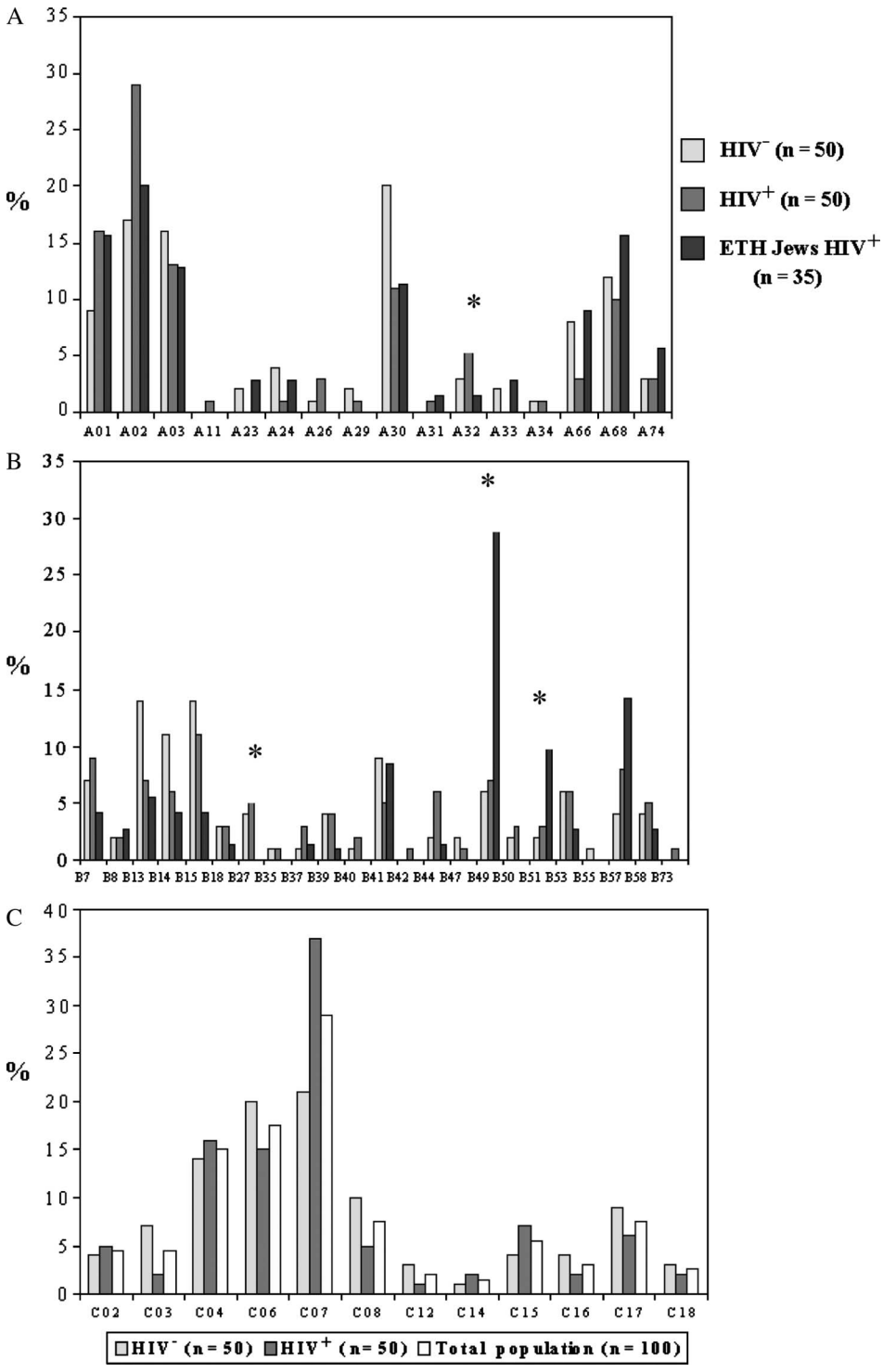

FIGURE 1. Frequencies of HLA-A, HLA-B, and HLA-C specificities in the ENARP cohorts. Frequencies of HLA-A (A), HLA-B (B), and HLA-C (C) specificities in the ENARP cohorts by HIV status and comparison with HIV-infected Ethiopian Jews (n = 35). HLA-C allelic frequency is not available for Ethiopian Jews. *Significant differences between Ethiopians in this study and Ethiopian Jews.

(6 of 28) showed a T-cell response directed to more than 2 peptides. Overall, responses were directed against 25 of the 49 peptides. Sixteen of the 25 peptides targeted by the study subjects were from the p24 region of Gag (Fig. 3). The absence or presence of HIV Gag-specific responses did not correlate with differences in $CD4^+$ T-cell counts, viral load, or T-cell maturation or activation status (data not shown). There was only 1 significant correlation with HLA type (Fig. 4). Individuals with HLA-B15 tended to be nonresponders (7 of 10 participants), whereas individuals with HLA-A32 tended to be responders (6 of 7 participants). Interestingly, HLA-A32 has been shown to be related to slow disease progression (relative hazard [RH] = 0.0; $P = 0.07$) among HIV-infected African Americans.[52]

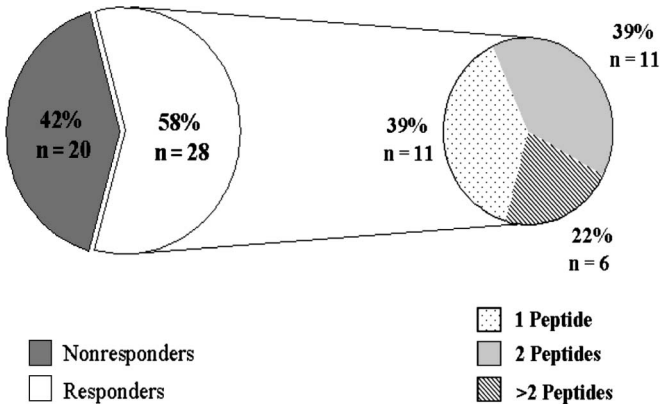

FIGURE 2. Proportion of responders and nonresponders to HIV-1 subtype C Gag peptides The left pie chart gives the number and percentage of individuals who responded (white area) or did not respond (gray area) to stimulation with Gag peptides (total pool) as measured by IFNγ Elispot assay. The small pie chart on the right gives the proportion of responders for the different numbers of Gag peptides targeted (based on the matrix approach described in the methods section).

In 5 individuals, we performed intracellular cytokine staining after Gag pool stimulation to investigate to what extent the T-cell responses observed in Elispot analysis were attributable to $CD8^+$ T-cell responses. In addition, we analyzed several time points during follow-up for an indication of the stability of the response. Proportions of IFNγ-producing $CD4^+$ T cells were generally low (see Figure 5A for a representative dot plot) and were always at least 4-fold lower than the proportion of IFNγ-producing $CD8^+$ T cells, reaching up to 0.19%. Predominant $CD8^+$ T-cell responses were observed in all subjects analyzed, with $CD8^+$ T-cell responses up to 0.92% (see Fig. 5B).

No Association Between Magnitude of T-Cell Response and Viral Load

The overall total magnitude of HIV-1 Gag-specific T-cell responses in the 28 responding subjects ranged from 126 to 5813 (median = 395) SFCs per million input PBMCs (Table 3). The highest magnitude was detected within the subset of peptides spanning Gag p24, ranging from 0 to 5813 (median = 428) SFCs/10^6 PBMCs. The magnitude of response in the p17 region ranged from 0 to 1530 (median = 302) SFCs/10^6 PBMCs, which is significantly lower than the response against peptides from the p24 region ($P = 0.04$; see Fig. 3).

We studied whether the magnitude of CTL response against the whole Gag peptide pool in the 28 responding subjects correlated with control of HIV infection. We found no significant correlation between the magnitude of CTL response and viral load, but there was a significant positive correlation between the magnitude of CTL response and $CD4^+$ T-cell numbers ($P = 0.01$; data not shown).

Identification of HIV-1 Subtype C Gag Peptides Targeted by T Cells in the Study Subjects

Based on the clade C subpool analysis, we were able to identify specific peptide responses. As summarized in Table 3 and Figure 2, of the 49 20-mer overlapping Gag peptides, the most frequent responses were directed to 4 peptides: IYKRWIILGLNKIVRMYSPV (amino acid [aa] ~261–280 [n = 8]), KTILKALGPGATLEEMMTAC (aa 330–349 [n = 8]), MFTALSEGATPQDLNTMLNT (aa 170–189 [n = 6]), and IKHLVWASRELERFALNPGL (aa 31–50 [n = 6]). Of the 25 peptides from Gag that induced a response in at least 1 individual, most (16 of 25) peptides came from the p24 region, including 3 of the 4 most dominant responses. Interestingly, 6 of the 8 subjects responding to the peptide IYKRWIILGLN-KIVRMYSPV were HLA-B27 carriers (3 of 8) or carriers of other HLA types belonging to the B27 supertype, such as

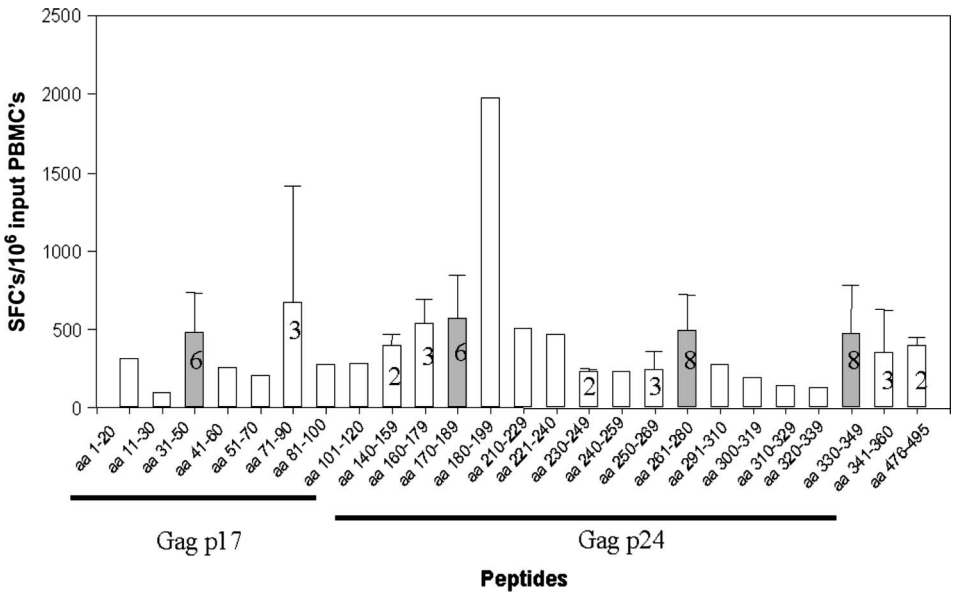

FIGURE 3. CTL response per peptide targeted. Mean number of SFCs/10^6 PBMCs per peptide targeted by the study subjects. Gray bars indicate the 4 main targeted peptides. The number of responding subjects is shown inside each bar and is not indicated when a response was observed in 1 subject only. The error bars indicate 1 SD above the mean.

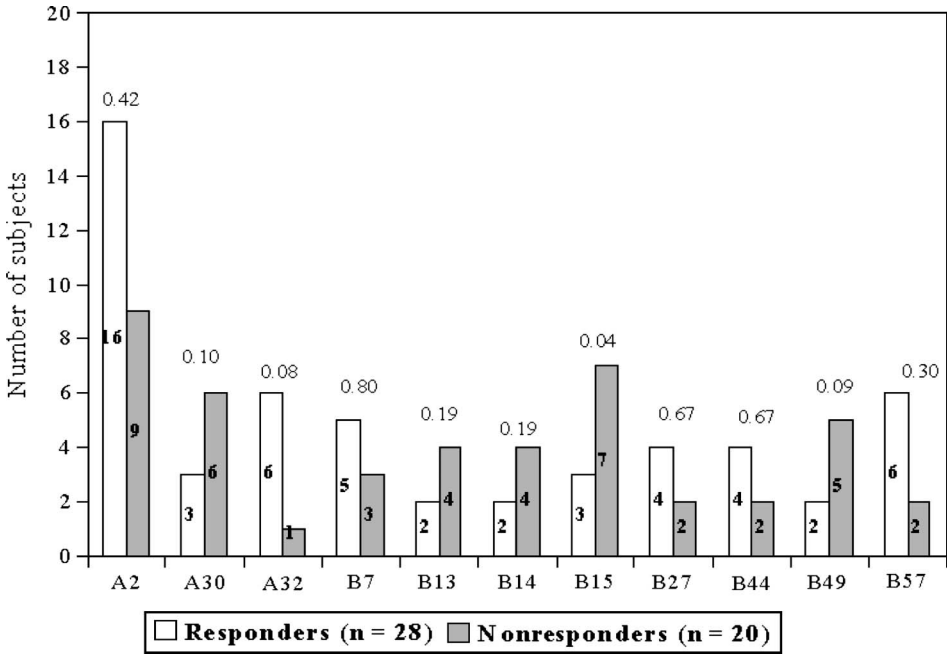

FIGURE 4. Distribution of selected HLA alleles among responders and nonresponders. Number of subjects responding (white bars) or not responding (gray bars) to Gag peptides in the Elispot assay in relation to their HLA types. The number of subjects in each group is indicated inside each bar, and *P* values are provided above the bars. Two-sided *P* values were calculated using the Student *t* test.

HLA-B14 and HLA-B39.[53] The remaining 2 subjects responding to this peptide were carriers of HLA-B15, which belongs to the supertypes B27, B58, and B62 according to Sette and Sidney's 1999 classification.[53] Responses to the KTILKALGPGATLEEMMTAC peptide were also common in our subjects. HLA-B7 is a common HLA supertype (including HLA-B7 and HLA-B53) in subjects responding against the latter peptide.

The second most frequent response was directed to the peptide MFTALSEGATPQDLNTMLNT containing the TPQDLNTML (TL9) epitope, which is the most dominant epitope in HIV-infected individuals from South Africa and Botswana, with HLA B*4201 and B*8201 restriction.[41] In the present study, only 1 of 6 responses against the peptide containing this epitope was associated with similar HLA restriction. Two of the subjects who targeted the peptide containing the TL9 epitope were carriers of HLA-B14-Cw8, an HLA association previously reported by Goulder et al.[41] Equally dominating as the TL9 responses were responses directed against the peptide IKHLVWASRELERFALNPGL in p17 Gag. Two of the responding subjects were HLA-B44 carriers, and 1 was B37-positive (a member of the B44 supertype). No specific HLA association could be identified for presentation of this peptide in the other 3 subjects.

Interestingly, despite the high frequency of HLA-A2 in the study group (25 [52%] of 48 subjects), only 3 HLA-A2–positive subjects responded to the peptide (aa 71–90) containing the most dominant A2-restricted white epitope SLYNTVATL (SL9).

DISCUSSION

Host genetics, through the distribution of HLA alleles, and viral genotype determine the pattern of T-cell response against HIV. The circulating HIV clade in Ethiopia has been well characterized, but there is only little information on HLA class I frequencies in Ethiopians. Only 1 previous publication has reported HLA-A and HLA-B frequencies among 35 HIV-positive Ethiopian Jews who immigrated to Israel.[45] The differences in HLA background between the previous study and the current study among 50 HIV-infected and 50 noninfected Ethiopians are likely attributable to differences in ethnic background. Because we included many different ethnic groups and approximately 70% of individuals were from the 2 major ethnic groups, Oromo and Amhara, we think the HLA distributions reported in the current study may be more representative of the general Ethiopian population.

HLA typing has been shown to be useful in predicting the percentage of the general population that could be targeted by an HIV vaccine designed on the basis of identified common HLA-A and HLA-B specificities.[49] Novitsky et al[49] have shown that a vaccine designed on the basis of 4 common HLA-A and 7 common HLA-B specificities in Botswana could target 97.5% of the Botswana population. Here, we found that 94% of the Ethiopian population carried 1 or more of the 10 most common HLA-A and HLA-B specificities, while 84%, 50%, and 18% of the study population carried, respectively, 2, 3, and 4 of these common HLA types, which is close to the estimates of 83.9%, 52.8%, and 12.4% reported in the Botswana population.[49]

The IFNγ Elispot assay has been shown to be a quick and reliable tool to identify CTL epitopes.[48] We therefore used this assay to identify immunodominant regions in HIV-1 subtype C Gag in HIV-infected Ethiopians in relation to their HLA background. Earlier subtype C Gag-specific CTL response studies have confirmed the $CD8^+$ specificity of the IFNγ response by performing $CD4^+$ and $CD8^+$ T-cell depletion and enrichment experiments using CD4 and CD8 magnetic

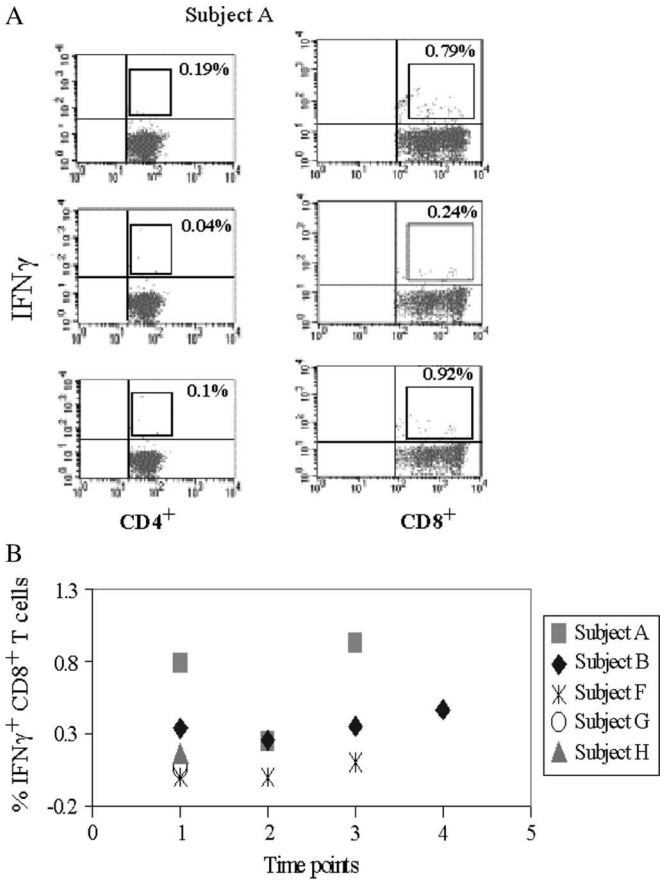

FIGURE 5. IFNγ-positive $CD8^+$ and $CD4^+$ T cells using intracellular staining after stimulation with the Gag peptide pool. A, Frequency of IFNγ-positive $CD8^+$ and $CD4^+$ T cells as assessed by intracellular staining of IFNγ in a subject with a slow $CD4^+$ T-cell decline at 3 different time points (20, 33, and 39 months after enrollment). B, Frequency of IFNγ-positive $CD8^+$ T cells in 5 subjects at several time points after enrollment in the ENARP cohort.

beads.[37,41,42] Here, we confirmed that $CD4^+$ T-cell responses were much lower than $CD8^+$ T-cell responses after stimulation with Gag peptide pools and intracellular cytokine staining, substantiating that the Elispot assay mainly measures $CD8^+$ T-cell responses.

Overall, HIV-1 Gag-specific $CD8^+$ T-cell responses in these Ethiopians were observed in a lower proportion of individuals than reported in other studies. Up to 48% of the subjects had no detectable HIV Gag-specific CTL response, whereas a lack of CTL responses directed to Gag proteins has been observed in only 16% of HIV-1C–infected children and adults from the Durban cohort,[41] in <14% of HIV-1B–infected individuals,[54] and in only 21.6% of HIV-1C–infected Africans.[37] We repeated the Elispot assay in 13 of the 20 nonresponders (data not shown), which confirmed the absence of detectable responses. Low responsiveness was not attributable to low viability of cells, because most individuals responded well to mitogens (PHA). It has been reported that T-cell responses to overlapping 20-mer peptides are generally lower than responses against 15-mer peptides. Together with the fairly conservative definition of positivity of the Elispot assay of >100 SFCs per million PBMCs, this may explain the relatively low number of responses observed, but it does not explain the discrepancies with previous studies, because they were based on the same 20-mer overlapping peptide pools and definition of Elispot assay positivity.[28,41,45,49] Another factor that may have contributed to the low fraction of individuals responding to Gag is the use of consensus HIV sequences, which may have underestimated the actual breadth and magnitude of the CTL response against autologous viruses.[55] Even though Gag is relatively conserved, there is a more than 10% amino acid difference between the available Gag sequences of Ethiopian HIV-C[56] (accession number U46016) and the Zambian Gag sequence that was used in the current study and in previous studies (96ZM651). After sequence alignment of different clade C strains, the Ethiopian clade C sequences were found to be similar to the South African clade C sequences (data not shown), however, thereby indicating similar sequence divergence in South African clade C compared with the Zambian clade C. Thus, differences in anti-Gag responses between Ethiopians and HIV-1C–infected South Africans cannot be explained by the use of the 96ZM651 Zambian clade C consensus.[41] Ethiopians therefore seem to have an intrinsic tendency to respond less to Gag peptides than other populations. The fact that the absolute frequencies of SFCs/10^6 PBMCs in the Ethiopians who did respond to Gag were similar to values that have previously been reported[28,41,45,49] suggests that we do not underestimate the anti-Gag response in our Ethiopian cohort. The relative paucity of Gag-specific T-cell responses among Ethiopians does not exclude the existence of broad T-cell responses to other HIV proteins across the viral genome, because broad responses against all HIV proteins have previously been demonstrated.[37,42,54,57] In addition, the Ethiopian Jews studied by Ferrari et al[45] were all individuals who had migrated to Israel and were no longer living in Ethiopia. It is suggested that individuals living in Ethiopia are continuously exposed to bacteria and parasites,[58,59] which has a profound effect on the level of T-cell activation and naive T-cell numbers.[16] This may further constitute differences in immunologic characteristics and explain lower responsiveness to Gag peptides in Ethiopians who are still exposed to a high antigen burden.[60]

We hypothesize that differences in CTL responses against Gag between different populations may be attributable to specific immunologic differences, including the HLA background. Indeed, the HLA background of our Ethiopian study population differs significantly from that of other African study populations, among which are individuals from Botswana, Zambia, and South Africa. Ethiopians have a relative higher frequency of HLA-A1, HLA-A2, HLA-A3, HLA-Cw7, and HLA-Cw15 and a lower frequency of HLA-A23, HLA-A29, HLA-A30, HLA-Cw2, and HLA-Cw16 compared with individuals from Botswana, Zambia, and South Africa. Most diversity among the 4 African countries was observed in the distribution of HLA-B alleles. Ethiopians have relatively higher frequencies of HLA-B13, HLA-B27, HLA-B41, and HLA-B49 and lower frequencies of HLA-B42,

TABLE 3. Summary of Magnitude of HIV-Specific CTL Responses Directed Against Overlapping Peptides Spanning Gag and HLA Types in the Study Subjects (n = 28)

Peptide Sequence	ID	HLA Type	SFCs/10^6 PBMCs
IKHLVWASRELERFALNPGL (p17/aa 31–50)	078	A2; B41/44; C17/07	293
	818	A*0201; B44/57; C04/07	763
	298	A32/66; B37/53; C06	574
	289	A1/A*0201; B13/15; C14/16	763
	143	A01/0201; B57/58; C07	302
	408	A01/03; B7; C07	190
GTEELRSLYNTVATLYCVHE (p17/aa 71–90)	886	A*0205/68; B51/44; C14/04	1530
	289	A1/A*0201; B13/15; C14/16	255
	763	A01/0205; B7/18; C07	239
QMVHQKLSPRTLNAWVKVIE (p24/aa 140–159)	236	A30; B14; C08	449
	818	A*0201; B44/57; C04/07	355
EKAFSPEVIPMFTALSEGAT (p24/aa 160–179)	961	A*0201/0202; B27/39; C02/12	428
	236	A30; B14; C08	490
	546	A2/68; B57/58; C03/07	710
MFTALSEGATPQDLNTMLNT (p24/aa 170–189)	650	A24/31; B42/50; C06/07	332
	818	A*0201; B44/57; C04/07	742
	961	A*0201/0202; B27/39; C02/12	530
	236	A30; B14; C08	910
	546	A2/68; B57/58; C03/07	729
	178	A03/32; B8/14; C07/08	191
PRGSDIAGTTSTLQEQIAWM (p24/aa 230–249)	236	A30; B14; C08	243
	469	A03/68; B49/57; C07/18	220
TSNPPIPVGDIYKRWIILGL (p24/aa 250–269)	961	A*0201/0202; B27/39; C02/12	352
	236	A30; B14; C08	259
	917	A32/66; B8/41; C07/17	126
IYKRWIILGLNKIVRMYSPV (p24/aa 261–280)	099	A26/74; B27/49; C02/07	656
	961	A*0201/0202; B27/39; C02/12	454
	236	A30; B14; C08	697
	138	A*0201/03; B07/15; C07	256
	180	A03/32; B27/40; C04/07	712
	178	A03/32; B8/14; C07/08	126
	376	A01/30; B13/39; C06/17	362
	766	A*0201/74; B15/44; C04	686
KTILKALGPGATLEEMMTAC (p24/aa 330–349)	318	A*0201/03; B7/15; C07	340
	023	A*0205/68; B41/53; C04/17	270
	138	A*0201/03; B7/15; C07	260
	156	A01/0205; B7/27; C02/07	1189
	162	A*0202/0205; B18/58; C03/07	511
	321	A29/32; B53/57; C04	480
	376	A01/30; B13/39; C06/17	441
	069	A01/0201; B18/51; C15	334
ATLEEMMTACQGVGGPSHKA (p24/aa 341–360)	318	A*0201/03; B7/15; C07	133
	156	A01/0205; B7/27; C02/07	654
	069	A01/0201; B18/51; C15	285
DREALTSLKSLFGSDPLSQ (p2p7p1p6/aa 476–495)	961	A*0201/0202; B27/39; C02/12	436
	647	A01/*0202; B57/B37; C07/06	360

Peptides targeted by at least 2 subjects are indicated.
ID indicates identification.

HLA-B45, and HLA-B58 than Zambians, South Africans, and people from Botswana. Differences in HLA background may also explain the discrepancies in CTL responses among Ethiopian Jews who immigrated to Israel and the multiethnic Ethiopian cohort studied here.

CTLs have been suggested to play a key role in controlling viral replication. However, existing data on CTL and viral load correlations are inconclusive. Some studies demonstrated an inverse correlation;[30,49,61] although others,[37,54,57,62] including a comprehensive epitope analysis of the entire HIV

genome, did not. In the group of responders, we observed a significant correlation between the magnitude of the anti-Gag CTL response and the $CD4^+$ T-cell count but no correlation with HIV viral load.

Our results on the cumulative Gag-specific CTL responses in Ethiopians are in line with those of previous studies[41,42] demonstrating distinctive differences in the dominant Gag-specific responses between subtype B–infected whites (mainly targeting the p17 region) and subtype C–infected Africans (mainly targeting the p24 region). Sixteen of the 25 peptides targeted by the study subjects, including 3 of the 4 dominant peptides, were from the p24 region of Gag. Because less than half (22 of 49) of the peptides tested came from p24, the subtype C–infected individuals in our study population also had a tendency to respond to p24. In addition, a peptide (DREALTSLKSLFGSDPLSQ, aa 476–495) in the p2p7p1p6 region of Gag was targeted by 2 of our subjects. The predominance of Gag p24-specific T-cell responses in our Ethiopian subjects and in other HIV-1C–infected Africans diverges from the results of the study among Ethiopian Jews in Israel,[45] which showed dominance of response against p17 Gag. Conversely, the most frequently targeted peptides in Ethiopian Jews overlapped with the most frequently targeted peptides in the Ethiopian subjects described in the present study, except for 1 dominant response in p17 Gag (aa 51–110) and another dominant response in the p24 Gag region (aa 201–260). Again, differences between the 2 studies may be attributable to differences in HLA background of the 2 Ethiopian populations.

We found a high frequency of CTL responses to the peptide containing a rarely reported epitope (GLNKIVRMY) in other subtype C–infected Africans.[41] A recent study on subtype C–infected subjects from 4 southern African countries identified immunodominant epitope-rich long amino acid stretches containing multiple epitopes that are frequently targeted by HIV-infected Africans.[37] Two of these immunodominant epitope-rich amino acid stretches are from the p17 Gag region (aa 9–46 and 71–95), and 3 are from p24 Gag (aa 155–194, 257–275, and 289–322). One of the peptides commonly targeted by our subjects (IYKRWIILGLNKIVRMYSPV, aa ~261–280) partially overlaps with the previously identified 257 to 275 amino acid stretch from p24.[37] The latter peptide contains a number of subtype B–specific epitopes. Interestingly, subjects responding to this 20-mer peptide share HLA molecules from the B27 supertype, suggesting that the specific epitope targeted is the previously described B27-restricted epitope KRWIILGLNK.[63]

Our finding of a dominant response to the peptide MFTALSEGATPQDLNTMLNT (aa 170–189), which contains the epitope TPQDLNTML (TL9), is in line with results from HIV-1C–infected South Africans. Using high-resolution HLA typing, Novitsky et al[42] specified HLA-B*4201 and B*8101 as HLA alleles that might restrict the CTL epitope TL9. In the present study, we observed heterogeneity in the HLA types among subjects responding to MFTALSEGATPQDLNTMLNT. Goulder et al[41] have additionally identified an HLA-B14-Cw08–restricted response to TL9. Indeed, 2 of our subjects targeting the peptide containing the TL9 epitope are carriers of HLA-B14-Cw08, substantiating earlier observations. The TL9 epitope and the peptide IKHLVWASRELERFALNPGL (aa 31–50), which was targeted by 6 of our subjects, are part of the recently identified immunodominant HIV Gag regions, suggesting that vaccines designed and developed for subtype C–infected southern Africans may be of use for Ethiopians as well. We cannot exclude the possibility that a large number of Ethiopians responding to the peptide IYKRWIILGLNKIVRMYSPV (aa ~261–280) targeted an epitope at the end of this peptide, however, which is not included in the 257 to 275 amino acid stretch from p24 that has been identified in the South African studies. Peptides inducing a response in most clade C–infected subjects suggest general high conservation in these epitopes, making it likely that strong T-cell responses against these peptides have an impact on the virus by control of replication or reduction of viral fitness. Therefore, combining these epitopes into a vaccine would provide the best means of inducing a potentially protective T-cell response in Africans in whom subtype C predominates.

In summary, in this report, we described CTL responses in relation to HLA type in the East African country of Ethiopia, where subtype C is the main circulating HIV clade, in a diverse group of Ethiopians, including the most frequent ethnic groups. Although overall HIV-1 Gag-specific CTL responses were relatively infrequent in these HIV-infected Ethiopians, the most frequently targeted Gag peptides overlapped with those previously described for HIV-1 subtype C–infected southern Africans, and therefore can be used in multiepitope vaccines.

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A. Tsegaye designed and performed the research, analyzed and interpreted the data, and wrote the paper. L. Ran performed the research and analyzed the data. D. Wolday helped with research design, interpretation of the data, and writing of the paper. B. Petros participated in scientific discussions and interpretation of the data. W. Dorigo helped with patient selection and design of the research. E. Piriou helped with design and performance of the research and interpretation of the data. T. Messele participated in scientific discussions and interpretation of the data. E. Sanders participated in scientific discussions and interpretation of the data. T. Tilahun performed part of the research. D. Eshetu

performed part of the research. H. Schuitemaker participated in scientific discussions and interpretation of the data. R. Coutinho participated in scientific discussions and interpretation of the data. F. Miedema participated in scientific discussions and interpretation of the data. J. Borghans helped with analysis of the data, interpretation of the data, and writing of the paper. D. van Baarle designed the research, analyzed the data, interpreted the data, and wrote the paper.

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