Broadly Immunogenic HLA Class I Supertype-Restricted Elite CTL Epitopes Recognized in a Diverse Population Infected with Different HIV-1 Subtypes[1]

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The genetic variations of the HIV-1 virus and its human host constitute major obstacles for obtaining potent HIV-1-specific CTL responses in individuals of diverse ethnic backgrounds infected with different HIV-1 variants. In this study, we developed and used a novel algorithm to select 184 predicted epitopes representing seven different HLA class I supertypes that together constitute a broad coverage of the different HIV-1 strains as well as the human HLA alleles. Of the tested 184 HLA class I-restricted epitopes, 114 were recognized by at least one study subject, and 45 were novel epitopes, not previously described in the HIV-1 immunology database. In addition, we identified 21 "elite" epitopes that induced CTL responses in at least 4 of the 31 patients. A majority (27 of 31) of the study population recognized one or more of these highly immunogenic epitopes. We also found a limited set of 9 epitopes that together induced HIV-1-specific CTL responses in all HIV-1-responsive patients in this study. Our results have important implications for the validation of potent CTL responses and show that the goal for a vaccine candidate in inducing broadly reactive CTL immune responses is attainable. *The Journal of Immunology,* **2008, 180: 5092–5100.**

The major obstacle in the development of new treatment and vaccine strategies for HIV-1 is the virus' remarkable genetic variability and ability to mutate. HIV-1 genetic variants appear rapidly within an individual, resulting in numerous closely related viral variants. Globally, the genetic variation of HIV-1 has resulted in the emergence of nine subtypes (A-D, F-H, J, and K), as well as circulating recombinant forms (CRF),[3] emphasizing the need for a vaccine to induce broadly reactive immune responses. The cellular immune response exerted by $CD8^+$ CTLs is known to be a crucial part of the immune defense against HIV-1 infection. In vivo evidence for the impact of Ag-specific CTL responses on HIV-1 evolution, viral replication, and disease progression comes from several studies (1–4). Although the characteristics of a protective HIV-1-specific CTL response are still unknown, several studies indicate that recognition of promiscuous epitopes in conserved regions is preferred (5–7). Such epitopes might be less sensitive or prone to induce mutations and might also be recognized independent of the HIV-1 subtype.

Ag-specific CTLs recognize Ags presented as 8- to 11-mer peptides (epitopes) by the HLA class I molecule on the APC. The HLA system is extremely polymorphic, and has a diverse distribution among different ethnic groups (8). Thus, the genetic variation of the host contributes to an ever more challenging situation in terms of identifying a broadly reactive CTL response. Recently, however, an analysis of HLA class I molecules with significant overlap among peptide-binding specificities has allowed a functional classification of HLA class I molecules into 12 distinct supertypes (9). Instead of taking into consideration several hundred HLA alleles, assembling HLA alleles into HLA class I supertypes, based on their ability to bind and present similar epitopes (8, 9), would enable large cohort studies of the association between immune responses against a limited number of HLA class I supertype-restricted epitopes and HIV-1 disease progression (10). Importantly, such an approach should allow for identification of promiscuous HLA class I epitopes eliciting broad population coverage.

In this study, bioinformatic prediction methods were used to select a set of 184 HLA class I supertype-restricted epitopes from the Gag, Pol, Env, Nef, and Tat regions for maximum coverage of HIV-1 strains belonging to the five HIV-1 subtypes, A, B, C, D, and CRF01_AE. Identification of an anti-HIV-1-specific CTL response restricted to seven HLA class I supertypes was determined using the IFN-γ ELISPOT assay in 31 HIV-1-infected patients. Using this approach, we have verified responses against 114 of the predicted epitopes. Some epitopes were highly immunogenic and induced a response in $>10\%$ of the study subjects. Of the immunogenic epitopes, we identified nine epitopes, which together gave 96% coverage of the study population. The epitopes restricted to the seven HLA class I supertypes were identified in patients with different genetic backgrounds, and infected with different HIV-1 subtypes, thus proving the success of an advanced bioinformatics epitope prediction and selection tool to identify immunogenic elite HLA class I supertype-restricted epitopes.

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[3] Abbreviations used in this paper: CRF, circulating recombinant form; CEF, CMV, EBV, and flu; SEB, staphylococcal enterotoxin B; SFU, spot forming unit; IQR, interquartile range.

Table I. *Clinical and virological characteristics of the study population at sampling time point*

Patient	ID	Ethnicity	Country of Origin	Sex[a]	Age	*HLA-A* Allele[b]	*HLA-A* Allele	*HLA-B* Allele	*HLA-B* Allele	*HLA-C* Allele	*HLA-C* Allele	$CD4^+$ T Cells/mm^3	HIV RNA Copies/ml	Treatment History[c]	Subtype[d]
1	2866	Caucasian	Sweden	M	57	ND	ND	ND	ND	ND	ND	400	400	Treated	B
2	2840	African	Uganda	F	35	*3104*	*3001*	*1301*	*3537*	*0602*	*0401*	130	200	Treated	D
3	2891	African	Zambia	F	45	*2402*	*2301*	*8201*	*4101*	*0302*	*0802*	720	5,900	Naive	C
4	2295	Caucasian	Sweden	F	69	*1101*	*0201*	*4001*	*3501*	*0303*	*0401*	480	2,400	Treat. exp	C
5	2275	Latin	Chile	M	49	*0101*	*0201*	*4402*	*0801*	*0701*	*0501*	630	35,000	Treat. exp	B
6	2717	African	Ghana	F	32	ND	ND	ND	ND	ND	ND	920	800	Naive	AG
7	2312	Caucasian	Sweden	M	70	*0201*	*0201*	*4001*	*1501*	*0303*	*0303*	800	195,000	Naive	B
8	2902	Caucasian	Sweden	M	43	*0201*	*2402*	*4001*	*0801*	*0701*	*0303*	190	1,100	Treated	B
9	2264	Caucasian	Sweden	M	40	ND	ND	ND	ND	ND	ND	560	17,000	Naive	B
10	2792	Caucasian	Finland	M	51	*2402*	*3201*	*4002*	*0801*	*0702*	*0202*	390	11,000	Naive	B
11	2426	African	Ethiopia	M	43	*6801*	*0202*	*1801*	*5101*	*0704*	*1604*	200	1,000,000	Treat. exp	C
12	2059	Caucasian	USA	M	55	*0301*	*0201*	*0702*	*5501*	*1203*	*0702*	450	600	Treated	B
13	2284	Caucasian	Sweden	M	49	*0201*	*0201*	*4001*	*4402*	*0704*	*0303*	330	100,000	Treat. exp	A1
14	2799	Caucasian	Iran	M	59	*3201*	*3201*	*5101*	*5101*	*1604*	*1502*	490	3,000	Treated	B
15	2521	Caucasian	Lithuania	M	34	*0301*	*1101*	*0702*	*3501*	*0702*	*0401*	550	<50	Treated	ND
16	2838	Caucasian	Sweden	M	42	ND	ND	ND	ND	ND	ND	240	100,000	Naive	AE
17	2395	Caucasian	Sweden	M	42	*0201*	*2402*	*5701*	*0702*	*0702*	*0702*	520	237,000	Naive	B
18	3076	African	Somalia	F	47	*0301*	*0103*	*0702*	*7301*	*1505*	*0702*	280	10,000	Naive	A1
19	2255	Caucasian	Sweden	F	52	*1101*	*1101*	*0702*	*4402*	*0501*	*0702*	70	38,000	Treated	B
20	2892	Caucasian	Sweden	F	38	*0301*	*0201*	*0702*	*3501*	*0702*	*0401*	320	37,000	Naive	AE
21	2016	African	Togo	F	49	*0301*	*0102*	*0702*	*1510*	*0804*	*1505*	110	1,200	Treated	CPX06
22	2795	Caucasian	Sweden	F	51	*2601*	*0101*	*4002*	*2705*	*0202*	*0303*	950	500	Naive	AG
23	2492	Caucasian	Sweden	M	63	*0201*	*0201*	*0702*	*2705*	*0702*	*0102*	1,150	<50	Naive	ND
24	2915	Caucasian	Sweden	F	40	*1101*	*2902*	*0702*	*0702*	*0702*	*0702*	160	800	Treated	C
25	2004	African	Ethiopia	F	35	*6601*	*0201*	*4901*	*4901*	*0701*	*1402*	550	<50	Treated	A1
26	2632	African	Uganda	F	28	*0301*	*3402*	*8101*	*4403*	*0804*	*0401*	440	14,000	Treat. exp	A1
27	2437	African	Angola	F	45	*2301*	*2301*	*4101*	*4501*	*1601*	*0802*	280	500	Treated	C
28	2384	Caucasian	Sweden	M	58	*0201*	*0201*	*1501*	*1501*	*0303*	*0303*	320	65,000	Treat. exp	B
29	2639	African	Ghana	M	37	ND	ND	ND	ND	ND	ND	390	290,000	Treat. exp	C
30	2146	African	Ethiopia	F	31	*0205*	*0201*	*4101*	*4101*	*1701*	*1701*	370	17,000	Treat. exp	C
31	2919	Unknown	Tunisia	M	38	*0202*	*3001*	*5703*	*5001*	*0701*	*0602*	410	200,000	Naive	A1

[a] F, Female; M, male.
[b] High-resolution HLA-typing results were obtained through sequencing.
[c] Treat. Exp = Treatment experienced, the patient has been treated during a short period of time, but was not treated at the sample time point.
[d] Circulating recombinant forms (CRF) and complex forms (CPX) of HIV-1: CRF02_AG, CRF01_AE, CRF06_cpx.

Materials and Methods

Study subjects

Thirty-one-treated and untreated HIV-1-infected subjects from the Karolinska University Hospital (Solna, Sweden) were identified for the study (Table I). To enable sample collection from a diverse patient population, our only inclusion criterion was a detectable plasma viral load measurement >50 RNA copies/ml obtained within 6 mo before inclusion in the study. This criterion was set to ensure detectable CTL responses (11). The study was approved by the Regional Ethical Council (Stockholm, Sweden (2005/1162)) and all subjects provided written informed consent.

Prediction, selection, and identification of HLA supertype-restricted HIV epitopes

The CTL epitope predictions were made based on a dataset consisting of 322 HIV-1 strains for which the Gag, Pol, Env, Tat, and Nef proteins had been completely sequenced and which were publicly available in the HIV Molecular Immunology database (www.hiv.lanl.gov). The number of strains for each of the five HIV-1 subtypes used in the prediction were: 40 subtype A (A1 and A2), 119 subtype B, 149 subtype C, 4 subtype D, and 10 subtype CRF01_AE. Epitopes restricted to either of the following HLA class I supertypes were predicted: A1, A2, A3, A24, B7, B44, and B58 using the NetCTL 1.0 method (12) (available at www.cbs.dtu.dk/services/NetCTL). In the NetCTL method, each possible nonamer in a protein is assigned a score based on a combination of prediction of proteasomal cleavage, TAP-transport efficiency, and HLA class I affinity with the highest weight assigned to the HLA class I affinity. For each of the seven HLA class I supertypes and for each of the five HIV-1 proteins from each of the 322 HIV-1 strains, between one and five (depending on the size of the protein) of the top-scoring nonamers were selected as the predicted epitopes. This resulted in a total of 5652 unique predicted epitopes. To reduce this set, a novel algorithm called EpiSelect, was used. The EpiSelect algorithm aims at selecting a given number of predicted epitopes in a way so that most strains are covered with the largest number of epitopes, while maximizing the coverage of the viral strain with the smallest number of epitopes. The algorithm is implemented as follows: for each viral strain, a coverage value is recorded as the number of selected epitopes targeting this viral strain. This number is initially zero for all viral strains. Also, a list of promiscuity values P_i^j is recorded for each epitope as the set of viral strains (i) covered by a given predicted epitope j. Initially, the epitope that covers the largest number of different viral strains is selected, and the coverage values are updated accordingly. The next epitope is then selected by choosing the epitope that maximizes the relation

$$\sum_i \frac{P_i^j}{(C_i + \delta)}$$

where the sum is over the set of viral strains. P_i^j is the promiscuity value for epitope j targeting the viral strain i (this value is 1 if the epitope is present in the viral strain i, otherwise 0), C_i is the coverage value for the viral strain i, and δ is an offset value defining the penalty for leaving viral strains untargeted. Small values of δ will focus the epitope selection toward maximal coverage of the different viral strains, whereas large values of δ will focus the selection toward epitopes present in most different viral strains. The value for $\delta = 0.001$ was determined empirically to achieve optimal balance between viral coverage and viral promiscuity. Next, the coverage values are updated accordingly. This procedure is repeated until the desired number of predicted epitopes has been selected. Using this algorithm, a total of 174 predicted epitopes were selected (supplementary table 1).[4] The final set included an additional 10 epitopes that were known from the literature as immunogenic CTL epitopes restricted by HLA-A2 (13) or HLA-B57/58 (supplementary table 1). The peptides were synthesized by Cambridge Research Biochemicals (Cleveland, U.K.) on a submicroscale in parallel synthesis. Stocks of peptides were dissolved in DMSO and kept at −70°C at concentrations of 10 mg/ml. Final concentrations of 2 μg/ml of each peptide were used in the assays.

Pan-specific prediction of peptide-HLA binding

We predict peptide-HLA binding using the pan-specific NetMHCpan method that allows for quantitative binding predictions for peptides to all HLA class I molecules of known sequence (14). The NetMHCpan method is a data-driven method trained on quantitative peptide primary HLA sequence data. In the original publication predictions were limited to HLA-A

[4] The online version of this article contains supplemental material.

and HLA-B loci molecules. Here, we extend the method to also include the HLA-C loci.

Immunological measurement

PBMC were isolated by Ficoll gradient centrifugation and stored in liquid nitrogen until used. The frozen PBMC were gently thawed, washed, and resuspended in RPMI 1640 Glutamax (Invitrogen Life Technologies) containing 10% FCS and 0.1 U/ml penicillin and 0.1 μg/ml streptomycin at a concentration of 1–2 $\times$ 10^6 cells/ml. The cell concentration and the viability were evaluated using a Nucleocounter (ChemoMetec) based on a fluorescence microscope counting propidium iodide-labeled cell nuclei and analyzed by the software Nucleo View. The cell viability was above 88%, with a mean of 93%.

The production of IFN-γ by Ag-specific T cells was detected using the ELISPOT assay (15). Flat-bottom 96-well Multiscreen IP plates (MSIP4510; Millipore) were activated by adding 15 μl 35% EtOH per well during 1 min, washed four times in PBS, and coated with an anti-IFN-γ mAb, 1-D1K (Mabtech), 5 μg/ml, at 4°C for 4 h. The plates were washed four times in PBS. The PBMCs were plated in duplicate at a concentration of 1–2 $\times$ 10^5 cells/well along with peptides at a final concentration of 2 μg/ml per peptide, and incubated at 37°C for 16 h. As a negative control, cells were incubated with medium only to determine the background responses for each patient. The HIV-Gag p55 peptide pool (BD Biosciences) was included as a control of the HIV-1-specific response, and a CMV, EBV, and Flu (CEF) control peptide pool (National Institutes of Health, Germantown, MD) as well as staphylococcal enterotoxin B (SEB; Sigma-Aldrich) were added as positive controls. The plates were washed and incubated with a biotinylated anti-IFN-γ mAb, 7-B6-1 (Mabtech), 1 μg/ml, for 1 h at 37°C. The wells were washed, and alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories) was added and the plate incubated in room temperature for 1 h in dark. Before adding the substrate, the plate soaked for 1 h in PBS with 0.1% Tween 20 (Sigma-Aldrich) to decrease background signal. The plates were developed by a 20-min incubation with the substrate Vector Blue Alkaline Phosphatase Substrate kit III (SK-5300; Vector Laboratories), and the spot-forming units (SFU) (3) were counted by computer-assisted image analysis using the software KS ELISPOT 4.9 and an Axioplan 2, Imaging system 2 microscope (Carl Zeiss). The net number of SFU per million PBMC was calculated as follows: (number of spots against relevant peptide) $-$ (number of spots against medium only) $\times$ (10^6 $\times$ concentration of cells in the well).

To establish background responses in our ELISPOT assay against the HIV-1 peptides, we analyzed PBMC samples from eight healthy volunteers. The background response induced by medium alone was low with a median of 64 SFU per million PBMC (interquartile range (IQR): 37–82). Neither of the peptide pools induced responses in any of the eight HIV-negative donors (median 70, IQR: 43–116 SFU/million cells). Likewise, the background response in our 31 HIV-1-infected study subjects against medium alone was generally low (median: 36, IQR: 22–72 SFU/million PBMC). A sample was considered positive when the responses were at least three times the experimental background and above 80 SFU/million PBMC. Also, due to limitations in detecting >400 SFU per well, the maximum response in the assay was 2000 SFU/million PBMC. All study subjects had detectable CTL responses against the control Ag SEB (supplementary table 1).

HIV-1 sequencing and phylogenetic analysis

HIV-1 RNA was extracted from 200 μl plasma using the QIAamp MinElute Virus Spin kit (Qiagen). Extracted viral RNA was eluted in 30 μl of sterile water with the addition of 1 μl RNase inhibitor (Promega). To increase the viral copy numbers in patient samples with low viral load (<10.000 HIV-1 RNA copies/ml); virus was extracted from 1 ml of plasma.

cDNA synthesis was performed using the SuperScript III RT kit (Invitrogen Life Technologies) with gene-specific primer 5′-TGC TGT CAT CAT TTC TTC TAR TGT-3′ (HXB2 nt position 1836–1813) (0.15 μM), and oligo deoxythymine (oligo dT) (0.15 μM). Our primers were selected to amplify the HIV-1 Gag p17 region (HXB2 nt position 790-1431), using a nested PCR (13). Excess primers, nucleotides, and enzymes were removed using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare).

The sequencing reaction was conducted using the BigDye Terminatior version 3.1 Cycle Sequencing kit (Applied Biosystems), purified through Sephadex G-50 (GE Healthcare) in an Multi Screen-HV plate (Millipore), and detected in the ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). Sequences were imported and manually edited using Sequencher software (Gene Codes). For the phylogenetic analysis, sequences were aligned with reference sequences for each subtype obtained from the HIV Sequence database (www.cbs.dtu.dk/services/NetCTL) using BioEdit (Citeline). Phylogenetic trees were constructed using the software MEGA3.1 (Center for Evolutionary Functional Genomics, The Biodesign Institute, Tempe, AZ). The sequences have been submitted to GenBank (GenBank accession numbers: EU300947–EU300975).

HLA genotyping

Isolation of genomic DNA from patient PBMC samples was performed using the Qiagen EZ1 BioRobot. PCR was conducted to amplify HLA-A, -B, -C, genes using gene-specific primers for each HLA gene (16). Following PCR, samples were examined by agarose gel electrophoresis and purified using Millipore's Montage PCR96 plates. The purified PCR products were sequenced using an ABI PRISM BigDye Terminator Cycle sequencing system and analyzed with ABI Prism 3100 Genetic Analyzer or 3700 Genetic Analyzer. High-resolution class I HLA genotyping was conducted using computer software based on a taxonomy-based sequence analysis method (17).

Statistical analyses

Statistical analysis and graphical presentations were performed with GraphPad Prism software. Correlations were calculated using Spearman's nonparametric rank test (two-tailed), and comparisons using the Mann-Whitney nonparametric *t* test. Analysis with a p value of <0.05 was considered statistically significant.

Results

PBMC and plasma samples were obtained from 16 men and 15 women who had been HIV-1 infected through different routes. Our 31 study-subjects composed a genetically diverse population originating from 13 countries, carrying 18 *HLA-A* alleles, 24 *HLA-B* alleles, and 19 *HLA-C* alleles. They also varied broadly in terms of age, stage of infection, and treatment history, and were infected by different HIV-1-subtypes and CRF (Table I). Eleven patients were infected with subtype B, 5 with subtype A1, 7 with subtype C, 1 with subtype D, 2 with CRF02_AG, two with CRF01_AE, and 1 with the complex CRF06_cpx (AJGK). A template amplification and subsequent HIV subtype analysis from plasma was unsuccessful for 2 patients due to low viral loads. The broad genetic background and distribution of different HIV-1-subtypes made the cohort well-suited for this study, aiming at identifying candidate components in a vaccine and simplify identification of correlates for an effective immune response in a global perspective.

Prediction of HLA class I supertype-restricted epitopes

To identify broadly immunogenic HIV-1-specific CTL epitopes, we first used a bioinformatics method (NetCTL) to predict HLA class I supertype-restricted epitopes. We then selected a number of predicted epitopes that together constitute a broad overall coverage among different HIV-1 subtypes using a newly developed algorithm: EpiSelect. A total of 174 predicted and 10 experimentally verified HLA class I supertype-restricted epitopes located in Gag, Pol, Env, Nef, and Tat were selected (Table II, and supplementary table 1). To reach an optimal coverage among subtypes our final set included 85 variants optimized to reflect variations in 35 epitopes. The HLA-A1, -A2, -A3, -A24, -B7, -B44, and -B58 supertypes were chosen, because they together will give an optimal coverage in the human population (>98%) independent on ethnicity (8). On average, each of the 322 reference HIV-1 strains (subtypes A, B, C, D, and CRF01_AE) used for the prediction was covered by 54 epitopes (minimum 29). The peptides were collected into 24 pools, with 7–8 peptides per pool, and distributed so that each HLA class I supertype was represented once or twice. Two additional pools were added and analyzed separately: one containing only the immunodominant HLA-A2-restricted epitope ^{77}SLYNTVATL^{85} (SL9), located in the HIV-Gag p17 region, and

Table II. *Number of predicted HLA supertype-restricted epitopes within the HIV-regions Gag, Pol, Env, Nef, and Tat*[a]

	Gag	Pol	Env	Nef	Tat	Total:
HLA-A1	8	9	3	4	1	25
HLA-A2	6	10	9	4	1	30
HLA-A3	6	9	6	3	1	25
HLA-A24	6	7	10	1	1	25
HLA-B7	6	8	8	3	0	25
HLA-B44	5	9	8	1	2	25
HLA-B58	11	10	5	2	2	30

[a] CTL epitopes restricted to the A1, A2, A3, A24, B7, B44, and B58 HLA class I supertypes were predicted for the Gag, Pol, Env, Tat, and Nef proteins by using the NetCTL method. The table shows the location and HLA-supertype restriction of the 185 predicted epitopes.

the other containing five other well-identified immunogenic HLA-A2-restricted epitopes (Gag-^{433}FLGKIWPSHK^{442}, Env-^{121}KLTPLCVTL^{129}, Env-^{813}SLLNATAIAV^{822}, Nef-^{83}GALDLSHFL^{91}, and Nef-^{83}AAVDLSHFL^{91}).

Immunogenicity of HLA class I supertype-restricted epitopes

Ag-specific CTL responses were determined using PBMCs from 31 HIV-1-infected individuals by detection of IFN-γ production in the ELISPOT assay (Fig. 1*A*, and supplementary table 1). All but one subject tested responded positively to the HIV-p55 control peptide pool, CEF peptide pool, and against at least one of the peptide pools containing predicted HLA class I supertype-restricted epitopes (including pools A2 and SL9). One patient did not respond to any of the 184 selected epitopes nor did he show a positive response against the HIV-p55 control peptide pool, thus indicating a very low HIV-1-specific CTL reactivity. To identify the specificity of the responses, the individual peptides from the pools giving responses were analyzed in a second ELISPOT assay (Fig. 1*B*). All study subjects showed a clear HIV-1 specific CTL response toward one or more peptides within each responding pool (Fig. 1*B*, and supplementary table 1). In most patients, broad responses against several individual epitopes were confirmed; a median of six epitopes per patient (IQR 4–11) were recognized (Fig. 2*A*).

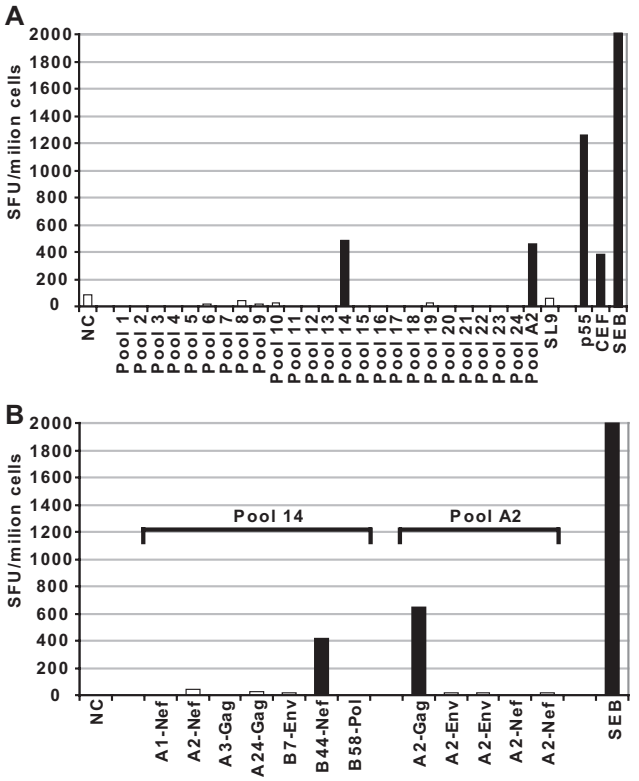

FIGURE 1. Identification of HIV-1-specific CTL response. To identify the specificity of the responses, the production of IFN-γ by Ag-specific T cells was first detected using the ELISPOT assay against pools of peptides and control Ags (*A*), and second against the individual peptide within the pools (*B*). Most of the study subjects responded to peptides restricted to several HLA supertypes. The figure shows the results of HIV-specific CTL responses in patient 13.

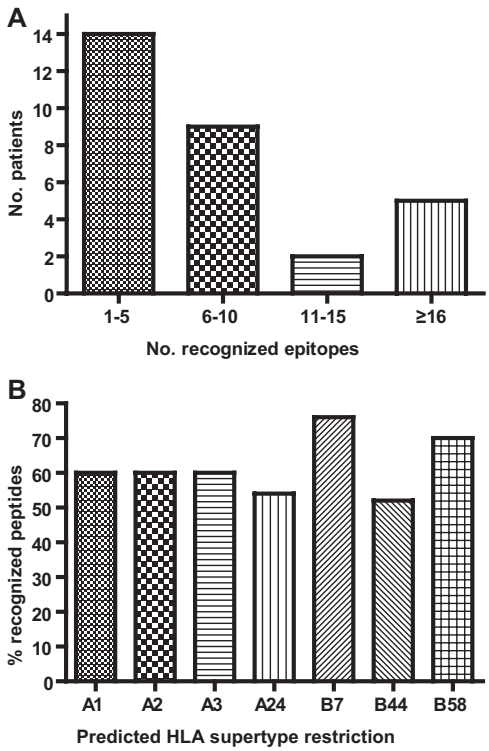

FIGURE 2. Broad CTL responses detected against epitopes restricted by the seven HLA supertypes. We found HIV-specific CTL responses toward a median of six epitopes per patient (*A*). The immunogenicity frequency of epitopes restricted by the seven HLA supertypes (i.e., percent of epitopes recognized in at least one patient) were similar (*B*).

Of the tested 184 HLA class I supertype-restricted epitopes, as many as 114 (62%) were recognized by at least one study subject, and 45 were novel epitopes, not previously described in the HIV Molecular Immunology database (18). Targeted epitopes were equally distributed among the 7 HLA class I supertypes included in the study and were not skewed toward a specific HLA class I supertype (Fig. 2*B*). The magnitude of the epitope-specific CTL responses was generally high with a median response per peptide of 587 SFU/million PBMC (IQR 319–686) (supplemental table 1). The majority of the predicted HLA supertype-restricted epitopes in Gag, Pol, Env, and Nef were shown to be immunogenic in at least one subject (Fig. 3). However, only 3 (38%) of the tested Tat epitopes induced a HIV-specific CTL response (Fig. 3*A*). Overall, the magnitude of the combined responses detected against HLA class I supertype-restricted epitopes in HIV-Gag (median 732 SFU/million PBMC) was not significantly different to that detected against the HIV-p55 control peptide pool (median 777 SFU/million PBMC) ($p = 0.971$, Mann-Whitney t test). Similarly to the HIV-Gag epitopes, strong responses were detected against Nef epitopes with a median response of 708 SFU/million PBMC

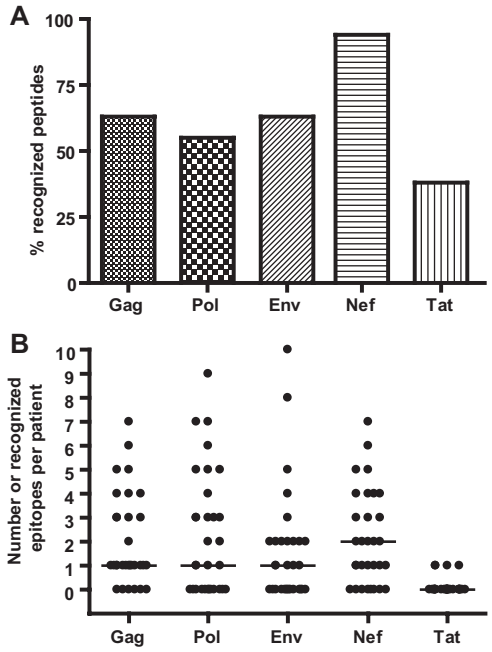

FIGURE 3. HLA supertype-restricted CTL responses detected against the majority of predicted Gag, Pol, Nef, and Env epitopes. Of the predicted epitopes restricted to the Gag, Pol, Env, Tat, and Nef regions, the Nef epitopes showed to be highly immunogenic in terms of percent (96%) of predicted epitopes recognized (*A*). Each subject responded to the median of two epitopes located in the HIV-Nef region and one epitope located in the HIV-Gag, -Pol, and -Env regions (*B*).

($p = 0.704$, Mann-Whitney t test) followed by Pol-specific responses at a median of 500 SFU/million PBMC ($p = 0.236$, Mann-Whitney t test). Although strong responses against Env epitopes were detected, the median response for all subjects (84 SFU/million PBMC) were significantly lower than against the Gag epitopes ($p = 0.025$, Mann-Whitney t test). The epitope-specific CTL responses detected against Tat was also significantly lower than against Gag ($p < 0.0001$, Mann-Whitney t test).

Cross-clade recognition of highly immunogenic epitopes

We next evaluated the immunogenicity of the 114 HLA class I supertype-restricted epitopes and identified 21 "elite" epitopes that induced CTL responses in at least four patients, corresponding to 13% of the study subjects (Fig. 4). Taken together, we found that 90% of the HIV-specific CTL responding study population recognized one or more of these highly immunogenic epitopes. When comparing the breadth of the response detected in our study subject toward the number of "elite" epitopes to the response against the total set of 184 predicted epitopes, we found a strong significant correlation ($p < 0{,}001$, $r^2 = 0{,}760$, Spearman rank correlation) (Fig. 5). These results prove that by using a limited set of optimal epitopes, it is possible to obtain information on specificity, magnitude, and breadth of individual HIV-specific CTL responses.

The elite epitopes contained seven subtype variants of three epitopes restricted by HLA-A2 (Nef), -A3 (Nef), and -B7 (Env) (Fig. 4). In response to HLA-A2-restricted epitopes in Nef (^{83}AAVDLSHFL^{91} and ^{83}GALDLSHFL^{91}), five subjects infected with subtypes A, B, and CRF02_AG cross-recognized the two variants. One subject, infected with a subtype C virus, recognized the Nef-^{83}GALDLSHFL^{91} variant only. Similarly, in response to the B7-Env epitope variants, three subjects infected with subtypes A, B, and CRF01_AE showed cross-reactive responses, while one subject infected with CPX06 and one infected with subtype C recognized one variant each. In response to the three A3-Nef variants, we found that patients infected with subtypes A, B, and D, cross-recognized the ^{84}AVDLSHFLK^{92} and ^{84}ALDLSHFLK^{92} variants, but not the ^{84}AFDLSFFLK^{92} variant more prevalent in subtypes C and CRF01_AE subtype variants of the same epitope (Fig. 4).

To evaluate the ability of cross-clade recognition of the immunogenic epitopes, we conducted a phylogenetic analysis to establish the infecting viral subtype for each study subject and looked at the frequency at which the epitope sequence was present in our reference set of viral strains belonging to a certain subtype. Importantly, most of the 21 "elite" epitopes were recognized by patients infected with different subtypes, but we found some differences in cross-subtype recognition (Fig. 4). Four patterns of cross-subtype recognition were found: 1) fairly conserved epitopes

Epitope sequence	HLA-supertype & protein region	The subtypes of the responders	Frequency of the epitope sequence in subtype[1]: A	B	C	D	AE
QVPLRPMTY	A1-Nef	B, B, C, D, AE, nd					
LTDTTNQKT	A1-Pol	B, B, B, C, C, AE					
KIQNFRVYY	A1-Pol	B, D, AE, nd					
FLGKIWPSHK	A2-Gag	A1, A1, A1, B, B, B, B, C, AE, nd					
SLYNTVATL	A2-Gag	A1, B, B, B, C, C, C					
GALDLSHFL	A2-Nef, var. 1[2]	A1, B, B, B, C, AG					
AAVDLSHFL	A2-Nef, var. 2	A1, B, B, B, AG					
ILKEPVHGV	A2-Pol	B, B, B, B, C, C, nd					
QLTEAVQKI	A2-Pol	B, B, B, C, C					
AVDLSHFLK	A3-Nef, var. 1	A1, B, D, nd					
ALDLSHFLK	A3-Nef, var. 2	A1, B, D, nd					
AFDLSFFLK	A3-Nef, var. 3	B, C, C, C, C, AE, AE					
WYIKIFIII	A24-Env	B, C, C, AE, AE					
HYMLKHLVW	A24-Gag	A1, B, B, C					
IPRRIRQGL	B7-Env, var 1	A1, B, C, AE					
IPRRIRQGF	B7-Env, var 2	A1, B, AE, CPX06					
HPVHAGPVA	B7-Gag	A1, B, C, D					
RALGPGATL	B7-Gag	A1, B, C, D					
TPQDLNTML	B7-Pol	A1, B, C, C					
SPAIFQSSM	B7-Pol	A1, A1, B, C, C, D, AE					
QEILDLWVY	B44-Nef	A1, A1, B, B, B, C					

FIGURE 4. Cross-clade recognition of HLA supertype-restricted elite epitopes. A total of 21 of the HLA supertype-restricted epitopes were highly immunogenic and induced a CTL response in at least four subjects. Shown is the subtype the responding subjects were infected with and at which frequency the epitope sequence is found among the HIV-1 subtype reference strains. 1, The color represents the frequencies of the exact epitopes sequence in the different subtypes; white: 0%, light grey: 1–24%, dark grey: 25–49%, and black: ≥50%. 2, Subtype variants of the same epitope; nd, not determined.

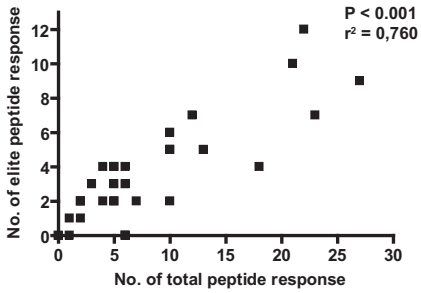

FIGURE 5. The breadth of responses detected against the 21 elite epitopes correlated significantly with the total breadth of responses. There was a significant correlation between the response to the 21 elite peptides and the entire set of 184 tested epitopes, suggesting that the elite peptides can be used as a tool to measure the breadth, magnitude, and functional characteristics of CTL responses against epitopes located in different HIV regions.

among all tested subtypes were recognized by patients infected with several different subtypes (e.g., A1-Nef ^{73}QVPLRPMTY^{81}), 2) the epitope sequence was not frequently found in the reference subtype strains but was still recognized in some patients infected by several different subtypes (e.g., A24-Env ^{680}WYIKIFIII^{688}), 3) epitopes more prevalent in a certain subtype were recognized in patients infected with several different subtypes (e.g., B7-Env ^{843}IPRRIRQGF^{851}), and 4) highly subtype-specific sequences and responses were seen against some epitopes indicating the inability of cross-clade recognition or presentation (e.g., A2-Pol ^{464}ILKEPVHGV^{472}). Overall, our results are very promising as the majority of the highly immunogenic epitopes were recognized in individuals infected with a broad range of HIV-1 subtypes.

Identification of a limited set of HLA-restricted epitopes for optimal coverage

As described above, the EpiSelect algorithm was first used to select a set of predicted epitopes that gave a good coverage of a large set of HIV-1 strains. After obtaining the immunogenicity data for the predicted epitopes in each of the patients in the study, we again used the algorithm; this time to select the epitopes that gave the best coverage of the patients in terms of experimentally verified immunogenicity. Fig. 6 shows the 20 first peptides selected by EpiSelect and the cumulative number of responses they gave rise to in each of the 31 patients in the study. The figure shows that a set of only nine peptides can cover 30 of 31 (96%) of the patients.

HLA type and responses to predicted HLA supertype-restricted epitopes

The majority of subjects with multiple responses recognized epitopes predicted to bind to several HLA class I supertypes. To evaluate how well the predicted epitope restriction explained responses detected in our study subjects, we successfully obtained high-resolution HLA typing results from 26 subjects (Table I). In total, our subjects recognized 225 epitopes of which 149 (66%) could be explained by the donor HLA allele belonging to the predicted HLA supertype. Next, we extended the analysis beyond the supertypes and investigated whether the recognized epitopes could be explained directly in terms of restriction to one of the donor HLA class I alleles. We predicted the rank of the epitope in the source protein using the pan-specific NetMHCpan prediction method (14) for the six HLA class I alleles of the donor. If the restriction allele could place the epitope within the top 10% we say we can successfully explain the epitope, if not that we have failed. By using this method, we can explain 85% of the identified 9-mer epitopes from the HLA types. These results show that by using bioinformatic tools, we have successfully been able to predict, and

				1	2	3	4	5	6	7	8	9	10	11	12	13	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	FLGKIWPSHK	A2	Gag	0	0	0	1	1	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0	1	0	1	1	0	1	0	0	0
2	AFDLSFFLK	A3	Nef	0	0	1	1	2	0	0	0	0	0	1	1	1	0	1	1	0	0	2	0	0	1	1	1	1	1	1	0	0	0
3	QVPLRPMTY	A1	Nef	0	1	1	2	2	0	0	0	0	1	1	1	1	1	1	1	0	0	3	0	0	1	1	1	1	1	2	1	0	0
4	GALDLSHFL	A2	Nef	1	1	1	3	2	0	1	0	0	1	1	1	1	1	1	1	0	0	3	0	1	1	1	1	2	1	3	1	0	0
5	ILKEPVHGV	A2	Pol	1	1	1	3	3	0	1	0	0	1	2	2	1	1	1	2	0	1	3	0	1	2	1	1	2	1	3	1	1	0
6	IPRRIRQGF	B7	Env	1	1	1	3	3	0	1	0	0	1	2	3	1	1	1	2	1	1	4	1	1	2	1	1	2	1	3	1	1	0
7	AISPRTLNAW	B58	Gag	1	1	1	3	3	1	1	0	0	1	2	3	1	1	1	2	1	1	4	1	1	2	1	1	2	1	3	1	1	1
8	RALGPGATL	B7	Gag	1	2	1	4	3	1	1	0	1	1	2	3	1	1	1	2	1	1	4	1	1	2	1	1	3	1	3	1	1	1
9	GEIYKRWII	B44	Gag	1	2	1	4	4	1	1	1	1	2	2	3	1	1	1	2	1	1	4	1	1	2	1	1	3	1	3	1	1	1
10	QEILDLWVY	B44	Nef	1	2	1	4	4	1	1	1	1	3	2	4	2	1	2	2	1	2	4	1	1	2	1	2	3	1	3	2	1	1
11	AAVDLSHFL	A2	Nef	2	2	1	4	4	1	2	1	1	3	2	4	2	1	2	2	1	2	4	1	2	2	1	2	4	1	4	2	1	1
12	TPQDLNTML	B7	Gag	2	2	1	4	4	1	2	1	1	3	2	5	2	1	2	2	1	2	4	1	2	2	2	2	5	2	4	2	1	1
13	RPMTYKGAL	B7	Nef	2	2	2	4	4	1	2	1	1	3	2	6	2	2	2	2	1	2	4	1	2	2	2	2	5	2	4	2	1	1
14	SPAIFQSSM	B7	Pol	2	3	2	5	4	1	2	1	1	3	2	7	2	2	2	2	2	2	5	1	2	2	2	2	6	2	4	3	1	1
15	SLYNTVATL	A2	Gag	2	3	2	6	5	1	3	1	1	3	2	8	2	2	2	2	2	2	5	1	2	2	2	3	6	2	4	3	2	1
16	QATQEVKGW	B58	Gag	2	3	2	6	5	2	3	1	1	3	2	8	2	2	2	3	2	2	5	1	2	2	2	3	6	2	4	3	2	1
17	RPQVPLRPM	B7	Nef	2	4	2	6	5	2	3	1	1	3	2	8	2	2	2	3	2	2	5	2	2	2	2	3	7	2	4	3	2	1
18	YFPDWQNYT	B58	Nef	2	4	2	6	5	2	3	1	1	3	2	8	2	2	2	3	2	2	5	2	2	2	2	3	8	2	4	3	2	2
19	LTDTTNQKT	A1	Pol	2	4	2	6	6	2	4	1	1	3	3	9	2	2	2	3	2	2	6	2	2	2	2	3	8	2	4	3	3	2
20	WYIKIFIII	A24	Env	2	4	3	6	6	2	4	1	1	3	3	10	2	2	3	3	2	2	7	2	2	2	2	3	8	2	4	4	3	2

FIGURE 6. The top 20 peptides selected by the EpiSelect algorithm to provide optimal coverage in terms of immunogenicity. From *top* to *bottom*, the rows indicate the order in which the peptides were selected. The columns 1–4 give the number, sequence, HLA supertype restriction, and HIV proteins sequence, respectively, for each peptide. The columns 5–34 each correspond to one of the patients in the study. Each row gives, for each patient, the accumulative number of peptides that are immunogenic in each of the patients.

most importantly detect, broadly immunogenic HLA class I-restricted CTL responses.

Discussion

The HLA alleles have the highest diversity of all human genes and allow cellular immune responses in different persons to be directed against different epitopes. They are responsible for the wide variety of T cell epitopes in HIV-1 and also determine the specificity and repertoire of the CTL response. In addition to the genetic differences of the host, the high evolutionary rate of HIV-1, influenced by changes in the environmental selection pressures, greatly influences the efficacy and specificity of HIV-specific CTL responses. Together, the genetic variation of the host and the virus constitute major obstacles for obtaining and evaluating the potency of HIV-specific CTL responses. We developed and used an algorithm capable of selecting epitopes that together constitute a broad coverage of the different HIV-1 strains. In a highly diverse group of patients infected with different HIV-1 subtypes we have successfully identified a limited set of highly immunogenic epitopes, inducing cross-clade HIV-specific CTL responses.

In this study, we applied a novel algorithm, EpiSelect, to select HLA class I supertype-restricted epitopes in HIV-Gag, Pol, Env, Nef, and Tat to reach a wide-ranging coverage among different HIV-1 subtypes. The set of HLA class I supertype-restricted epitopes were predicted using a program combining predictions of HLA-I binding, TAP transport, and proteasomal cleavage (12), a proven method for screening for immunogenic HLA class I restricted CTL epitopes in viral infections and of potential utility as candidates of virus diagnostics and vaccines (19). Using this approach, we were able to detect epitope-specific CTL responses in 30 of 31 subjects, showing an HIV-specific immune response against the majority of the 184 predicted or experimentally identified epitopes, of which 39% are novel epitopes. By obtaining high resolution HLA typing, we could confirm that 66% of the responses could be attributed a HLA allele belonging to a certain HLA supertype. In addition, a total 85% of the detected epitope-specific T cell responses could be explained by the predicted binding of the epitope to one of the donors HLA allele. Our results support a recent finding that HLA supertypes often provide an oversimplification of the HLA specificity space (20). However, and more importantly, we demonstrate that the observed epitope promiscuity is indeed a direct result of overlapping HLA specificities and that these overlaps are predictable using advanced bioinformatical methods for pan-specific HLA-peptide binding. Longitudinal studies will have to be performed to evaluate the efficacy of individual responses. The design of a vaccine candidate (protein or DNA) which facilitates processing and presentation of such epitopes from full-length regions, would increase the probability of eliciting CTL responses in a global setting, especially because the genetic background of the population might be an important factor for vaccine efficacy.

The efficacy of an HIV-specific CTL response is dependent on the intricate kinetics between viral evolution causing immune escape, the fitness cost of the mutation, and recognition of escape variants. Recently, Fernandez et al. (21) showed that moderate levels of vaccine-induced wild-type specific CTLs capable of killing transiently reverting virus-infected cells, after infection with an escape mutant, were more beneficial in terms of control of viremia than the fitness cost induced by the escape mutant. Complete maintenance of escape virus seen in subjects with high-level immunodominant T cell responses against the wild-type epitope did not result in improved virological control, pointing out that vaccines inducing broad subdominant responses may be more beneficial (21, 22). Lately, studies have shown that a broad cross-recognition is associated with a slower disease progression and a better control of the virus (6, 7). Also, the high mutation rate of HIV-1 makes it impossible to identify CTL epitopes that are conserved among all subtypes, which is why the identification of cross-reactive between-clade T cell responses would be important to achieve in vaccine models (23, 24). In our study, we successfully identified several broadly immunogenic elite epitopes, including six with sequence variants recognized in a diverse patient population, which constitute promising candidates for vaccine-induced cellular immune responses. Immunogenicity analysis of the variant epitopes tested in this study show that three (A2-Nef, A3-Nef, and B7-Env) were cross-recognized by responding subjects infected with different subtypes. However, while two of three A3-Nef variants (AVDLSHFLK and ALDLSHFLK) were cross-recognized by HLA-A3-positive subjects infected with subtypes A, B, and D virus, the third variant (AFDLSFFLK) was not. The AFDLSFFLK variant is predominant in viral strains of subtypes C and CRF01_AE, and four of the six responding subjects recognizing this variant were infected with exactly these subtype variants of the same epitope. The differential targeting of the A3-Nef AFDLSFFLK variant indicates that this epitope sequence is presented, but that the differences may alter the TCR contact in such way, that another T cell repertoire is needed for recognition. Alternatively, this epitope is not efficiently presented by the same HLA alleles as the two other variants, and is thus not recognized by the same subjects (5). This notion is supported by that only 50% of subjects recognizing this epitope variant were HLA-A3 positive. Three additional broadly immunogenic epitopes (A24-Env WYIKIFIII, A24-Gag HYMLKHLVW, and B7-Pol SPAIFQSSM) had subtype variants that were cross-recognized by less than half of the responding subjects. Additional in-depth functional studies will have to be conducted to rule out whether these changes hampered binding to the HLA class I, or prohibited recognition by the TCR of the responding T cell population. Together, our results emphasize the complex interaction between the responding TCR repertoire and the configuration of the HLA-peptide complex, because some epitope variants are efficiently targeted by responding T cells, while others are not (13, 25–27). Our results are nonetheless promising as they show that many HLA class I supertype-restricted epitopes are recognized in subjects infected with different subtypes. Some of these epitopes are present at a low frequency in most subtypes, while some were more conserved between the subtypes.

In line with the results obtained in different cohorts of clade B- and C-infected subjects using overlapping peptides, we found a hierarchical targeting of our selected set of HLA class I supertype-restricted epitopes in Gag, Nef, Pol, Env, and Tat (28–33), listed in order of immunogenicity. Almost all of the studied Nef-epitopes were recognized in at least one patient. The identification of Nef epitopes to be stimulated by a preventive HIV vaccine may be of particular interest, as the HIV-Nef region has been shown to be preferentially targeted in primary HIV-1 infection (34, 35). Also, obtaining Gag-specific T cell responses is highly relevant as the breadth of HIV-Gag-specific T cell responses has lately been associated with lower viral loads in subjects infected with subtype B or C virus (30, 32, 36–40). Although Env, due to its high variability, is not thought to be an optimal T cell Ag, we have identified at least two cross-clade recognized epitope sequences that could be used to optimize a future vaccine candidate, especially, because Env Ag optimization is required for obtaining Ab responses (41) and is thus likely to be included if both B and T cell immunity is to be reached. HIV-Pol responses may have been studied to a lesser extent as a T cell immunogens, although Pol responses may have important implications. New treatment regimens have had a dramatic impact on the mortality among HIV patients,

but resistance to antiretroviral therapy is rapidly emerging during low adherence or suboptimal therapy (42). As a consequence, the risk for transmission of resistant HIV variants is a major threat against the last few years' success with antiretroviral therapy (42–46). Importantly, we and others have shown that treatment-mediated selected mutations act as novel epitopes (47–52). Considering the important role of the cellular immune responses in a durable control of drug-resistant HIV-1 (53), we suggest that vaccines targeting epitopes in Pol may be important.

Today, the comparison of induced immune responses by different vaccine candidates is severely hampered by the limitations of the commonly used overlapping peptide sets to identify the breadth and specificity of the response, as they invariably will underestimate or even fail to detect responses against putative autologous epitopic variants (13, 31, 54, 55). Methods have been developed for designing polyvalent T cell vaccines based on designing mosaic proteins with maximal peptide coverage of protein sequences (23, 56) or selecting CTL epitopes with maximum coverage (57). In the study by Fischer et al. (23), sets of one, three, four, or six mosaic proteins with maximal 9-mer peptide coverage were designed for each of the subtypes B and C, and for the M group. Here, we have, as suggested earlier, designed polyvalent T cell vaccines using sets of CTL epitopes with maximum coverage of the genomic variation of the pathogen (57). We compared our 21 elite epitope sequences to the sets of mosaic proteins and found that 12, 13, and 13 of the elite epitopes could be found in the subtypes B and C, and M group sets, respectively, with just one mosaic protein. Compared with the sets containing six mosaic proteins, the vast majority; 18, 16, and 21, of the 21 elite epitope sequences could be found in the subtypes B and C, and M group sets, respectively. The large overlap between the elite epitope set and the mosaic protein sets suggested by Fischer et al. (23) strongly supports the notion that sequence conservation is a key feature in defining broadly reactive T cell epitopes. However, it is important to stress that the mosaic protein construction is a whole protein approach that does not consider Ag presentation and peptide immunogenicity. Furthermore, the use of a limited set of epitopes to evaluate the function and phenotype of specific T cells is of particular relevance to gain insight into the mechanisms important for immune control.

Using a very limited set of HLA class I supertype-restricted epitopes, we have shown that it is possible to detect and evaluate both the magnitude and breadth of epitope-specific CTL responses in a genetically diverse population infected with different HIV subtypes. Our findings are of great relevance for the detection of HIV-specific CTL responses for the validation of vaccines designed to generate promiscuous CTL responses with a global coverage and to simplify the identification of the efficacy of responses restricted by a multitude of HLA alleles.

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Disclosures

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