Enhanced Detection of Human Immunodeficiency Virus Type 1 (HIV-1) Nef-Specific T Cells Recognizing Multiple Variants in Early HIV-1 Infection[∇]†

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A human immunodeficiency virus (HIV)-preventive vaccine will likely need to induce broad immunity that can recognize antigens expressed within circulating strains. To understand the potentially relevant responses that T-cell based vaccines should elicit, we examined the ability of T cells from early infected persons to recognize a broad spectrum of potential T-cell epitopes (PTE) expressed by the products encoded by the HIV type 1 (HIV-1) *nef* gene, which is commonly included in candidate vaccines. T cells were evaluated for gamma interferon (IFN-γ) secretion using two peptide panels: subtype B consensus (CON) peptides and a novel peptide panel providing 70% coverage of PTE in subtype B HIV-1 Nef. Eighteen of 23 subjects' T cells recognized HIV-1 Nef. In one subject, Nef-specific T cells were detected with the PTE but not with the CON peptides. The greatest frequency of responses spanned Nef amino acids 65 to 103 and 113 to 147, with multiple epitope variants being recognized. Detection of both the epitope domain number and the response magnitude was enhanced using the PTE peptides. On average, we detected 2.7 epitope domains with the PTE peptides versus 1.7 domains with the CON peptides ($P = 0.0034$). The average response magnitude was 2,169 spot-forming cells (SFC)/10^6 peripheral blood mononuclear cells (PBMC) with the PTE peptides versus 1,010 SFC/10^6 PBMC with CON peptides ($P = 0.0046$). During early HIV-1 infection, Nef-specific T cells capable of recognizing multiple variants are commonly induced, and these responses are readily detected with the PTE peptide panel. Our findings suggest that Nef responses induced by a given vaccine strain before HIV-1 exposure may be sufficiently broad to recognize most variants within subtype B HIV-1.

Developing a vaccine that protects against the wide range of human immunodeficiency virus type 1 (HIV-1) circulating strains is one of the greatest public health challenges ever confronted. The enormous genetic diversity of HIV-1 creates a major barrier to both the design and the evaluation of HIV-1 vaccines, and it is widely recognized that a globally efficacious vaccine will most likely need to elicit broadly reactive immune responses (31, 34, 35). Group M HIV-1, which dominates the worldwide pandemic, consists of several distinct subtypes (24). Proteins within subtypes vary by approximately 5 to 25% and vary between subtypes by approximately 10 to 40%, with the lower bound representing conserved proteins and the upper bound representing variable proteins (26). A successful vaccine is likely to require an immunogenic sequence or a combination of sequences that can induce T cells recognizing epitopes that are similar to or cross-react with those in circulating viruses. To better understand the potentially relevant responses that T-cell-based vaccines should elicit, we examined the ability of T

cells from early infected persons to recognize multiple epitope variants within subtype B HIV-1. We chose subjects with acute and early HIV-1 infection since the immune responses that are measured in these subjects are more likely to be relevant for immune protection and immune control, compared to later stages of infection when the viruses may have already escaped key cytotoxic T-lymphocyte (CTL) responses. Since HIV-1 Nef contains highly immunogenic regions of HIV-1 (1) and is commonly included in candidate HIV-1 vaccines, we focused our investigations on the detection of T-cell responses to a broad spectrum of potential T-cell epitopes (PTE) expressed by the products encoded by the HIV-1 *nef* gene.

Today, numerous candidate T-cell-based vaccines representative of multiple HIV strains and several subtypes are under evaluation in clinical trials. Vaccine-induced T cells are typically identified by their ability to produce cytokines when stimulated with overlapping 15-mer peptides whose sequences are based on vaccine strains. This approach provides the greatest sensitivity for defining the immunogenicity of the vaccine preparation but does not adequately assess specificities of the immune responses that may be potentially relevant to protection against circulating strains worldwide. The use of peptides based on centrally derived computational sequences has the potential to increase the sequence similarity between the antigen expressed in a vac-

cine strain and that of circulating viruses. For example, the consensus (CON) method has at each site the most common nucleotide or amino acid residue across a sequence alignment, and this approach has been frequently utilized for the design of peptide reagents in the assessment of T-cell responses (15, 36). Although this method offers improvements over the use of peptide panels based on a single reference strain or patient isolate, there are two disadvantages: first, the CON sequences are artificially derived and may not occur naturally in HIV-1 strains; and second, most T-cell epitopes identified by central-sequence peptides cluster in domains with low sequence variability, with few being detected in regions with moderate and high sequence variability (47).

An alternative strategy is the use of autologous sequence-based peptides. These peptide sets can improve the detection of T-cell determinants in variable regions of HIV-1 proteins and are useful in detailed studies of viral escape mutations resulting from immune pressure in HIV-1-infected subjects (2, 37). However, this approach is not relevant in the examination of immunogenicity in HIV-1-uninfected vaccine recipients and certainly is not feasible in large population studies. Thus, a standard HIV-1 peptide panel is needed that is more representative of the circulating virus strains vaccine recipients are likely to encounter and that permits affordable and objective comparisons of T-cell responses to a variety of candidate vaccines.

To address these issues, we developed a standardized peptide panel, designated PTE-B, that represented PTE contained in diverse circulating subtype B HIV-1 strains. Responses were assessed by gamma interferon enzyme-linked immunospot (IFN-γ ELISPOT) assays of two Nef peptide sets: a PTE-B panel designed to cumulatively cover 70% of PTE among subtype B isolates and the CON-B panel. We provide evidence that T cells in most newly infected subjects are better at recognizing multiple epitope variants than heretofore appreciated when using CON peptide panels. The data have wider implications in relation to vaccine immunogenicity, suggesting that vaccine-induced T cells may be able to recognize epitopes both in and outside the subtype represented in the vaccine. In addition, our findings indicate that the magnitude and breadth of responses detected with PTE-optimized peptide panels are significantly higher than those detected with CON peptides. While initially we used the PTE peptides to evaluate vaccine immunogenicity, this approach laid the framework for the design of a vaccine immunogen with expanded T-cell epitope coverage to provide protection against highly diverse viruses and rapidly changing virus populations.

MATERIALS AND METHODS

Study population. Subjects with primary HIV-1 infection were recruited and enrolled at the University of Washington Primary Infection Clinic. The duration of infection was typically defined as the time from the onset of clinical signs and/or symptoms suggestive of acute retroviral syndrome (4, 29, 43). All participants were men who have sex with men and presumed to be infected with clade B viruses (42). Some patients at various time points following diagnosis elected to receive combination antiretroviral therapy with nucleoside reverse transcriptase inhibitors and either a protease inhibitor or a nonnucleoside reverse transcriptase inhibitor. Uninfected volunteers at low risk for HIV-1 infection were recruited through the Fred Hutchinson Cancer Research Center HIV Vaccine Trials Unit to serve as donor controls in the evaluation. The Human Subjects

Review Board at the institutions approved the studies, and the volunteers provided written consent prior to participation.

Plasma HIV-1 RNA levels, T-cell subset analyses, and HLA typing. Plasma HIV-1 RNA was determined by quantitative branched-chain DNA (bDNA; Chiron, Emeryville, CA) and ultrasensitive reverse transcriptase-PCR (RT-PCR; Roche Molecular Systems, Branchburg, NJ) assays. Levels were expressed as copies/ml, and the lower levels of sensitivity were 500 copies/ml (bDNA assay) and 50 copies/ml (RT-PCR assay) (12). Absolute blood CD4$^+$ T-cell counts were measured by consensus flow cytometry methodology. HLA typing was performed at the Puget Sound Blood Center by sequence-specific primer PCR as previously described (7).

Analysis of PTE from circulating strains and PTE coverage. Given that major histocompatibility complex (MHC) class I molecules typically present antigenic peptides that are 8 to 10 amino acids (aa) in length (33, 38, 46), we considered 9 aa as the minimal epitope length and defined PTE as all possible unique 9-mers contained in the virus sequences. The PTE peptide set was designed to capture the most prevalent CTL epitopes among circulating HIV-1 strains (27), with each stretch of nine amino acids within a protein sequence having the potential to be a T-cell epitope. Therefore, for a given HIV-1 protein whose sequence was x amino acids in length, the number of PTEs that could be extracted was $x - 8$. All possible PTEs from a collection of virus sequences (e.g., all clade B viruses) were combined, and any repeat PTEs were removed. Within this mixture of unique PTEs, each was then classified by its frequency of occurrence relative to the other PTEs reported for HIV-1 strains in the Los Alamos database. We then arbitrarily decided on the extent of coverage desired across all these sequences. For instance, 70% coverage would represent a peptide set that contains 70% of all the potential epitopes within the group of virus sequences under consideration (e.g., clade B).

We then derived the synthetic peptides from the PTE sequences. Either peptides corresponding to the extracted 9-mer PTEs or longer peptides can be made. We chose to synthesize 15-mer PTEs that contained one or more overlapping PTEs and yet retained the natural virus sequence, for the following reasons: (i) peptides of 15 amino acids in length conform to the size of common peptide reagents in use (e.g., consensus peptides) and are less expensive to synthesize than 9-mers; (ii) 15-mer PTEs are preferable to use in screening assays over smaller peptides (e.g., 9-mers) because the latter more often stimulate non-HIV-1-specific T-cell reactivities; and (iii) 15-mers should be recognized by both CD4$^+$ and CD8$^+$ T cells. To design longer peptides such as 15-mers, each unique 15-amino-acid sequence embedded in the group of virus sequences was extracted. These naturally occurring 15-mers were then ordered by their PTE coverage level, which is the summation of the PTE frequencies associated with each of the individual embedded 9-mers. Thus, the higher the PTE coverage, the greater the likelihood that the peptide represented the virus sequences. A peptide reagent set was then built in a stepwise fashion. The first 15-mer was selected as the one with the highest coverage. The PTEs covered by this 15-mer were removed from the set, the coverage of each of the candidate 15-mers was recomputed, and the second 15-mer was selected as the one with the highest (updated) coverage.

In this study, a total of 46,000 unique 9-mers were extracted from subtype B Nef proteins ($n = 110$) in the Los Alamos HIV-1 sequence database, and a 15-mer peptide set was then constructed by the forward stepwise algorithm described above. The stepwise selection process was repeated until a mean cumulative PTE coverage of 70% was reached (Fig. 1; also see Table S1 in the supplemental material).

Synthetic HIV-1 peptides. Synthetic overlapping peptides corresponding to gene products from the HIV-1 Nef CON subtype B were obtained through the NIH AIDS Research and Reference Reagent Program (NARRRP, Bethesda, MD), and the HIV-1 Nef PTE peptides were synthesized at Mimotopes (Raleigh, NC). It should be noted that in validation studies, expected positive and negative responses to both peptide sets were observed for HIV-infected and uninfected donors, respectively. In addition, four frequently recognized Nef CON peptides (aa 69 to 83, PVRPQVPLRPMTYKA; aa 73 to 87, QVPLRPM TYKAAVDL; aa 81 to 95, YKAAVDLSHFLKEKG; and aa 129 to 143, PGP GIRYPLTFGWCF) were obtained from both sources (NARRRP and Mimotopes) and compared for T-cell recognition. T-cell responses to these peptide pairs in both infected and uninfected donors were comparable in breadth and magnitude (data not shown).

For the PTE peptide set ($n = 90$ peptides) there were 18 pools, and each pool consisted of 9 to 10 peptides (9-by-10 matrix). For the CON peptide set ($n = 49$ peptides), there were 14 pools, and each pool consisted of 7 peptides (7-by-7 matrix). The peptides were pooled in a matrix format so that each peptide was contained in two pools (5, 21). Additional experiments were performed comparing responses to individual PTE peptides, with variant peptides contained in

TABLE 1. Demographic, clinical, and virological characteristics of the study population

Subject[a]	Age (yr)	Ethnicity[b]	Enrollment time point			Sampling time point				HLA class I type		
			Days postinfection	HIV-1 RNA copies/ml	CD4+ T cells/mm³	Days postinfection	Treatment duration (days)	HIV-1 RNA copies/ml	CD4+ T cells/mm³	A	B	Cw
1188	23	H	79	73,074	409	93	5	<500	375	23	35, 15	02, 07
1212	37	C	11	5,060,000	757	16	No[c]	102,045	925	02, 23	44, 62	03, 04
1216	33	C	129	26,000	300	134	2	38,400	255	03, 24	07, 39	07
1238	42	C	45	99,600	367	45	No	99,600	367	01, 03	07, 14	07, 08
1242	30	C	42	96,900	874	105	57	343	787	03, 33	14, 35	04, 08
1362	24	C	8	8,240,000	878	8–680	No	117,077	871	02, 25	18, 51	01, 12
1484	27	C	12	4,340,700	300	19	No	4,340,700	830	02, 03	07, 35	04, 07
1490	41	C	55	286,272	568	62	No	286,272	568	02, 11	15, 35	03, 04
1509	37	C	191	271	486	343	No	65	426	01, 02	15, 57	03, 06
1576	32	C	22	3,717,000	332	63	42	4,129	552	11, 33	49, 51	07, 15
1577	32	C	89	701	746	177	No	24	603	2	27, 49	02, 07
1585	39	C	123	58	659	150	No	56	554	10, 68	03	03, 06
1596	36	C	19	260,605	700	20	No	260,605	265	03, 26	38, 35	04,12
1599	30	C	46	404,463	265	46	No	404,463	265	01, 02	08, 18	7
1686	38	H	55	155,545	186	70	No	219,312	241	23, 25	35, 40	03, 04
1689	28	H	11	2,240,958	1206	11	No	94,708	745	02, 03	35, 40	03, 04
1690	42	C	21	1,031,477	396	21	No	400,163	528	01	08	07
1691	23	B	29	26,713	694	29	No	7,382	758	30	42, 53	06, 17
1692	42	B	50	2,461	612	50	No	2,461	612	23, 30	08, 42	07, 17
1693	41	H	7	377,063	559	7	No	1,912,195	472	01, 03	08, 35	04, 07
1698	45	H	11	734,413	458	11	No	262,437	531	NT[d]	NT	NT
1699	43	C	28	215	1,94	28	No	296	945	01, 02	08, 57	06, 07
1706	28	C	25	354,860	420	25	No	6,457	521	02, 32	08, 55	03, 07

[a] All subjects were male.
[b] Ethnicity: C, Caucasian; H, Hispanic; B, black.
[c] No, no treatment.
[d] NT, not tested.

single pools to ensure that there were no significant antagonist effects on T-cell responses (data not shown). All peptides were used at a final concentration of 2 μg/ml, unless stated otherwise. The final concentration of dimethyl sulfoxide never exceeded 1% in the assays (34).

IFN-γ ELISPOT assay. ELISPOT assays were performed to detect HIV-1-specific IFN-γ-secreting cells as previously described (9), with modifications. Peripheral blood mononuclear cells (PBMC) were plated (1×10^5 to 2×10^5 cells/well) in 96-well hydrophobic polyvinylidene difluoride-backed plates (Mil-
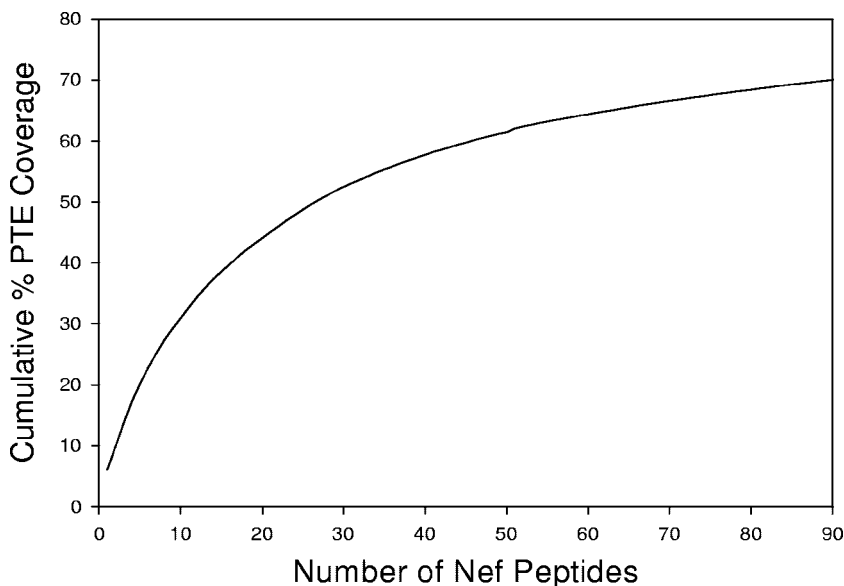

FIG. 1. Schematic representation of the PTE frequency coverage plot for the Nef PTE peptide set ($n = 90$ peptides). The PTE frequency coverage plot shows the number of 15-mer peptides relative to the cumulative PTE coverage for 110 Nef sequences. Ninety peptides were required to achieve a cumulative coverage of 70%. The PTE peptides with lower numbers are those that are present at the highest frequency among the virus sequences in the database.
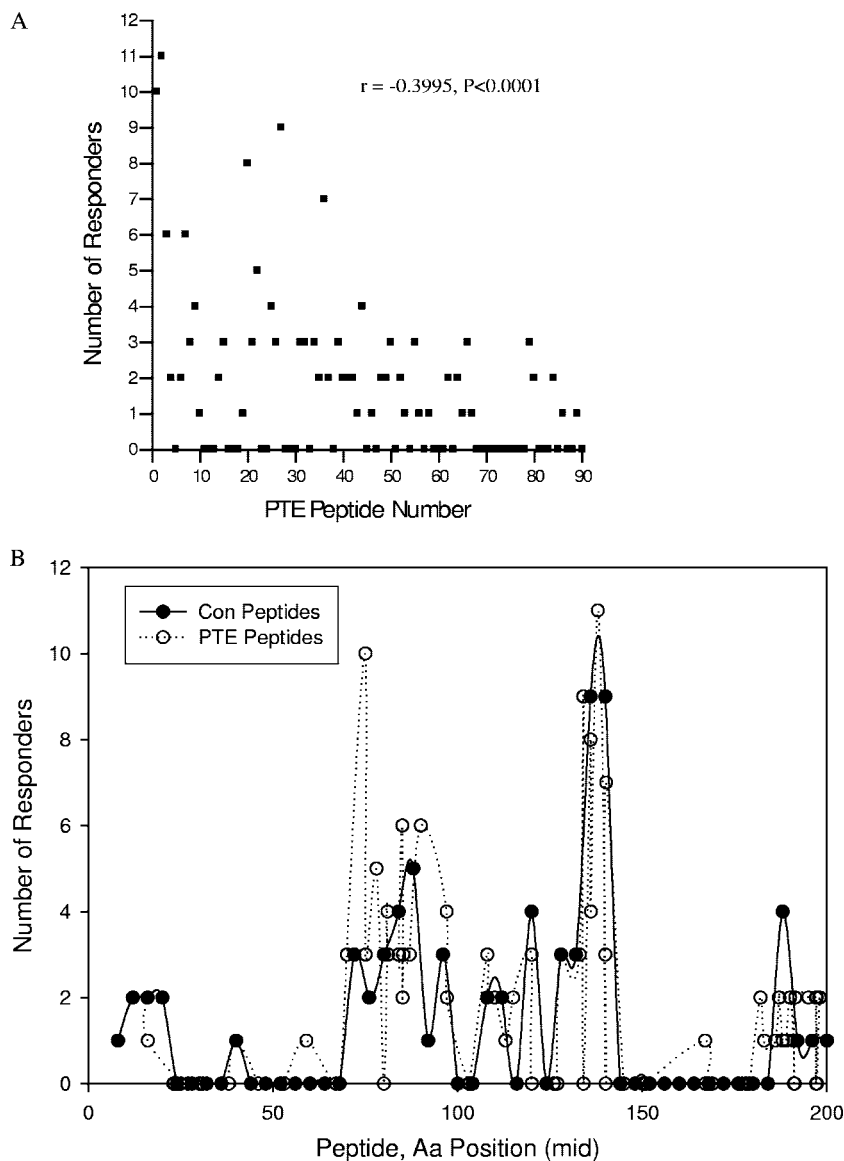
FIG. 2. Relationship between responder frequency with PTE peptide order (A) and peptide distribution within the Nef protein (B). PBMC were stimulated with HIV-1 PTE and CON peptide sets in a matrix format and tested for IFN-γ production using an ELISPOT assay. Responses >3-fold above background and >50 SFC/10^6 cells above background were scored positive. All responses were confirmed at the single-peptide level. Shown are the number of subjects responding to each peptide. In panel A, the highest ranking (lower numbers) PTE peptides are those that are present at the highest frequency among the clade B virus sequences in the database. In panel B the position of each CON and PTE peptide is imputed upon the HXB2 position for the middle amino acid in each of the peptides.

lipore, Bedford, MA) precoated with 50 μl of 10 μg/ml anti-IFN-γ monoclonal antibody (1-D1K, mouse immunoglobulin G1; Mabtech, Nacka, Sweden). Phyto-hemagglutinin (Murex Biotech, Dartford, United Kingdom) stimulation at 2 μg/ml was used as a positive control, and no peptide stimulation was used as a negative control.

Plates were incubated overnight at 37°C in 5% CO_2 and then developed. Spot-forming cells (SFC) were counted using an Immunospot (Cellular Technology Ltd., Cleveland, OH) optical reader. The number of antigen-specific IFN-γ-secreting cells was calculated by subtracting the SFC in the negative control wells from those in the antigen-stimulated wells. The peptides were first tested in matrix pools, and all responses were confirmed at the single-peptide level (5, 21). Responses >3-fold above background and >50 SFC/10^6 cells above background were scored positive. We detected no false-positive responses in 12 HIV-1-seronegative control subjects with either the CON or the PTE peptide set.

The breadth of the Nef-specific response for an individual subject was defined as the number of distinct epitopic domains recognized (1), and the total magni-

tude of response was determined by the sum of the response (IFN-γ SFC/10^6 PBMC) to all epitopic domains. The recognition of two adjacent CON peptides was imputed as one epitopic domain, and the greater of the two responses was enumerated in the total magnitude. Similarly, the recognition of multiple variant PTE peptides with an amino acid overlap of ≥10 aa was imputed as one epitopic domain, and the highest IFN-γ SFC frequencies among all variant epitope responses were used to enumerate the total magnitude of response.

To determine the functional avidity of the T-cell response, the standard IFN-γ ELISPOT assay was performed with the optimal epitopic peptides at serial concentrations between 10,000 and 0.1 ng/ml. The SFC frequencies were plotted against the log_{10} peptide concentration with ORIGIN 6.0 professional software (Microcal Software, Inc., Northampton, MA). The effective peptide concentration that elicited 50% of the maximum T-cell response, defined as the EC_{50}, was determined with a Sigmoidal Fit tool in the software.

Sequencing of autologous viruses. DNA was purified from 2 × 10^6 cryopreserved PBMC using a QIAamp DNA blood mini-kit (QIAGEN, Valencia, CA),
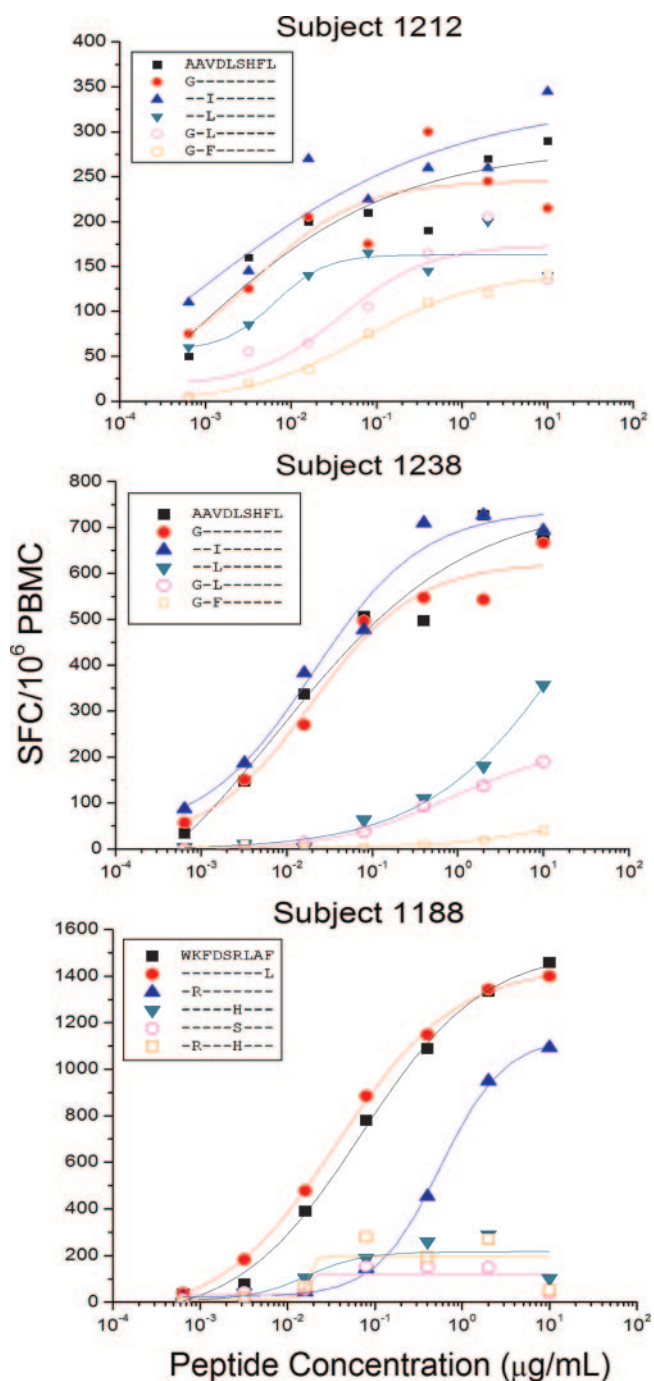
FIG. 3. Characterization of the T-cell responses to epitope variants AL9/B62 in subject 1212 (top panel), AL9/Cw08 in subject 1238 (middle panel), and WF9/B1503 in subject 1188 (bottom panel). A standard IFN-γ ELISPOT assay was performed by stimulation with the optimal epitopic peptides and variants at serial concentrations between 10,000 and 0.1 ng/ml. Shown here are PBMC responses from subjects 1238 and 1212 (AL9 epitope variants) and subject 1188 (WF9 epitope variants). The SFC frequencies were plotted against the $\log_{10}$ peptide concentration, and the effective peptide concentration that elicited 50% of the maximum T-cell response was determined using ORIGIN 6.0 professional software. Epitope mapping and HLA restriction data were published previously (9).

according to the manufacturer's recommendations. Alternately, RNA was purified from the plasma virions using a QIAamp viral RNA mini-kit. The HIV-1 Nef sequences were amplified by standard nested PCR as previously described (28). Primers DS7 (F) 8169-8198 and TMMNEF6 (R) 9607-9631 were used in the first-round PCR, resulting in a 1.5-kb product. Primers DS9 (F) 8678-8697 and DS8 (R) 9527-9550 were used in the second-round PCR, resulting in an 872-bp product. The PCR products were cloned into the TA vector using a TOPO TA cloning kit according to the manufacturer's protocol (Invitrogen, San Diego, CA). Plasmids from individual clones were isolated and sequenced using the M13 355-370 and T7 328-347 forward and M13 205-221 and T3 243-262 reverse primers with ABI Prism BigDye Terminator cycle sequence reagents (Applied Biosystems, Foster City, CA). Computational analysis of the sequences was performed using ExPASy Translate Tool (http://us.expasy.org/tools/dna.html), ClustalW (http://www.ebi.ac.uk/clustalw/), and BioEdit.

Scoring of changes in amino acid sequences. Amino acid substitutions between sequences were scored for similarity using a BLOSUM matrix (20). The matrix is derived from blocks of aligned sequences from homologous proteins with a certain level of identity. Conservative changes have positive scores, and nonconservative changes have negative scores, with the scale extending from −4 to +3. Conservative amino acid substitutions are likely to conserve the physical and chemical properties necessary to maintain the structure and function of the protein, whereas nonconservative substitutions are likely to disrupt essential structural and functional features of the protein.

Statistical analysis. T-cell responses from CON and PTE peptide sets were compared by the use of two-tailed paired nonparametric t tests with linear (breadth) and log-transformed (magnitude) data. PTE peptide order number versus T-cell responder frequency was examined using the Spearman correlation coefficient.

RESULTS

Study population. The study population consisted of 23 HIV-1-infected subjects enrolled during primary infection (Table 1). All subjects were men, and the median age was 36 years (range, 23 to 45 years). At enrollment, the subjects had been infected for a median duration of 29 days (range, 8 to 191 days). The median plasma HIV-1 RNA level was 260,605 copies/ml, and CD4$^+$ T-cell counts were 559 cells/mm^3. Subjects in this analysis were chosen based on PBMC specimen availability from time points within 1 year of infection. At the time of analysis of the immune response, the subjects had been infected for a median duration of 46 days; and two subjects had received antiretroviral therapy for >1 week (duration, 42 and 57 days, respectively) (Table 1).

Distribution of reactive PTE peptides versus frequency distribution in virus isolates. T-cell reactivity among the 23 subjects was quantified using an IFN-γ ELISPOT assay of the PTE and CON peptide sets. IFN-γ-secreting Nef-specific T-cell responses were detected in 18 of 23 HIV-1-infected subjects by using the PTE peptide set and in 17 of 23 subjects by using the CON peptide set (see Table S2 in the supplemental material). The five patients whose T cells failed to recognize the PTE Nef peptides were the same five (of 6) patients who failed to recognize the Con B Nef peptides.

Here, we examined the relationship between the PTE peptide rank and the detection of T-cell responses to the peptide (Fig. 2A). As predicted, the number of responses detected correlated with the frequency ranking of the PTE peptides ($r = -0.3995$; $P < 0.0001$). Thus, the greatest frequency of T-cell responses detected was following stimulation with the most frequent PTE among subtype B virus isolates. We also investigated whether T-cell reactivity to certain Nef domains was more likely to be observed than others. We observed clustering of reactive peptides, with major clusters spanning amino acids 65 to 103 and 113 to 147 and minor clusters between amino

TABLE 2. IFN-γ ELISPOT responses to epitope variants with PBMC from HIV-1 infected subjects[d]

PTE peptide[a]	HXB2 aa position	Epitope sequence[b]	BLOSUM score(s)[c]	1212/AL9/B62			1238/AL9/Cw08			1188/WF9/B1503		
				SFC/10⁶ PBMC 15-mer	SFC/10⁶ PBMC 9-mer	EC$_{50}$ ng/ml 9-mer	SFC/10⁶ PBMC 15-mer	SFC/10⁶ PBMC 9-mer	EC$_{50}$ ng/ml 9-mer	SFC/10⁶ 15-mer	SFC/10⁶ 9-mer	EC$_{50}$ ng/ml 9-mer
Epitope AL9												
YKAAVDLSHFLKEKG	081–095	AAVDLSHFL		191	270	0.42	287	726	7.99			
PMTYKAAVDLSHFLK	078–092	....		222	270	0.42	690	726	7.99			
RPMTYKGAVDLSHFL	077–091	G........	0	402	245	3.87	700	542	19.40			
RPMTYKAALDLSHFL	077–091	..L......	1	276	200	0.55	Not reactive	179	202.00			
TRRAAIDLSHFLREK	080–094	..I......	3	Not reactive	260	6.46	95	726	20.30			
PMTFKGALDLSHFLR	078–092	G.L......	0, 1	Not reactive	205	39.23	Not reactive	136	909.00			
GALDLSHFLKEKGGL	083–097	G.L......	0, 1	Not reactive	205	39.23	Not reactive	136	909.00			
PMTYKGAFDLSHFLK	078–092	G.F......	0, −1	61	120	70.80	Not reactive	Not reactive				
Epitope WF9												
EREVLEWKFDSRLAF	177–191	WKFDSRLAF								2,826	1,335	69.60
VLVWKFDSRLAFRHM	180–194	....								1,436	1,335	69.60
VLMWKFDSRLAFHHI	180–194	....								1,013	1,335	69.60
WRFDSRLAFHHMARE	183–197	.R.......	2							949	950	563.43
EVLQWKFDSRLALRH	179–193	L	0							753	1,343	37.55
LVWKFDSSLAFHHRA	181–195	.....S...	−1							350	148	17.23
LVWKFDSHLAFHHMA	181–195	....H....	0							123	290	16.44
VWRFDSHLAFRHMAR	182–196	.R...H...	2, 0							106	270	17.16

[a] Amino acid differences from the CON sequence are underscored. The optimal epitope sequence is in bold type.

[b] The CON B sequence for each epitope is indicated on the top. Identity to the amino acid in the CON sequence is indicated by a dot. All epitopes shown were previously mapped and HLA restriction was performed (9).

[c] Conservative amino acid substitutions are denoted by positive scores and nonconservative substitutions by negative scores. The scoring is on a scale of −4 to +3.

[d] All SFC data shown were obtained from IFN-γ ELISPOT assays performed at 2 μg/ml. Response levels of >50 SFC/10⁶ PBMC were designated reactive. EC$_{50}$, effective peptide concentration that elicited 50% of the maximum T-cell response.

TABLE 3. T-cell recognition of PTE peptide variants and the sequence of autologous viruses in subject 1188

Epitope restricting HLA allele[a]	ELISPOT responses to 15-mer PTE peptides					Autologous sequences	
	PTE peptide sequence[b]	HXB2 aa position	Epitope sequence[c]	BLOSUM score(s)[d]	SFC/10⁶ PBMC	Sequence[e]	Clonal frequency
	YPLTFGWCF					GPGIR **YPLTFGWCF** KLVPV	
YF9/B35	PGPGT<u>R</u>**FPLTFGWCF**	129–143	F........	3	1,046	...T. F........	13/13
	PGPGIR**YPLTFGWCF**	129–143	....	NA	523		
	PGPGIR**YPL<u>C</u>FGWCF**	129–143	....C....	−1	87		
	PGIR**YPLTFGWCF**KL	131–145	....	NA	1,096		
	G<u>V</u>R**YPL<u>T</u>LGWCF**KLV	132–146	...L.....	0	0		
	G<u>V</u>R**FPL<u>C</u>FGWCF**KLV	132–146	F..C.....	3, −1	9		
	T<u>R</u>**YPLTFGWC<u>Y</u>**KLVP	133–147	.......Y	3	409		
	WKFDSRLAF					REVLE **WKFDSRLAF** HHMAR	
WF9/B15	VL<u>M</u>**WKFDSRLAF**HHI	180–194	....	NA	1,013	....M	6/12
	EREVLE**WKFDSRLAF**	177–191	....	NA	2,826	....I ....L..	5/12
	EVL<u>Q</u>**WKFDSRLA<u>L</u>**RH	179–193	L	0	753	....M ...A.....	1/12
	VL<u>V</u>**WKFDSRLAF**<u>R</u>HM	180–194	....	NA	1,436		
	L<u>V</u>**WKFDS<u>H</u>LAF**HHMA	181–195	.....H...	0	123		
	L<u>V</u>**WKFDS<u>S</u>LAF**HH<u>R</u>A	181–195	.....S...	−1	350		
	VW<u>R</u>**FDS<u>H</u>LAF<u>R</u>HMAR	182–196	.R...H...	2, 0	106		
	W<u>R</u>**FDSRLAF**HHMARE	183–197	.R.......	2, 0	949		
	KRQDILDLWVY					IYSQ **KRQDILDLWVY** HTQG	
KY11/Cw07	IYS<u>Q</u>**KRQDILDLW<u>I</u>Y**	101–115	.........I.	3	1,256	....I.	9/13
	KRQDILDLWVYHTQG	105–119	....	NA	1,453	....	4/13
	IYS<u>R</u>**KRQ<u>E</u>ILDLW<u>I</u>Y**	101–115	R..E.....I.	2, 2, 1	773		
	S<u>Q</u>R**RQDILDLWVY**HT	103–117	R.........	2	1,516		
	RQ<u>E</u>ILDLWVYHTQGY	106–120	..E.......	2	647		

[a] Epitope WF9 was previously mapped, and HLA restriction was performed (9). Epitopes YF9 and KY11 are presumed based on the subject having the appropriate HLA allele and publication in the Los Alamos database (23).
[b] Amino acid differences from the CON sequence are underlined. The optimal epitope sequence is in bold type.
[c] The CON B sequence for each epitope is indicated on the top. Identity to amino acid in the CON sequence is indicated by a dot.
[d] Conservative amino acid substitutions are denoted by positive scores and nonconservative substitutions by negative scores. The scoring is on a scale of −4 to +3. NA, not applicable.
[e] The CON B sequence for the domain is indicated on the top. Identity to amino acid in the CON sequence is indicated by a dot. Numbers at the end of the sequences indicate the percentage of clones encoding the given variant. The compartment sampled was PBMC.

acids 1 to 27 and 177 to 206. Reactive domains detected with both the CON and PTE peptide sets were similar, with the greatest frequency of responses spanning amino acids 65 to 103 and 113 to 147, respectively (Fig. 2B).

Recognition of multiple epitope variants: magnitude and avidity of responses. For several of the Nef epitopes, recognition of multiple variants was detected with the PTE peptide set (see Table S2 in the supplemental material). We further characterized T-cell recognition of epitope variants, comparing recognition of the 15-mer PTE peptide with that of optimal 9-mer epitope truncations: the AL9/B62-restricted response in subject 1212, the AL9/Cw08-restricted response in subject 1238, and the WF9/B1503-restricted response in subject 1188 (9). The proportion of variants recognized varied for different epitopes and between subjects (Table 2). For example, four of six variants of the AL9 epitope (AAVDLSHFL, <u>G</u>AVDLSHFL, AA<u>L</u>DLSHFL, and <u>GAF</u>DLSHFL) contained within five 15-mer PTE peptides were recognized by PBMC from subject 1212 (Table 2). Notably, responses to all six optimal-length 9-mer variants were detected (Fig. 3, top panel), although responses to the variants containing two amino acid substitutions (<u>GAL</u>DLSHFL and <u>GAF</u>DLSHFL) each were 92- to 166-fold lower in avidity than responses to the AAVDLSHFL epitope.

PBMC from subject 1238 recognized three of six variants (AAVDLSHFL, <u>G</u>AVDLSHFL, and AA<u>I</u>DLSHFL) contained within four 15-mer PTE peptides (Table 2). When

tested with optimal-length 9-mer variants, low-frequency responses with 25- to 114-fold lower avidity were detected for an additional two variants (<u>GAL</u>DLSHFL, and AA<u>L</u>DLSHFL) (Fig. 3, middle panel). Finally, all five variants of the WF9/B1503 epitope contained within eight different 15-mer PTE peptides were recognized by T cells from subject 1188 (Fig. 3, bottom panel), although amino acid substitutions in presumed T-cell receptor (TCR) contact sites, e.g., WKFDS<u>S</u>LAF (R→S, position 6, BLOSUM score, −1) and WKFDS<u>H</u>LAF (R→H, position 6, BLOSUM score, 0), were associated with a 5- to 10-fold reduction in magnitude compared to responses to the WKFDSRLAF epitope. Smaller differences in the magnitude of responses observed for peptides containing identical optimal epitope sequences, for example, WKFDSRLAF, may be related either to variations in the epitope position within the 15-mer peptides or to the flanking amino acids.

In summary, T cells from early infected persons can recognize multiple epitope variants, although the magnitude and avidity of the responses can vary greatly across peptide variants. Multiple factors may influence the recognition of variants, including the number, the type (i.e., conservative versus nonconservative amino acid substitutions), and the position of the amino acid substitutions. Finally, while we detected IFN-γ-secreting cells in response to stimulation with peptide variants, further investigations are needed to define the quality of the T-cell response to the different variants.

A.

Days	Sequence	Frequency
8	PGPGIRYPLTFGWCFKLVP	10/10
22	------------------	14/15
	----V-------------	1/15
50	------------------	13/14
	----V-------------	1/14
76	------------------	13/14
	----G-------------	1/14
113	------------------	12/12
155	------------------	9/10
	----V-------------	1/10
190	------------------	14/15
	----T-------------	1/15
344	------------------	15/15
580	------------------	23/23
769	------------------	7/7
832	------------------	9/9
1035	------------------	13/13

B.

Sequence, Optimal Epitope	BLOSUM Score	Predominant Clades	Sequence, 15-mer Peptide Variants	SFC/10⁶ PBMC
YPLTFGWCF		B, C, A	PGIR**YPLTFGWCF**KL	1230
F--------	3	A, B, C	PGPGT<u>R</u>**<u>F</u>PLTFGWCF**	1203
--------Y	3	A, C	T<u>R</u>**YPLTFGWC<u>Y</u>**KLVP	1086
----L----	0	F	PGIR**YPLT<u>L</u>GWCF**KL	0
F--C-----	3, -1	Circulating recombinant forms	GVR**<u>F</u>PL<u>C</u>FGWCF**KL	0
---C-----	-1	B	PGPGIR**YPL<u>C</u>FGWCF**	0

FIG. 4. YPLTFGWCF epitope evolution (A) and recognition of epitope variants (B) in subject 1362. Evolutionary changes at the Nef epitope in autologous viral sequences from 12 time points between days 8 and 1,035 after infection are shown. Identity to amino acid in the consensus sequence is indicated by a dot. Numbers at the end of the sequences indicate the proportion of clones from the plasma encoding the given variant. The recognition of YPLTFGWCF variants by PBMC from subject 1362 was assessed by IFN-γ ELISPOT assay. Reactivity to six 15-mer peptides containing the variants was examined, and responses >3-fold background and >50 SFC/10⁶ cells above background were scored positive. The optimal epitope sequence is in bold type. Amino acid differences from the CON sequence are underlined.

Recognition of epitope variants and autologous virus sequence analyses. To determine the relationship of the autologous Nef sequences to the epitope variants recognized by the PBMC from an individual, we analyzed an average of 13 Nef sequences from subject 1362 at 12 time points spanning days 8 through 1,035 following infection (Fig. 4). The Nef-specific T-cell response restricted by HLA B18 was previously fine mapped to YPLTFGWCF (9). Although no YPLTFGWCF epitope variants were detected at any of the time points (Fig. 4A), the T cells reacted with three of six 15-mer peptide variants (Fig. 4B). These data indicate that the T-cell clonotypes generated in response to the infecting virus sequence YPLTFGWCF were able to recognize a range of epitope variants.

Autologous viruses from subject 1188 were sequenced from day 93 following infection and analyzed for heterogeneity in three epitopes: YF9/B35, WF9/B15, and KY11/Cw07 (Table 3). While all 13 sequences contained the <u>F</u>PLTFGWCF sequence (a Y1F substitution, compared to that of CON), PTE peptide variants containing three different epitope sequences were recognized. In addition, while 11 of 12 WF9 autologous sequences matched the CON B sequence, six different variants of the epitope were recognized. Similarly, while the KY11 autologous sequences contained a mix of only two sequences (KRQDILDLWVY and KRQDILDLW<u>I</u>Y), all five epitope variants tested showed reactivity (Table 3). In summary, multiple epitope variants were recognized by the PBMC even when these were not contained among the autologous virus sequences.
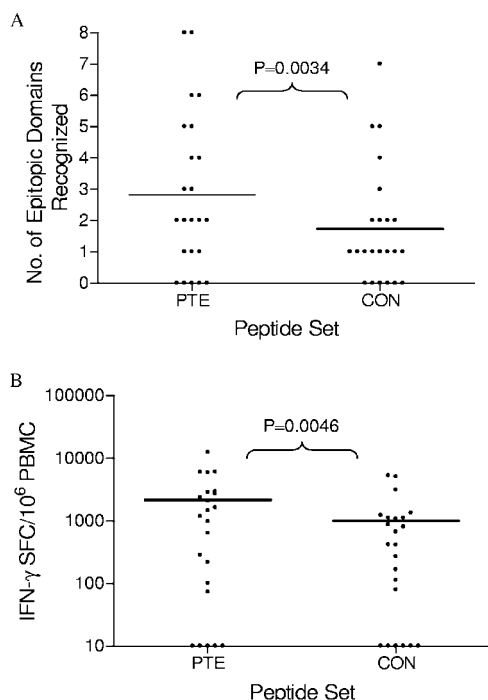
FIG. 5. Breadth (A) and magnitude (B) of the Nef-specific T-cell responses in 23 HIV-1 infected subjects, using PTE and CON peptide sets in IFN-γ ELISPOT assays. The breadth of response for an individual subject was defined as the sum of the recognized peptides, and the total magnitude of response was determined by the sum of the response to all epitopic regions. The recognition of two adjacent CON peptides was imputed as one epitopic region, and the greater of the two responses was enumerated in the total magnitude. Similarly the recognition of multiple variant PTE peptides with an amino acid overlap of ≥10 amino acids was imputed to be one epitopic region, and the greatest response was enumerated in the total magnitude. T-cell responses between CON and PTE peptide sets were compared by the use of two-tailed paired t tests on linear (breadth) and log-transformed (magnitude) data.

Breadth and magnitude of response to Nef CON and PTE peptide sets. Among the 23 subjects, a mean of 2.7 (median, 2; range, 0 to 8) epitope domains were detected with the PTE peptide set compared to 1.7 (median, 1; range, 0 to 7) epitope domains with the CON peptide set ($P = 0.0034$) (Fig. 5A). The average (median) magnitude of response was 2,169 SFC/10^6 PBMC (1,177) with the PTE peptide set compared to 1,010 SFC/10^6 PBMC (419) with the CON peptide set ($P = 0.0046$) (Fig. 5B). The recognition of multiple variant PTE peptides with an amino acid overlap of ≥10 aa was imputed as one epitopic domain.

To determine why an increased breadth of response was detected with the PTE peptide set versus the CON peptide set, we compared the sequence of the reactive PTE peptides with the CON peptides. Overall, an additional 29 epitopic domains not detected with the CON peptide set were detected with the PTE peptides (Table 4; also see Table S2 in the supplemental material). Among these, 18 epitopic domains contained one or more amino acid substitutions. In the remaining 11, the position of the domain within the 15-mer differed between the PTE and CON peptides. An additional five epitopic domains not detected with the PTE

peptide set were detected with the CON peptides. A compilation of all PTE and CON peptides recognized is presented in Table S2 in the supplemental material.

In summary, both the breadth and magnitude of responses detected by stimulation with the PTE peptide sets were significantly greater than the breadth and magnitude detected with the CON peptide set. The increase in the number of epitopic domains detected with the PTE peptides was a combination of the recognition of peptides whose amino acid sequence differed from the CON sequence and the differences in the position of the epitopic domain within the 15-mer peptides.

DISCUSSION

Our findings indicate that Nef is frequently targeted during early HIV-1 infection and that T cells in these newly infected subjects are capable of recognizing multiple epitope variants. Several variants were frequently recognized even when these were not present among the autologous virus populations. Thus, T-cell clonotypes capable of cross-reactivity exist within the memory/effector pool, despite not having encountered the variants previously. These findings have wider implications in relation to vaccine immunogenicity, suggesting that vaccine-induced T cells may be able to recognize epitopes both in and outside the subtype represented in the vaccine.

The use of multiple peptide variants for variable epitopic domains, as in the PTE peptide set, provided important insights into TCR cross-reactivity, but it also raised important questions. T-cell responses that reacted with multiple epitope variants often showed significant differences in the magnitudes of their responses to the variants. Importantly, reductions in magnitude were not always accompanied by reductions in the avidity of the T-cell responses. In subject 1188, for example, while the magnitude of the response to the WF9 variant-R6S versus the WF9 epitope was nearly 1 log-fold lower, the avidity of the response was 4-fold greater (Table 2). It is possible that the immune response constitutes several T-cell clonotypes, both dominant and subdominant (39), and that different sets of clonotypes may react with the epitope and its variants. Studies to interrogate the constituent T-cell clonotypes within the memory T-cell pools are currently in progress.

The quality of the virus-specific T-cell response is variously determined by the avidity of the TCR-peptide MHC interaction, the duration and intensity of antigen stimulation, and the presence of CD4 help. The relative avidity of the TCR-peptide MHC interaction may influence the cytokine response profiles (25, 39). Epitope variants may deliver negative signals to T cells specific for the agonist peptide-MHC complexes. For example, altered class II anchor residues but identical TCR contact residues can induce T-cell anergy in an antigen-specific manner (32). Thus, while we detected IFN-γ-producing T-cell responses to multiple epitope variants, further investigation is needed to define the quality of the response to the different epitope variants. Such information will be insightful for the strategic design of vaccines that generate T-cell populations with optimal effector activities.

Furthermore, our data provide evidence for enhanced detection of T-cell responses with the Nef PTE peptides compared to that of CON peptides in subjects with early HIV-1

TABLE 4. Summary of epitopic domains identified with PTE or CON peptides, in subjects with HIV-1 subtype B infection

Subject	Presumed epitope/HLA restriction (aa)[a]	PTE				CON			
		Peptide	HXB2 aa position	Sequence[b]	SFC/10⁶ PBMC	Peptide no.	HXB2 aa position	Sequence	SFC/10⁶ PBMC
1188	RL9/B7-super (77–85)	9	074–088	IPLRPMTYKGALDLS	176				
		79	074–088	VPLRPMTYRAARDLS	313				
	TL10/B35 (128–137)	31	126–140	NYTPGPGTRYPLTFG	82				
		27	127–141	YTPGPGVRYPLTFGW	492				
	UNK	42	175–189	DPEREVLEWRFDSRL	53				
1212	UNK	64	009–023	SVVGWPAVRERMRRA	54				
	UNK	31	126–140	NYTPGPGTRYPLTFG	66				
	UNK	19	160–174	ENNSLLHPMSLHGMD	56				
1216	UNK	46	032–046	AVSRDLERHGAITSS	82				
	FL9/B7 (68–76)	39	063–077	EEEVGFPVKPQVPLR	117				
	UNK	80	188–202	SLAFHHRARELHPEY	57				
		6	190–204	AFHHMARELHPEYYK	262				
		40	190–204	AFRHVARELHPEYFK	167				
1238	UNK					1	001–015	MGGKWSKRSVVGWPT	53
	UNK					9	033–047	VSRDLEKHGAITSSN	73
	FR10/B7 (68–77)					17	065–079	EVGFPVRPQVPLRPM	87
1362	UNK	37	108–122	DILDLWIYHTQGYFP	1,430				
1484	FL9/B35/B7 (68–76)	39	063–077	EEEVGFPVKPQVPLR	618				
1490	AK10/A11 (83–92)	7	078–092	PMTYKAAVDLSHFLK	60				
		3	083–097	GALDLSHFLKEKGGL	67				
	UNK	2	131–145	PGIRYPLTFGWCFKL	57				
1576	UNK	34	101–115	IYSQKRQDILDLWIY	67				
1585	UNK	80	188–202	SLAFHHRARELHPEY	57				
		52	191–205	FHHKARELHPEYYKD	73				
1596	UNK	2	131–145	PGIRYPLTFGWCFKL	462				
1599	UNK	1	068–082	FPVRPQVPLRPMTYK	775				
	AL8/A2 (84–91)	3	083–097	GALDLSHFLKEKGGL	72				
	UNK	25	090–104	FLKEKGGLEGLIYSQ	867				
		41	090–104	FLREKGGLEGLVYSQ	157				
	UNK	27	127–141	YTPGPGVRYPLTFGW	467				
	UNK					46	181–195	LEWKFDSRLAFHHMA	105
						47	185–199	FDSRLAFHHMARELH	58
1686	VY8/B35 (74–81)	9	074–088	IPLRPMTYKGALDLS	829				
		79	074–088	VPLRPMTYRAARDLS	1,976				
1690	UNK	2	131–145	PGIRYPLTFGWCFKL	690				
	EL8/B8 (182–189)	42	175–189	DPEREVLEWRFDSRL	227				
		35	180–194	VLVWKFDSRLAFRHM	77				
	AY8/A01 (195–202)	52	191–205	FHHKARELHPEYYKD	170				
1692	UNK	1	068–082	FPVRPQVPLRPMTYK	101				
	AL9 (190–198)	10	184–198	KFDSRLAFHHVAREL	51				
		62	184–198	KF*SRLAFHHIAREKH	88				
1693	WM8/B0801 (13–20)					4	013–027	WPTVRERMRRAEPAA	110
	UNK	43	052–066	NAACAWLEAQEEEEV	193				
	UNK	39	063–077	EEEVGFPVKPQVPLR	1,810				
		1	068–082	FPVRPQVPLRPMTYK	180				

[a] HLA restriction was inferred based on the subject's known HLA type and published MHC class I restricted CD8⁺ T-cell epitopes (23), except for epitopes indicated with an asterisk. Optimal epitopes and HLA restrictions for the latter were confirmed using peptide truncations and partially matched and mismatched antigen-presenting cells. UNK, epitope unknown.

[b] Amino acid differences from the CON sequence are underlined.

subtype B infection. The data support the model for which the per-epitope magnitude of response is comparable across both peptide sets but the PTE set provides greater numbers of reactive epitopes. Thus, the gain is primarily in the enhanced detection of breadth, with the increased magnitude of the total response being a secondary effect. Since the PTE peptides are designed to maximally cover known circulating viral sequences, they are especially well suited for the analysis of potentially protective immune responses in recipients of HIV-1 preventive vaccines and for objective comparisons of T-cell responses to various vaccine candidates. We are currently using the PTE peptides to screen vaccine candidates in HIV vaccine trials (phase I-IIB), and we continue to observe enhanced detection of responses with the PTE compared to those of CON peptides to gene products other than HIV-1 Nef as well. In addition, the use of these peptides has been associated with substantial cuts in peptide synthesis costs. The PTE peptide sets are also an affordable alternative to autologous sequence-based peptides for the evaluation of immune responses in HIV-1-infected subjects.

Overall, an additional 29 epitopic domains not detected with the CON peptide set were detected with the PTE peptides. The greater breadth of T-cell responses detected with the PTE than with the CON peptide set can be attributed to several factors. Most importantly, the PTE peptide set is larger and specifically optimized for the representation of viral population diversity. The PTE peptide set contains several peptide variants corresponding to the more variable domains. Such variants differed from those of the CON sequence by one or more amino acids, either within the presumed epitope or in the flanking sequences. Of note, 18 of the 29 epitopic domains detected with the PTE, but not the CON, peptides contained one or more amino acid substitutions. In the remaining 11, the position of the domain within the 15-mer differed between the PTE and CON peptides. Here, differences in T-cell recognition may be related to the position of the epitope within the 15-mer peptides, which may influence binding to the HLA molecule and complexing with the TCR. However, when differences attributable to epitope location are excluded from the analysis, the breadth of responses detected with the PTE peptide set still remains significantly higher than that detected with the CON peptide set (two-tailed paired nonparametric t test, $P = 0.013$). Importantly, the 15-mer PTE peptide set is designed to efficiently capture the most prevalent CTL epitopes among circulating HIV-1 strains using the fewest numbers of peptides. In other words, the primary emphasis in the PTE peptide design is to encompass the maximal number of 9-mer PTE within as few 15-mer peptides as possible. Thus, while epitope location within the 15-mer may be important, the 9-mer PTE are not positioned in any preferred location within the 15-mer. Finally, we speculate that other differences may be due to some epitope lengths being greater than 11 amino acids, since such epitopes may not be represented at all in a CON peptide set that consists of 15-mers overlapping by 11 amino acids.

The decision to construct a clade B Nef PTE reagent with 70% coverage was arbitrary. The potential benefit of higher-percentage (80 to 90%) coverage is largely outweighed by the low probability of sufficient numbers of donor cells being available to complete the testing. Of note, since 220 peptides are needed for 80% and 740 for 90% coverage, the processes of

synthesizing, quality controlling, and pooling are costly and labor intensive. Of our two options to design either a 9-mer or a 15-mer peptide set, constructing a 9-mer peptide set required the synthesis of several hundred peptides, which was neither practical nor economically feasible. Therefore, we designed a 15-mer peptide set with each peptide containing either one or more overlapping high-frequency PTE, such that gene sequence colinearity was preserved. The ability to capture PTE, both $CD4^+$ and $CD8^+$, that may be longer than 9-mers, is another advantage of this approach. CTL epitope length restrictions are clearly not strict, and many epitopes of 11 to 14 residues elicit dominant responses (8, 11, 17, 18, 40, 44, 45). The frequency at which long epitopes are recognized is likely to be underestimated since widely used algorithms to predict CTL epitopes typically include peptides of up to 10 amino acids in length (19, 41).

Our findings demonstrate a highly characteristic pattern of PTE peptide recognition among the subjects. The greatest number of subjects' T cells recognized the highest ranking peptides, and few recognized the low-ranking peptides. In other words, the most recognized peptides were also the ones containing the PTE present at the greatest frequency among the virus isolates. This finding is not surprising, since the high-ranking PTE are also the ones that are the most conserved among the different virus sequences. However, not all the high-ranking peptides were recognized. For example, PTE peptides 5, 11, 12, 13, 16, 17, and 18 did not react with PBMC from any of the 23 subjects. We speculate that some of these peptides encompass domains that, although highly conserved, do not contain T-cell epitopes. These findings are in agreement with prior observations for a nonuniform epitope distribution in HIV with distinct clusters separated by regions where no epitopes have been identified (14, 47). Mechanisms proposed to account for the distribution of epitopes include the processing preferences for proteasomal cleavage (6, 10, 22), the frequency of C-terminal amino acids that inhibit HLA binding (3, 6) and C-terminal anchor amino acids (13), and finally, the constraints on the amino acids that can be accommodated in the peptide-binding groove of the HLA molecules (16, 30, 38). Thus, while we detected a significantly greater breadth and magnitude of T-cell response with the PTE peptide set than with the CON set, the maps of epitope distributions with the two peptides sets had a striking resemblance, with similar epitope-rich and epitope-sparse domains. Additionally, we speculate that the pattern of greater reactivity to the more conserved PTE is likely a function of studying infected subjects. Thus, the pattern may be different in HIV-1-uninfected vaccinees, in whom the antigen stimulating the immune response is fixed for all study participants.

During early HIV-1 infection, Nef-specific T cells are commonly induced that are capable of recognizing multiple variants within subtype B HIV-1. Our findings suggest that when induced by a given vaccine strain before HIV-1 exposure, the T-cell responses may be sufficiently broad to recognize many variants within subtype B HIV-1. Our study confirms the enhanced detection of immune responses using PTE peptides in comparison with that of the CON peptides, which to date has been the single best strategy put forward to maximize coverage. While in this study we have employed the PTE peptides to

evaluate vaccine immunogenicity, the approach lays the framework for the design of a vaccine immunogen with expanded T-cell epitope coverage for protection against highly diverse and rapidly changing virus populations.

ACKNOWLEDGMENTS

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