Comprehensive Analysis of Human Immunodeficiency Virus Type 1 (HIV-1)-Specific Gamma Interferon-Secreting CD8⁺ T Cells in Primary HIV-1 Infection

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Human immunodeficiency virus type 1 (HIV-1)-specific CD8⁺ T cells provide an important defense in controlling HIV-1 replication, particularly following acquisition of infection. To delineate the breadth and potency of these responses in patients upon initial presentation and before treatment, we determined the fine specificities and frequencies of gamma interferon (IFN- γ)-secreting CD8⁺ T cells recognizing all HIV-1 proteins in patients with primary infection. In these subjects, the earliest detected responses were directed predominantly against Nef, Tat, Vpr, and Env. Tat- and Vpr-specific CD8⁺ T cells accounted for the greatest frequencies of mean IFN- γ spot-forming cells (SFC). Nef-specific responses (10 of 21) were more commonly detected. A mean of 2.3 epitopes were recognized with various avidities per subject, and the number increased with the duration of infection ($R = 0.47$, $P = 0.031$). The mean frequency of CD8⁺ T cells (985 SFC/10⁶ peripheral blood mononuclear cells) correlated with the number of epitopes recognized ($R = 0.84$, $P < 0.0001$) and the number of HLA-restricting alleles ($R = 0.79$, $P < 0.0001$). Neither the total SFC frequencies nor the number of epitopes recognized correlated with the concurrent plasma viral load. Seventeen novel epitopes were identified, four of which were restricted to HLA alleles (A23 and B72) that are common among African descendents. Thus, primary HIV-1 infection induces strong CD8⁺-T-cell immunity whose specificities broaden over time, but their frequencies and breadth do not correlate with HIV-1 containment when examined concurrently. Many novel epitopes, particularly directed to Nef, Tat, and Env, and frequently with unique HLA restrictions, merit further consideration in vaccine design.

The emergence and preservation of CD8⁺ cytotoxic T lymphocytes (CTL) are fundamental in the host defense against human immunodeficiency virus type 1 (HIV-1) infection. In primary infection, the appearance of CTL coincides with a rapid decline of plasma viremia, and the early responses correlate with slower declines in CD4⁺-T-cell counts as the viral set point is established over the ensuing months (6, 23, 27, 31). In addition, vigorous HIV-1-specific CTL responses are maintained in most long-term nonprogressors (17, 20, 29) and decline in persons experiencing disease progression (9, 20, 32). In recent studies of antiretroviral treatment interruptions, significant increases in CTL responses were observed when viremia rebounded, suggesting an accelerated defense against viral replication (30, 33). In vitro experiments provide further evidence that antigen-specific CD8⁺ T cells can suppress HIV-1 replication by major histocompatibility complex (MHC)-restricted cytolytic and noncytolytic mechanisms (38, 39). Perhaps the strongest support for the crucial role of CTL in controlling acute infection is the demonstration in the macaque model that depletion of CD8⁺ T cells following simian immunodeficiency virus (SIV) challenge results in reduced suppression of the initial plasma viremia (19, 36).

Despite the recognized importance of adaptive CD8⁺-T-cell immunity in acute infection, there is very little information concerning the overall breadth, specificity, frequency, and functional avidity of the T-cell response early after acquisition compared to responses after viral set point is achieved. Defining these issues requires a heightened awareness among clinicians to suspect acute HIV-1 infection and refer patients for enrollment in immunopathogenesis studies. Moreover, many patients with acute infection elect to initiate antiretroviral therapy, if available, soon after diagnosis, which can induce alterations in the frequencies of HIV-1-specific CD8⁺ T cells (29). Thus, most HIV-1-specific CTL epitopes identified thus far have been those recognized by CD8⁺ T cells from patients with chronic infection (22), and recent studies suggest that differences in specificities may occur (14).

In the macaque model, the predominant CD8⁺ CTL responses controlling acute SIV infection may be directed against accessory proteins rather than structural proteins that have been well characterized in chronic SIV infection (1). Furthermore, some CTL responses may be more important than others in controlling SIV replication (28), an issue that has not been fully explored in HIV-1 infection. To understand the extent to which CD8⁺ memory T cells exert immune pressure and determine viral set point, additional studies to characterize the CD8⁺-T-cell response with respect to epitope specificity and effector function are needed first.

In this investigation, we performed a comprehensive analysis

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TABLE 1. Demographic and baseline clinical and virological characteristics of subjects with primary HIV-1 infection

Subject no.	Age (yr)	Ethnicity	Seroconversion ^a		dpi	Baseline values		
			Last negative EIA (dpi)	First positive EIA (dpi)		Plasma viral load (RNA counts/ml)	CD4 count (cells/ μ l)	CD8 count (cells/ μ l)
1168	20	Caucasian	-26	18	9	556,728	548	429
1184	28	Hispanic	None	7	26	4,110	601	789
1188	23	Hispanic	-28	62	79	73,074	409	1,172
1201	27	Caucasian	-129	24	4	>750,000	775	1,072
1212	37	Caucasian	11	17	5	>750,000	755	481
1224	41	Caucasian	-28	7	7	43,500	537	1,039
1237	32	Black	7	24	7	83,100	680	333
1238	42	Caucasian	-31	35	45	99,600	367	367
1242	30	Caucasian	-125	24	42	96,900	880	1,500
1245	32	Caucasian	4	59	67	39,562	777	587
1252	38	Caucasian	-24	30	3	3,250,000	381	127
1261	23	Caucasian	-306	16	16	25,000	990	855
1282	31	Caucasian	4	24	4	800,000	639	867
1294	26	Hispanic	-43	48	44	1,749,236	718	1,144
1362	24	Caucasian	8	22	8	>800,000	876	786
1372	36	Caucasian	0	19	19	39,746	939	755
1394	45	Caucasian	-2	75	75	3,428	645	510
1396	39	Caucasian	27	32	32	750,000	686	1,346
1397 ^b	36	Caucasian	-42	44	48	752	757	426
1408	33	Caucasian	3	14	3	>500,000	397	629
1410	39	Caucasian	-89	74	91	31,537	412	528
Mean	32		-40	32	37	497,442	656	750

^a EIA, enzyme immunoassay; dpi, days postinfection.

^b Subject 1397 was the only study participant who was not diagnosed with acute infection on the basis of acute symptoms. His date of infection was designated as the midpoint between the last negative and the first positive enzyme immunoassay.

of the HIV-1 epitopes recognized by CD8⁺ T cells in 21 untreated subjects presenting with early infection. Overall, the early CD8⁺ T responses were narrow with respect to the number of epitopes recognized per patient and exhibited various functional avidities. The CD8⁺-T-cell epitopes, including many previously undefined, were commonly found within regulatory and accessory genes. Furthermore, the frequencies of HIV-1-specific gamma interferon (IFN- γ)-secreting T cells did not correlate with the concurrent levels of plasma viremia.

MATERIALS AND METHODS

Study subjects. Twenty-one patients enrolled with newly diagnosed HIV-1 infection and followed longitudinally at the University of Washington Primary Infection Clinic were evaluated in this study. These subjects were selected based upon the availability of cryopreserved peripheral blood mononuclear cells (PBMCs) from leukapheresis performed during primary infection. Primary infection was documented by signs and symptoms consistent with an acute retroviral syndrome for 20 subjects (3, 34, 35). The duration of infection in these subjects was designated from the date of onset of acute symptoms (Table 1). In the case of subject 1397, the duration of infection was estimated as the midpoint between a prior documented negative HIV-1 enzyme immunoassay and a recent positive confirmatory test. Blood samples analyzed for viral load and T-cell counts were collected before initiation of antiretroviral therapy. The University of Washington and Fred Hutchinson Cancer Research Center institutional review boards approved the study, and all subjects provided written informed consent for participation in the study.

Plasma HIV-1 levels, T-cell subset counts, and HLA typing. Plasma HIV-1 RNA was determined by quantitative branched-DNA assay (Chiron, Emeryville, Calif.) (26) and/or reverse transcription-PCR assay (Roche Molecular Systems, Branchburg, N.J.) (11), having sensitivities of 500 and 50 copies/ml, respectively. Peripheral blood CD4⁺ and CD8⁺-T-cell counts were determined by flow cytometry with consensus methods (11) and expressed as cells per microliter. HLA typing was performed at the Puget Sound Blood Center by sequence-specific primer PCR as previously described (8).

Synthetic peptides. To express HIV-1 epitopes in Elispot assays, overlapping peptides that spanned the HIV-1 genes encoding the Gag (122 15-mers), Pol (100 20-mers), Env (80 8- to 20-mers), Nef (20 20-mers), Tat (23 15-mers), Vpr (22 15-mers), Rev (27 15-mers), Vif (47 15-mers), and Vpu (six 9-mers and 13 15-mers) proteins were synthesized. All 20-mers overlapped by 10 amino acids, and the 15-mers overlapped by 11 amino acids. The sequences of the Gag, Nef, Pol, and Tat peptides were based on HIV-1_{HXB2}, the Env peptides were based on HIV-1_{MN}, and the Vpr, Rev, Vif, and Vpu peptides were based on the HIV-1 clade B consensus sequence (22). The SynPep Corporation (Dublin, Calif.) synthesized the Gag peptides, and the Shared Resources Center at Fred Hutchinson Cancer Research Center synthesized the Tat peptides. The National Institutes of Health AIDS Research and Reference Reagent Program (Bethesda, Md.) provided all other peptides.

Peptides corresponding to each protein were tested initially in pools: Gag, five pools; Pol, 10 pools; Env, eight pools; Nef, Vif, and Vpu, two pools each; and Tat, Vpr, and Rev, one pool each. In addition, 8- to 11-mers corresponding to approximately 100 described class I HLA-restricted HIV-1 CTL epitopes (14) were tested either singly or in pools according to an individual's HLA type. Four peptides derived from human actin and one from the HLA class I heavy chain were synthesized and used as negative peptide controls.

IFN- γ Elispot assay. Cryopreserved PBMCs were thawed at 37°C and resuspended (2×10^6 cells/ml) in medium (RPMI-HEPES with 10% fetal bovine serum supplemented with L-glutamine, and penicillin-streptomycin). Cell viability and recovery were both determined after initial thawing and overnight incubation at 37°C and 5% CO₂. To determine the specific T-cell subset secreting IFN- γ , PBMCs were separated into enriched CD4⁺ and CD4⁻ (i.e., CD8⁺) T-cell populations with anti-CD4 monoclonal antibody-coated immunomagnetic beads (Miltenyi Biotec, Auburn, Calif.) according to the manufacturer's instructions.

Multiscreen (Millipore, Bedford, Mass.) filtration plates were coated with 100 μ l of the anti-IFN- γ monoclonal antibody 1-D1K/well (10 μ g/ml; Mabtech, Nacka, Sweden), incubated overnight at 4°C, washed with phosphate-buffered saline, and blocked (37°C for 1 h) with RPMI-HEPES-0.5% bovine serum albumin. PBMCs were washed, and 200,000 cells suspended in 75 μ l of medium were plated in each well. Peptides, either alone or in pools, were suspended in 25 μ l of RPMI-HEPES and added to appropriate wells at a 2- μ g/ml final concentration. The peptide solvent, dimethyl sulfoxide, was kept below 1% final con-

TABLE 2. HIV-1-specific CD8⁺-T-cell responses: breadth, HLA class I usage, and total frequency^a

Subject no.	Time of analysis (dpi)	No. of subjects responding to:									No. of epitopes recognized per subject	No. of restricting class I alleles	Total IFN- γ SFC/10 ⁶ PBMCs	Concurrent plasma viral load (RNA copies/ml)
		Gag	Pol	Env	Nef	Tat	Vpr	Rev	Vpu	Vif				
1168	32			1							1	1	208	8,469
1184	46										0	0	NR	657
1188	92		3	2	1						6	3	2,665	58,434
1201	40	1									1	1	1,045	1,240
1212	16				1						1	1	1,060	102,045
1224	43	2									2	2	482	5,000
1237	24	1									1	1	145	12,500
1238	47	2		1	2		1				6	4	1,930	99,600
1242	46			2	2						4	2	1,525	96,900
1245	79										0	0	NR	36,800
1252	15										0	0	NR	800,000
1261	35			1	1					1	3	2	1,061	9,550
1282	31			1	1						2	2	770	78,400
1294	49	1		3	2						6	5	3,018	222,000
1362	34					1	1			ND	2	2	1,973	152,000
1372	36						1		ND	ND	1	1	250	13,601
1394	83	1						1			2	2	1,155	2,545
1396	62	1			1						2	2	538	8,199
1397	62	2	1		1						4	4	1,169	<50
1408	19			1		1					2	2	1,035	305,990
1410	92	2			1			ND	ND		3	3	660	31,537
Mean	47										2.3	1.9	985	97,406
Total ^b		9	2	8	10	2	3	1	0	1				

^a NR, no response detected; ND, not determined.^b Total number of subjects responding to each HIV-1 protein.

centration in the peptide mixtures. Cells were stimulated with the peptide pools or individually overnight at 37°C (5% CO₂). IFN- γ secretion was detected colorimetrically by sequential incubations with secondary biotinylated anti-IFN- γ 7-B6-1 monoclonal antibody (1 μ g/ml; Mabtech), avidin-peroxidase (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, Calif.), and peroxidase substrate (Vector Laboratories). Phytohemagglutinin (2 μ g/ml; Murex Biotech, Dartford, United Kingdom) or staphylococcal enterotoxin B (2 μ g/ml; Sigma, St. Louis, Mo.) stimulations were used as a positive control. For the negative control, no peptide or a pool of five irrelevant peptides (2 μ g/ml) was used.

Spots formed by IFN- γ -secreting cells were counted with an automated ImmunoSpot plate reader (Cellular Technologies, Cleveland, Ohio), and results are presented as spot-forming cells (SFC) per 10⁶ PBMCs. A response was considered positive when the mean SFC for the experimental wells was at least twofold greater than the mean SFC for the negative control wells and the mean SFC/10⁶ cells in the experimental wells was >50 after subtraction of the mean SFC/10⁶ cells of the negative control wells. Duplicate experimental and control wells were used in each experiment, and all positive responses reported here were confirmed in separate assays.

Determination of avidity of T-cell responses. The standard IFN- γ Elispot assay was performed with the defined optimal epitopic peptide at the following concentrations: 0.64, 3.2, 16, 80, 400, 2,000, and 10,000 ng/ml. The SFC frequencies per 10⁶ PBMCs were plotted against the log₁₀ peptide concentration with Origin 6.0 professional software (Microcal Software, Inc. Northampton, Mass.). The effective peptide concentration that elicited 50% of the maximum T-cell response, defined as the EC₅₀, was determined with the Sigmoidal Fit tool in the software. The molar peptide concentration was then calculated based on the molecular weight of each peptide and presented as a concentration.

Class I MHC restriction analysis. Epstein-Barr virus-transformed B-lymphoblastoid cell lines (B-LCLs) were established from study subjects and seronegative individuals (5). Autologous and allogeneic mismatched B-LCLs (10⁶/ml) were incubated in medium (RPMI-HEPES-10% fetal bovine serum supplemented with L-glutamine, and penicillin-streptomycin) with or without the peptide (2 μ g/ml) for 90 min at 37°C and 5% CO₂ and then washed three times. The peptide-pulsed B-LCLs (10,000 cells) were added to PBMCs or the T-cell subset (200,000 cells), and the Elispot assay was then performed as described above. Wells containing B-LCLs without peptide pulsing and the same donor's PBMCs served as the negative controls.

Statistical analysis. Spearman's rank correlation coefficient (*R*) was used to describe the degree of association between T-cell responses (total SFC/10⁶

PBMCs) and the number of epitopes, the number of class I HLA alleles, and the avidity of the response between the number of epitopes and sampling time and between T-cell response and plasma HIV-1 RNA copies. All correlation and associated *P* values were calculated by using SAS version 6.12. Graphic presentation was done with Excel 97 (Microsoft) and Canvas 7 (Deneba Systems). Results are shown as the mean $\pm$ standard error.

RESULTS

Study population. Twenty-one male subjects with a median age of 32 years were evaluated during acute and early HIV-1 infection. Eight (subjects 1168, 1201, 1212, 1237, 1252, 1282, 1362, and 1408) were enrolled before seroconversion (Table 1). The mean baseline plasma viral load of this study population was 497,442 HIV-1 RNA copies/ml at the mean time of 37 days after infection, ranging from 3 to 91 days (Table 1). The mean baseline CD4⁺- and CD8⁺-T-cell counts were 656 and 750 per μ l, respectively. To perform a comprehensive analysis of the earliest HIV-1-specific T-cell responses, cryopreserved PBMCs from leukapheresis were used, which was performed within 3 months of the onset of acute retroviral symptoms (mean, 47 days; range, 15 to 92 days; Table 2).

Mapping HIV-1-specific T-cell epitopes in a representative subject with acute infection. To determine the magnitude and specificities of the HIV-1-specific T-cell responses in primary infection, we screened each subject by IFN- γ Elispot assay with synthetic peptides spanning HIV-1 Gag, Pol, Env, Nef, Tat, Vpr, Rev, Vif, and Vpu. Cryopreserved PBMCs were stimulated initially with pools of overlapping 15- to 20-mer peptides. As shown for subject 1282, Nef pool 1 alone was recognized (Fig. 1A and data not shown for Vpr, Rev, Vif, and Vpu peptide pools). In addition, each subject's PBMCs were evaluated according to his class I HLA type for selective recogni-

FIG. 1. Mapping and characterization of HIV-1-specific T-cell response in acute infection. The HIV-1-specific T-cell response from subject 1282 was determined with cryopreserved PBMCs by IFN- γ Elispot assay. The bars depict the average number of IFN- γ spot-forming cells (SFC) per well containing 200,000 PBMCs ($\pm$ standard error). (A) Initial mapping of the HIV-1-specific T-cell response to peptide pools spanning Gag, Pol, Env, Nef, and Tat. Eight previously defined HLA-A2-restricted (pool) and one HLA-A31-restricted (RLRDLLLVTR) optimal CTL epitopes were also tested. Phytohemagglutinin (PHA)-stimulated cells served as the positive control, and cells stimulated with irrelevant peptides (Neg) or no peptide served as negative controls. (B) Determination of the T-cell subset responsible for the IFN- γ secretion. T-cell-enriched (CD4⁺) or depleted (CD4⁻) cell populations were tested in an IFN- γ Elispot assay with Nef pool 1. Staphylococcal enterotoxin B (SEB) instead of phytohemagglutinin stimulation was used as a positive control in this assay. (C) Fine mapping of the T-cell response to Nef with individual 20-mers (1 to 10) included in Nef pool 1. The amino acid sequence of the positive 20-mer peptide Nef 8 is shown. (D) Determination of the optimal Nef epitope. Twelve overlapping 9-mers spanning Nef 8 were tested in the Elispot assay. The amino acid sequence of the recognized 9-mer Nef8-12 (AL9) is shown. (E) Peptide titration of the Nef-AL9 epitope. Standard IFN- γ Elispot assay was carried out with the indicated peptides at 0.64, 3.2, 16, 80, 400, 2,000, and 10,000 ng/ml. The regression curve is drawn with the Sigmoidal Fit tool in the Origin 6.0 software. (F) HLA class I restriction of the Nef-AL9 epitope. Six mismatched B-LCLs were pulsed with (black bars) or without (white bars) the Nef-AL9 peptide. The class I alleles matching subject 1282 (A2 and A31; B44 and B60; and Cw3 and Cw16) are indicated for each B-LCL. IFN- γ secretion was determined by Elispot assay after incubating 1282 PBMCs with these peptide-pulsed B-LCLs.

tion of 8- to 11-mers corresponding to previously defined class I MHC-restricted HIV-1-specific CTL epitopes (21). In the case of subject 1282, the known HLA-A31-restricted CD8⁺ CTL epitopic peptide in Env (HXB2 strain), RLRDLLLIVTR, was recognized (Fig. 1A). This Env-specific response was not detected with use of the HIV-1_{MN} Env 20-mer, whose amino acid sequence, LRSFLFSYHHRDLLLIAAR, differed at positions 1, 2, 9, and 10 in comparison to the HLA-31-restricted epitope. Of note, no response was detected against the peptide pool composed of eight defined HLA-A2-restricted CD8⁺ CTL epitopes.

The T-cell responses were further defined with enriched CD4⁺ and CD4-depleted cells and single peptides or peptide matrices within the recognized pools. In the case of subject 1282, the HIV-1 Nef- and Env-specific IFN- γ -secreting cells were CD8⁺ T cells (Fig. 1B). Stimulation of PBMCs with Nef peptide 8 within Nef pool 1 resulted in the highest IFN- γ secretion in comparison to the other peptides in the pool (Fig. 1C). Subsequently, the CD8⁺-T-cell responses were mapped to an optimal 8- to 11-mer epitope, and in the case of subject 1282, this was the 9-mer Nef 8-12 AAVDLSHFL (AL9) after testing all possible 9-mers within the 20-mer peptide (Fig. 1D). We further tested the two truncated 8-mers within the AL9 9-mer. No IFN- γ SFC were detected after stimulation of PBMCs from the same time point with either 8-mer at any concentration up to 10 μ g/ml (data not shown).

The defined optimal epitope was confirmed by assessment of responses following stimulation with this peptide and two closely related 9- to 10-mers in serially diluted concentrations (Fig. 1E). The functional avidity was then identified as the effective peptide concentration eliciting 50% of the maximal response (EC₅₀), which in the case of the Nef AL9 epitope was 63 nM (Fig. 1E). The KL10 peptide (KAAVDLSHFL) also stimulated a positive response but with a higher EC₅₀ value of 152 nM.

To determine the class I MHC-restricting molecule, the epitopes were presented with various class I partially matched allogeneic B-LCL to the subject's PBMCs or CD8⁺ T cells. The restricting class I allele for the Nef AL9-specific response in subject 1282 was assessed with Nef peptide-pulsed B-LCL from six seronegative donors partially matching the class I allele HLA-A2, -A31, -B44, -B60, -Cw3, or -Cw16. IFN- γ SFC were observed with donor B-LCL matched for the HLA-B60 allele (Fig. 1F), indicating that this Nef-specific response is restricted by HLA-B60. Of note, the HLA-B60-restricted Nef-AL9 epitope is reported here for the first time, and it has an alanine at position 2 instead of the common glutamate anchor residue.

Primary infection induces HIV-1-specific IFN- γ -secreting CD8⁺ T cells in the majority of subjects. HIV-1-specific T cells secreting IFN- γ were detected in PBMCs from 18 (86%) of the 21 subjects during acute and early infection (Table 2). After performing the initial screening with PBMCs, subsequent experiments assessed the specific T-cell subset secreting IFN- γ . All positive HIV-1-specific responses detected by the IFN- γ Elispot assay were exclusively reproduced with the CD4-depleted cell populations, i.e., CD8⁺ T cells, as shown for subject 1282 in Fig. 1B. No measurable IFN- γ SFC frequencies above control levels were detected to any peptides for three subjects (1184, 1245, and 1252). The cell recovery and viability ($\geq 85\%$)

of these specimens were similar to those from the 18 donors with detectable responses, and responses to staphylococcal enterotoxin B or phytohemagglutinin were noted.

To assess the overall quantity of virus-specific CD8⁺ T cells, we totaled the IFN- γ SFC observed following stimulation with epitopic peptides (see below) in the 18 subjects with responses (Table 2). The mean number of IFN- γ -secreting cells per subject ($n = 21$) was 985 IFN- γ SFC/10⁶ PBMCs and ranged from undetectable to 3,018 (Table 2). Second, the mean SFC frequency per 10⁶ PBMCs to each of the HIV-1 proteins (compiled from the sum of the positive responses following stimulation with the individual peptides for a given gene product) were as follows: Gag, 334 ($n = 13$); Pol, 558 ($n = 4$); Env, 338 ($n = 12$); Nef, 410 ($n = 13$); Tat, 867 ($n = 2$); Rev, 1030 ($n = 1$); Vpr, 586 ($n = 3$); and Vif, 325 ($n = 1$). By contrast, the mean number of IFN- γ SFC detected following stimulation overnight with the control peptides was 9.8 (median, 5.0 SFC/10⁶ PBMCs), similar to the levels shown in Fig. 1. The CD8⁺-T-cell responses among the acutely and early-infected subjects were most commonly detected against Nef (10 of 21, 48%) and Gag (9 of 21, 43%) proteins.

Novel and previously defined CD8⁺-T-cell epitopes. To determine the precise epitopes recognized, the PBMCs were stimulated with overlapping 20-mers and 15-mers and their truncations, as demonstrated for subject 1282 and shown in Fig. 1. A mean of 2.3 epitopes were recognized by the 21 subjects (Table 2), and a summary of the defined class I MHC-restricted HIV-1 epitopes is shown in Table 3 and depicted in Fig. 2A for Gag, Nef, and Env. Forty-one HIV-1-specific CD8⁺ epitopes were recognized among the 18 subjects with a positive response (Table 3). Twenty-four of these were distinct, previously defined class I MHC-restricted HIV-1-specific CD8⁺ epitopes (14), and additional overlapping truncated peptides were not tested.

When CD8⁺-T-cell responses were detected following stimulation with 15- or 20-mers for which there were no corresponding known optimal epitopes, the T-cell determinants were fine mapped with overlapping 8- to 10-mers. Seventeen (41%) of these epitopes have not been reported previously in the HIV database (21). These newly defined epitopes spanned seven of the nine HIV-1 proteins analyzed (Table 3). It is important to note that of the 17 newly described epitopes, 6 were within HIV-1 Nef, clustering predominantly in the region from amino acid 73 to 97 (Fig. 2A). An additional five were within Env, particularly directed at gp41 amino acids 760 to 795 (Fig. 2A). Both of these regions were shown previously to be rich in CTL epitopes (21). Also, one new Nef epitope (Nef-AL9) was recognized by CD8⁺ T cells from four subjects, and the restricting HLA class I alleles included B62, B60, and Cw8 (Table 3). Of note, the Nef-AL9 epitope recognized by CD8⁺ T cells of subjects 1238 and 1294 was experimentally determined to be restricted by HLA-Cw8 and not HLA-B14 (data not shown), which are known to be in strong linkage disequilibrium. The 10-mer Nef-Cw8 epitope KAAVDLSHFL (22) was also recognized but with lower avidity than Nef-AL9 for these two subjects (data not shown).

It is important to note that IFN- γ -secreting T cells recognized a large number of epitopes within regulatory and accessory gene products during early infection (Fig. 2B), as has been previously described for acute SIV infection in Mamu-A*01

TABLE 3. HIV-1-specific CD8⁺-T-cell responses in acute and early infection: epitope specificity, MHC restriction, frequency, and avidity

Subject no.	HLA class I type	Epitope and position ^a	Peptide sequence ^b	HLA restriction ^c	No. of SFC/10 ⁶ PBMC	EC ₅₀ (nM)
1168	A*0201, 29; B44, 60; Cw3, 16	gp120 (209–217)	SFEPIPIHY	A29	208	0.3
1188	A*2301; B*3501, 1503 (B72); Cw2, 7	gp41 (585–593) ^d gp41 (760–767) RT (118–127) RT (175–183) INT (263–271) Nef (183–191)	RYLKDDQQLL LRSFLFLS VPLDEDFRKY NPDIVIYQY RKAKIIRDY WRFDSRLAF	A*2301 A*2301 B*3501 B*3501 B*1503 B*1503	85 120 285 230 1,505 440	229.6 5.4 ND ^e 28.4 172.1 751.9
1201	A1, 26; B35, 57; Cw4, 0601	p24 (108–117)	TSTLQEQIGW	B57	1,045	629.1
1212	A*0201, 23; B44, 62; Cw3, 4	Nef (83–91)	AAVDLSHFL	B62	1,060	629.1
1224	A*0201, 3; B44, 57, Cw5, 6	p17 (20–28) p24 (30–40)	RLRPGGKKK KAFSPEVIPMF	A3 B57	247 235	87.6 199.8
1237	A1, 30; B42, 58; Cw7, 17	p24 (108–117)	TSTLQEQIGW	B58	145	714.3
1238	A1, 3; B7, 14; Cw*0702, 0802	p17 (20–28) p24 (166–174) Nef (73–82) Nef (83–91) gp41 (843–851) Vpr (53–63)	RLRPGGKKK DRFYKTLRA QVPLRPMTYK AAVDLSHFL IPRRIROQL TWAGVEAIIRI	A3 B14 A3 Cw*0802 B7 ND ^e	555 200 80 400 405 290	13.5 0.3 1.6 843.6 45.1 ND
1242	A3, 33; B14, 35, Cw*0401, 0802	gp120 (78–86) gp41 (606–614) Nef (134–141) Nef (79–85)	DPNPQEVVL TAVPWNASW RYPLTFGW RPMTYKAAV	B35 B35 A33 ND	100 75 1,055 295	0.6 ND 40.1 116
1261	A*0201, 29; B58, 62; Cw*0301 1601	gp120 (209–217) Nef (19–27) Vif (125–135)	SFEPIPIHY RMRAEPAA HIVSPRCEY	A29 B62 ND	475 303 325	0.1 ND ND
1282	A*0201, 31; B44, 60, Cw3, 16	gp41 (770–780) Nef (83–91)	RLRDLILLIVTR AAVDLSHFL	A31 B60	610 160	59.9 63
1294	A1, 1; B8, 14; Cw7, 8	p24 (166–174) Nef (83–91) Nef (108–115) gp41 (584–594) gp41 (787–795) gp41 (849–856)	DRFYKTLRA AAVDLSHFL DILDLDWIY ERYLKDDQQLLG RRGWEVLKY QGLERALL	B14 Cw8 Cw7 ND A1 B8	573 480 115 125 615 1,110	ND 61.7 ND ND 14.5 5.7
1362	A*0201, 2501; B18, 51, Cw*0102, 1203	Tat (30–37) Vpr (29–37)	CCFHCQVC EAVRHFPRI	Cw*1203 B51	755 1,218	212.3 17.8
1372	A*0201, 32; B49, 51, Cw1, 7	Vpr (59–67)	AIIRILQQL	A*0201	250	42
1394	A1, 0201; B44, 57; Cw5, 6	p24 (108–117) Rev (67–75)	TSTLQEQIGW SAEPVPLQL	B57 Cw5	125 1,030	370 ND
1396	A1, 3; B8, 35	p17 (20–28) Nef (74–81)	RLRPGGKKK VPLRPMTY	A3 B35	440 98	356.1 245.9
1397	A*0201, 11; B51, 61, Cw2, 14	p17 (77–85) p15 (63–71) Nef (84–92) RT (128–135)	SLYNTVATL CTERQANFL AVDLSHFLK TAFTIPSI	A*0201 B61 A11 B51	149 70 740 210	132.2 24.6 93.0 85.5
1408	A3, 26; B7, 3801; Cw*0702, 1203	Tat (30–37) gp120 (104–112)	CCFHCQVC MHEDIISLW	Cw*1203 B*3801	905 130	700.6 163
1410	A1, 3; B8, 62; Cw3, 7	p17 (20–28) p24 (137–145) Nef (90–97)	RLRPGGKKK GLNKIVRMV FLKEKGGL	A3 B62 B8	495 60 105	43.9 ND 1,397

^a Epitope position (HXB2 amino acid numeration) in HIV-1 proteins.^b Amino acid sequence of identified T-cell epitopes, with newly defined epitopes shown in bold.^c Epitope presenting HLA allele, with newly defined alleles shown in bold.^d The amino acid numeration for gp41 starts at gp160 initiation ATG codon.^e ND, not determined; experiments failed to clearly determine the EC₅₀ value or define the MHC restriction.

FIG. 2. Novel and previously defined CD8⁺-T-cell epitopes in acute and early infection. (A) Clustering of CD8⁺-T-cell epitopes in Nef and gp41. Gag-, Env-, and Nef-specific CD8⁺ epitopes revealed in this study are illustrated. The amino acid (a.a.) numeration of each protein is shown. The previously known epitopes are shown in solid bars, and the newly defined epitopes are shown in open bars. (B) Broad CD8⁺ response to eight HIV-1 proteins. CD8⁺ responses to each HIV-1 protein in color coding are illustrated for each subject. Subjects are presented in the order of day postinfection (dpi) when the T-cell analysis was performed. The frequency (C) and breadth (D) of CD8⁺ responses were analyzed based on the size of the protein. The mean frequency and number of epitopes were calculated for each HIV-1 protein and subjects recognizing epitopes within the particular protein, then divided by the size of the protein in number of amino acids, and multiplied by 100.

rhesus macaques (1). For example, the dominant response in subject 1212 was to Nef-AL9 (1,060 SFC/10⁶) and in subject 1408 to Tat-CC8 (905 SFC/10⁶) when examined within the first 3 weeks of infection (Fig. 2B; Table 3). The Tat-CC8 epitope was also recognized by patient 1362 (755 SFC/10⁶) at day 34 of infection (Table 3) and even earlier (day 9) upon more detailed analysis (data not shown). Both subjects had the common HLA allele Cw*1203, and this was the restricting allele for the Tat-specific responses in both subjects (data not shown). Considering that the regulatory and accessory proteins are typically smaller than those to Gag, Pol, and Env, we determined the distribution of IFN- γ SFC frequencies (Fig. 2C) and the number of CD8⁺-T-cell epitopes (Fig. 2D) per 100 amino acids of each protein. As shown in Fig. 2C, responses of the highest magnitude were directed to Tat- and Rev-specific T-cell epitopes. This trend of gene-specific responses could not be analyzed statistically due to the limited number of observations. Overall, there were significantly greater IFN- γ SFC frequencies specific for epitopes within regulatory and accessory proteins than against Gag, Pol, and Env proteins ($P < 0.0001$).

Magnitude of CD8⁺ IFN- γ -secreting T cells correlates with breadth but not functional avidity of primary response. Based on the comprehensive analysis of the CD8⁺-T-cell response detailed in Table 3, we next sought to understand the relationships between the magnitude, breadth of epitope specificities, patterns of MHC restriction, and functional avidity. Not surprisingly, there was a strong correlation between the total frequencies of IFN- γ -secreting CD8⁺ T cells and the number of epitopes recognized ($R = 0.84$, $P < 0.0001$) (Fig. 3A). Consistent with this finding is the correlation of the number of restricting alleles with the total frequency of HIV-1-specific CD8⁺ IFN- γ secreting cells ($R = 0.79$, $P < 0.0001$) (Fig. 3B). Thus, there is a strong association between the breadth and overall magnitude of the CD8⁺ immune response in primary infection.

To assess the ability of the IFN- γ -secreting CD8⁺ T cells to recognize the defined epitopes in primary infection, we determined the EC₅₀ of the epitopic peptides as a correlate of functional avidity (Fig. 1E). The EC₅₀ varied widely among the CD8⁺ T-cell epitopes recognized, with a mean of 241 nM and a median of 87 nM. Also, there was marked heterogeneity in avidity for epitopes recognized in a given individual, illustrated by both relatively strong (very low EC₅₀) and weak (intermediate and high EC₅₀) concurrent responses (Table 3). For example, the EC₅₀ of the five epitopes identified from subject 1238 were 0.3, 1.6, 14, 45, and 844 nM, with the greatest avidity for the Gag p24 and Nef epitopes (Table 3). The greatest functional avidity was observed in subjects 1261 and 1168 recognizing the gp120 SFEPPIHY epitope, with EC₅₀ values of 0.1 and 0.3 nM, respectively, and in subject 1238 recognizing the p24 DRFYKTLRA (EC₅₀ = 0.3 nM).

To determine the relationship between the functional avidity and the magnitude of the CD8⁺-T-cell response to a particular HIV-1 epitope, we compared the log₁₀-transformed EC₅₀ with the IFN- γ SFC frequency for each individual epitope. As shown in Fig. 3C, there was clearly no correlation between the efficiency of the response, i.e., the ability to recognize a given epitope at low peptide concentrations, and the overall abundance of the IFN- γ -secreting cells that recognized that epitope. Of note, the epitopes recognized in the earliest in-

FIG. 3. Magnitude of CD8⁺ IFN- γ T cells correlates with breadth but not functional avidity of primary response. The total frequency of virus-specific CD8⁺ T cells is plotted against the number of epitopes (A) and number of restricting HLA class I alleles (B) for each subject. (C) The frequency of virus-specific CD8⁺ T cells was plotted against the functional avidity (EC₅₀) for each epitope. The EC₅₀ value is shown in a log₁₀ scale. The R and P values are indicated for each graph.

fects subjects were not necessarily the most avid responses. Moreover, several subjects recognized the same epitope (restricted by the same or different MHC class I allele), but with a wide range of functional avidities (e.g., Nef-AL9 in donors 1212, 1238, 1282, and 1294), as detailed in Table 3.

Breadth and magnitude of CD8⁺-T-cell response in relation to duration of infection and plasma viral load. To understand

FIG. 4. Breadth and magnitude of CD8⁺-T-cell response in relation to duration of infection and plasma viral load. (A) The HIV-1-specific CD8⁺-T-cell response broadens with the duration of infection. The number of HIV-1-specific CD8⁺-T-cell epitopes was plotted for each subject against the sampling time, indicated as days postinfection (dpi). (B) No correlation between the frequency of CD8⁺ responses and the plasma viral load. The total HIV-1-specific CD8⁺-T-cell frequency was plotted against the plasma HIV-1 RNA load (in log₁₀ scale) for each subject. The R and P values are indicated for each graph.

the kinetics of the T-cell immune response in primary infection, we examined cross-sectionally the relationship between the number of epitopes recognized and the sampling time after infection. Rapid initiation of antiretroviral therapy in the majority of our study participants precluded examination of this effect in a longitudinal analysis. As shown in Fig. 4A, the number of HIV-1-specific CD8⁺ epitopes increased with the duration of infection, indicating a broadening of the CD8⁺-T-cell response during acute and early infection. Thus, there was a correlation (albeit mild) between the number of CD8⁺ epitopes and the duration of infection among the 21 subjects studied ($R = 0.47$, $P = 0.031$; Fig. 4A). However, when we analyzed the relationship between the total frequency of detectable HIV-1-specific CD8⁺-T-cell responses and the duration of infection, no statistically significant correlation was observed (data not shown).

To understand the potential effect of CD8⁺-T-cell responses on viral replication, we examined the association between the frequency and breadth of the earliest HIV-1-specific CD8⁺ responses and the concurrent plasma viral load (Table 2). No correlation was observed between the HIV-1 RNA copy number in plasma and the total frequency of HIV-1-specific CD8⁺-T-cell responses ($R = 0.25$, $P = 0.278$, Fig. 4B). Moreover, there was no association between the number of epitopes recognized and the concurrent viral load ($R = 0.08$, $P = 0.72$). Thus, neither the magnitude nor the breadth of the earliest CD8⁺-T-cell responses measured in these subjects correlated with the concurrent level of plasma HIV-1 RNA.

Class I HLA restriction of HIV-1-specific epitopes. We determined the HLA restriction for identified epitopes, including all previously described CTL epitopes recognized by subjects in this study (Table 3). In total, eight class I HLA-A antigens presented HIV-1 epitopes, and this corresponded to 67% of all A alleles represented by the cohort. Twelve HLA-B antigens (67%) and four HLA-C antigens (33%) also presented HIV-1-specific peptides (Table 3 and Fig. 5). A mean of 1.9 class I MHC antigens per subject were used in HIV-1 epitopic presentation (Table 2). The HLA-Cw*1203 allele characterized in this study has not been previously reported to present HIV-1-specific CD8⁺-T-cell epitopes (21). In addition, we identified new epitopes restricted to HLA molecules, such as A23, B38, and B72, in which only one to three CTL epitopes have been previously reported.

Interestingly, three of the six CD8⁺-T-cell epitopes identified in subject 1294 and four of the six epitopes in subject 1188 were novel. Both subjects reported Hispanic origin (Table 1). The four novel epitopes identified in subject 1188 were restricted by HLA-A23 and -B72 (B*1503), which are common alleles among Africans and descendants of Africans (25). Moreover, only one HIV-1-specific CTL epitope has been previously reported to be restricted by HLA-A23, and two for HLA-B70, which includes B71 and B72 (21). Although the number of study subjects was limited, the use of HLA-A2 alleles in presenting HIV-1 peptides is uncommon (Fig. 5). Only 2 (subjects 1372 and 1397) of the 10 HLA-A2 patients, all further typed as A*0201 in this study, recognized epitopes restricted by HLA-A2. These were of low frequency and directed to the Gag epitope SLYNTVATL (subject 1397) and the Vpr epitope AIIRILQQL (subject 1372) (Table 3). In addition, the HLA-A1, -A30, and -B44 alleles appear to be underrepresented. By contrast, HLA-A3, -B35, -B57, and -B62 were frequently used (Fig. 5). Although very few of the previously described HIV-1 epitopes are presented by HLA-C alleles, 5 of the 21 subjects in this study used in total four C alleles (Cw5, Cw7, Cw8, and Cw12) to present HIV-1 peptides (Table 3 and Fig. 5).

DISCUSSION

Our comprehensive analysis of T-cell responses to all nine proteins of the HIV-1 genome underscores the intense CD8⁺-T-cell immune response induced during primary HIV-1 infection. The predominant IFN- γ -secreting HIV-1-specific T cells were CD8⁺ rather than CD4⁺ T cells, and they accounted on average for 0.1% of the total PBMCs as defined by Elispot analysis. This is consistent with the relative proportions of

FIG. 5. HLA class I presentation of virus-specific epitopes in acute and early infection. Twelve HLA-A, 18 HLA-B, and 12 HLA-C alleles were represented in this study cohort of 21 subjects. These alleles are indicated on the x axis with the specific allele number. The frequency for each allele is shown as the total number of subjects having the allele (white bars) and number of subjects recognizing HIV-1 epitopes restricted to the particular class I allele (black bars).

these populations in the early adaptive immune responses induced in other viral infections (18). Eighteen of the 21 patients demonstrated CD8⁺-T-cell responses, but in three patients, IFN- γ -secreting HIV-1-specific T cells were absent despite comparable specimen quality. The latter finding may be attributable to the induction of very low frequency responses (<50 IFN- γ SFC/10⁶) below the level of detection of the assay. Alternatively, the three patients may have had HIV-1-specific CD8⁺ T cells that secreted cytokines other than IFN- γ , particularly with altered T helper function, indicating responses more consistent with Tc2 profiles. It is also possible that they recognized determinants neither identical to nor cross-reactive with the epitopic peptides used in this investigation. Future studies are planned to analyze T cells recognizing epitopes within autologous viruses during acute and chronic infection.

The HIV-1-specific T cells induced during early infection of 18 patients recognized 41 distinct epitopes spanning all HIV-1 proteins except Vpu and were restricted by 24 individual class I molecules. Interestingly, 17 (41%) epitopes identified were novel. Because of the comprehensive evaluation in patients with diverse HLA types, it is not surprising that so many new epitopes and unique restrictions were identified. Most of the novel epitopes were discovered in Nef ($n = 6$) and gp41 ($n = 4$). The cluster of Nef CD8⁺-T-cell epitopes between amino acids 73 and 97 (Fig. 2A) is located in the protein's core domain containing essential residues involved with cellular signaling interactions. These epitopes primarily lie within the region containing the repeat proline motifs (PxxP) resembling SH3 binding sites (13), as well as the early stretch of the well-conserved polypurine tract. The gp41 regions recognized are within and flank the highly conserved ectodomain and the regions encoding the lentivirus lytic peptides and calmodulin-binding sites (37). The novel Tat epitope is encoded within the cysteine-rich TAR-binding region. Of note, these epitopes lie

within conserved regions of the HIV-1 genome. Thus, we anticipate that acute responses to epitopes within variable regions are underrepresented in our analysis and may largely remain undiscovered. As underscored in macaque studies of acute SIV infection (1), this limitation can be overcome only by examination of responses to determinants within the infecting autologous virus rather than consensus viral strains.

Our findings highlight several characteristics that distinguish the T-cell response in early infection. In general, we observed a narrow response per individual, with 2.3 epitopes recognized per subject on average. However, there was a broadening of the T-cell immune response within the first 3 months of infection in these individuals. Thus, the number of epitopes correlated with the time after infection, which is consistent with previous studies (2). This finding presumably parallels increasing viral diversity and evolution as a result of the error-prone reverse transcription and may be influenced by replicative fitness. More importantly, the broadening of the T-cell repertoire may reflect the response of the immune system to viral escape during acute and early infection, as seen in SIV infection in macaques (1).

Second, some of the more common class I alleles were less frequently used in HIV-1-specific antigen presentation during primary infection. For example, alleles such as A3, B35, B57, and B62 were more frequently used than others such as A1, A2, A30, and B44. This may relate to the transmission of viruses that contain escape mutations within epitopes restricted by the more common alleles over the years (24). Regarding the most frequent class I allele A2, only two subjects (1372 and 1397) out of 10 HLA-A*0201 positive subjects demonstrated responses to A2-restricted epitopes (Table 3). The HLA-A*0201-restricted Gag epitope SLYNTVATL (SL9) is typically recognized in up to 75% of A2-positive individuals studied (6, 7, 15, 16). However, only one patient developed a

response to this epitope, and he had received an HIV-1 immunogen containing this epitope. Moreover, our preliminary studies indicate the absence of MHC peptide tetramer-binding CD8⁺ T cells recognizing the common HLA-A2-restricted Gag SL9 and Pol IV9 epitopes. These results are in concordance with previous findings by Goulder and colleagues (14).

We also observed significant usage of class I HLA-C alleles in HIV-1 antigen presentation. Five patients developed HLA-C allele-restricted CD8⁺-T-cell responses to Nef, Tat, and Rev epitopes (Table 3), and in total four C alleles (Cw5, -7, -8, and -12) were identified in antigen presentation, 33% of all available C alleles (Fig. 5). The novel CD8⁺ Tat epitope (CCFHCQVC) is restricted to HLA allele Cw*1203 (Table 3), which is not known to present any HIV-1 antigens. One can speculate that the higher frequency of C allele usage relates to the failure of Nef to downregulate HLA-C expression and that this effect may be most relevant during acute infection, when viral replication is profound (10).

Third, several investigations have pointed to the potential importance of functional avidity in exerting the optimal antiviral effect (28). Our findings indicate that not all early responses are highly avid, and the avidity does not directly correlate with the frequency of each specific response. We found a wide range of avidities among the T-cell determinants recognized, and only four responses were highly avid ($EC_{50} \leq 1.0$ nM). It may be important to sustain T-cell responses with a wide range of avidities in acute infection, based on recent studies examining the beneficial effects of CTLA-4 (12). T-cell frequencies are likely to be determined by other factors, such as rapid escape of the viral epitopes. However, a limitation of our approach was that the responses were identified with peptides based on strain MN, HXB2, and consensus sequences rather than the autologous viral sequences. In addition, the assay relies upon the rate of peptide loading and unloading from the class I molecule in vitro, the T-cell receptor affinity for the complex, the number of complexes present, and the level of CD8 expression on the effectors, all of which may warrant caution in the interpretation of these findings.

In contrast to earlier reports (2, 23), the CD8⁺-T-cell response, expressed as either the total frequency of IFN- γ secreting cells or the number of recognized epitopes, did not show significant correlation in this study with the concurrent plasma HIV-1 RNA viral load. These findings are in agreement with a recent publication (4) that reported no correlation between the viral load and the total frequency of HIV-1-specific CD8⁺ T cells in a similar comprehensive study of untreated patients with chronic infection. Our findings may relate in part to the limitations of assessing the total response to consensus rather than autologous HIV-1 strains and the bias toward detection of more conserved epitopes. In addition, this is a period in which the plasma viral load and virus-specific CD8⁺-T-cell counts can undergo intense dynamic changes.

It is likely that the effects of the CD8⁺-T-cell response on viral load cannot be fully understood by the single concurrent measurement in a cross-sectional analysis. This was apparent in our previous studies examining the role of virus-specific CTL in primary infection (4). Since the patients in this group elected to initiate antiretroviral therapy soon after our analysis, we were unable to examine the viral load effects beyond the concurrent measurements. However, since neither the strength

nor the breadth of the CD8⁺ IFN- γ -secreting response was associated with concurrent HIV-1 control, this argues that the quality of the effector response may be most important, as has been suggested in macaque SIV infection studies (1). This may relate to the specific antiviral function invoked (e.g., cytolysis, cytokine, or chemokine secretion), the avidity of the interaction between the peptide-MHC complex and the T-cell receptor, and the ability of the CD8⁺ T cells to exert immune pressure for viral escape. Studies are ongoing to determine the extent of concordance between IFN- γ secretion by the CD8⁺ T cells identified in this study and their ability to lyse autologous HIV-1-infected targets and limit viral replication in vitro.

In summary, our study revealed vigorous virus-specific CD8⁺-T-cell responses during primary HIV-1 infection, and a number of new class I MHC-restricted epitopes were found. These early responses demonstrated a wide range of functional avidities. The rapid decline of plasma viral load during acute infection suggests the high level of effectiveness of CD8⁺-T-cell immunity in this phase of HIV infection. However, it is clear that induction of a strong CD8⁺-T-cell response may not be sufficient to control the viral load. Studies are in progress to delineate the phenotype and functionality of these virus-specific CD8⁺ T cells and to elucidate the features that influence lowering viral set point and sustained viral control.

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REFERENCES

- Allen, T. M., D. H. O'Connor, P. Jing, J. L. Dzuris, B. R. Mothe, T. U. Vogel, E. Dunphy, M. E. Liebl, C. Emerson, N. Wilson, K. J. Kunstman, X. Wang, D. B. Allison, A. L. Hughes, R. C. Desrosiers, J. D. Altman, S. M. Wolinsky, A. Sette, and D. I. Watkins. 2000. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. *Nature* **407**: 386–390.
- Altfeld, M., E. S. Rosenberg, R. Shankarappa, J. S. Mukherjee, F. M. Hecht, R. L. Eldridge, M. M. Addo, S. H. Poon, M. N. Phillips, G. K. Robbins, P. E. Sax, S. Boswell, J. O. Kahn, C. Brander, P. J. Goulder, J. A. Levy, J. I. Mullins, and B. D. Walker. 2001. Cellular immune responses and viral diversity in individuals treated during acute and early HIV-1 infection. *J. Exp. Med.* **193**:169–180.
- Berrey, M. M., T. Schacker, A. C. Collier, T. Shea, S. J. Brodie, D. Mayers, R. Coombs, J. Krieger, T. W. Chun, A. Fauci, S. G. Self, and L. Corey. 2001. Treatment of primary human immunodeficiency virus type 1 infection with potent antiretroviral therapy reduces frequency of rapid progression to AIDS. *J. Infect. Dis.* **183**:1466–1475.
- Betts, M. R., D. R. Ambrozak, D. C. Douek, S. Bonhoeffer, J. M. Brenchley, J. P. Casazza, R. A. Koup, and L. J. Picker. 2001. Analysis of total human immunodeficiency virus (HIV)-specific CD4⁺ and CD8⁺ T-cell responses: relationship to viral load in untreated HIV infection. *J. Virol.* **75**:11983–11991.
- Blumberg, R. S., T. Paradis, R. Byington, W. Henle, M. S. Hirsch, and R. T. Schooley. 1987. Effects of human immunodeficiency virus on the cellular immune response to Epstein-Barr virus in homosexual men: characterization of the cytotoxic response and lymphokine production. *J. Infect. Dis.* **155**: 877–890.
- Borrow, P., H. Lewicki, B. H. Hahn, G. M. Shaw, and M. B. Oldstone. 1994. Virus-specific CD8⁺ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J. Virol.* **68**:6103–6110.
- Brander, C., K. E. Hartman, A. K. Trocha, N. G. Jones, R. P. Johnson, B. Korber, P. Wentworth, S. P. Buchbinder, S. Wolinsky, B. D. Walker, and

- S. A. Kalams. 1998. Lack of strong immune selection pressure by the immunodominant, HLA-A*0201-restricted cytotoxic T lymphocyte response in chronic human immunodeficiency virus-1 infection. *J. Clin. Invest.* **101**: 2559–2566.
8. Bunce, M., G. C. Fanning, and K. I. Welsh. 1995. Comprehensive, serologically equivalent DNA typing for HLA-B by PCR with sequence-specific primers (PCR-SSP). *Tissue Antigens* **45**:81–90.
9. Carmichael, A., X. Jin, P. Sissons, and L. Borysiewicz. 1993. Quantitative analysis of the human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection: differential CTL responses to HIV-1 and Epstein-Barr virus in late disease. *J. Exp. Med.* **177**:249–256.
10. Collins, K. L., B. K. Chen, S. A. Kalams, B. D. Walker, and D. Baltimore. 1998. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature* **391**:397–401.
11. Dewar, R. L., H. C. Highbarger, M. D. Sarmiento, J. A. Todd, M. B. Vasudevachari, R. T. Davey, Jr., J. A. Kovacs, N. P. Salzman, H. C. Lane, and M. S. Urdea. 1994. Application of branched DNA signal amplification to monitor human immunodeficiency virus type 1 burden in human plasma. *J. Infect. Dis.* **170**:1172–1179.
12. Egen, J. G., and J. P. Allison. 2002. Cytotoxic T lymphocyte antigen-4 accumulation in the immunological synapse is regulated by TCR signal strength. *Immunity* **16**:23–35.
13. Geyer, M., O. T. Fackler, and B. M. Peterlin. 2001. Structure-function relationships in HIV-1 Nef. *EMBO Rep.* **2**:580–585.
14. Goulder, P. J., M. A. Altfeld, E. S. Rosenberg, T. Nguyen, Y. Tang, R. L. Eldridge, M. M. Addo, S. He, J. S. Mukherjee, M. N. Phillips, M. Bunce, S. A. Kalams, R. P. Sekaly, B. D. Walker, and C. Brander. 2001. Substantial differences in specificity of hiv-specific cytotoxic t cells in acute and chronic HIV infection. *J. Exp. Med.* **193**:181–194.
15. Goulder, P. J., A. K. Sewell, D. G. Lalloo, D. A. Price, J. A. Whelan, J. Evans, G. P. Taylor, G. Luzzi, P. Giangrande, R. E. Phillips, and A. J. McMichael. 1997. Patterns of immunodominance in HIV-1-specific cytotoxic T lymphocyte responses in two human histocompatibility leukocyte antigens (HLA)-identical siblings with HLA-A*0201 are influenced by epitope mutation. *J. Exp. Med.* **185**:1423–1433.
16. Gray, C. M., J. Lawrence, J. M. Schapiro, J. D. Altman, M. A. Winters, M. Crompton, M. Loi, S. K. Kundu, M. M. Davis, and T. C. Merigan. 1999. Frequency of class I HLA-restricted anti-HIV CD8⁺ T cells in individuals receiving highly active antiretroviral therapy (HAART). *J. Immunol.* **162**: 1780–1788.
17. Harrer, T., E. Harrer, S. A. Kalams, P. Barbosa, A. Trocha, R. P. Johnson, T. Elbeik, M. B. Feinberg, S. P. Buchbinder, and B. D. Walker. 1996. Cytotoxic T lymphocytes in asymptomatic long-term nonprogressing HIV-1 infection. Breadth and specificity of the response and relation to in vivo viral quasispecies in a person with prolonged infection and low viral load. *J. Immunol.* **156**:2616–2623.
18. Homann, D., L. Teyton, and M. B. A. Oldstone. 2001. Differential regulation of antiviral T-cell immunity results in stable CD8⁺ but declining CD4⁺ T-cell memory. *Nat. Med.* **7**:913–919.
19. Jin, X., D. E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C. E. Irwin, J. T. Safritz, J. Mittler, L. Weinberger, L. G. Kostrikis, L. Zhang, A. S. Perelson, and D. D. Ho. 1999. Dramatic rise in plasma viremia after CD8⁺ T-cell depletion in simian immunodeficiency virus-infected macaques. *J. Exp. Med.* **189**:991–998.
20. Klein, M. V. B., C. A. Holwerda, et al. 1995. Kinetics of gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: A longitudinal analysis of rapid progressors and long-term asymptomatics. *J. Exp. Med.* **181**:1365–1372.
21. Korber, B., B. D. Walker, C. Brander, R. A. Koup, J. Moore, B. Haynes, and G. Meyer. 1999. HIV molecular immunology database 1999. Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, N.Mex.
22. Korber, B., C. Brander, B. Haynes, R. A. Koup, C. Kuiken, J. Moore, B. D. Walker, and D. Watkins. 2000. HIV molecular immunology database 2000. Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, Los Alamos, N.Mex.
23. Koup, R. A., J. T. Safritz, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol.* **68**:4650–4655.
24. Moore, C. B., M. John, I. R. James, F. T. Christiansen, C. S. Witt, and S. A. Mallal. 2002. Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science* **296**:1439–1443.
25. Mori, M., P. G. Beatty, M. Graves, K. M. Boucher, and E. L. Milford. 1997. HLA gene and haplotype frequencies in the North American population: the National Marrow Donor Program Donor Registry. *Transplantation* **64**:1017–1027.
26. Mulder, J., N. McKinney, C. Christopherson, J. Sninsky, L. Greenfield, and S. Kwok. 1994. Rapid and simple PCR assay for quantitation of human immunodeficiency virus type 1 RNA in plasma: application to acute retroviral infection. *J. Clin. Microbiol.* **32**:292–300.
27. Musey, L., J. Hughes, T. Schacker, T. Shea, L. Corey, and M. J. McElrath. 1997. Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection. *N. Engl. J. Med.* **337**:1267–1274.
28. O'Connor, D. H., T. M. Allen, T. U. Vogel, P. Jing, I. P. DeSouza, E. Dodds, E. Dunphy, C. Melsaether, B. R. Mothe, H. Yamamoto, H. Horton, N. Wilson, A. L. Hughes, and D. I. Watkins. 2002. Acute phase cytotoxic T lymphocyte escape is a hallmark of simian immunodeficiency virus infection. *Nat. Med.* **8**:493–499.
29. Ogg, G. S., X. Jin, S. Bonhoeffer, P. R. Dunbar, M. A. Nowak, S. Monard, J. P. Segal, Y. Cao, S. L. Rowland-Jones, V. Cerundolo, A. Hurley, M. Markowitz, D. D. Ho, D. F. Nixon, and A. J. McMichael. 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* **279**:2103–2106.
30. Oxenius, A., D. A. Price, P. J. Easterbrook, C. A. O'Callaghan, A. D. Kelleher, J. A. Whelan, G. Sontag, A. K. Sewell, and R. E. Phillips. 2000. Early highly active antiretroviral therapy for acute HIV-1 infection preserves immune function of CD8⁺ and CD4⁺ T lymphocytes. *Proc. Natl. Acad. Sci. USA* **97**:3382–3387.
31. Pantaleo, G., J. F. Demarest, H. Soudeyns, C. Graziosi, F. Denis, J. W. Adelsberger, P. Borrow, M. S. Saag, G. M. Shaw, R. P. Sekaly, et al. 1994. Major expansion of CD8⁺ T cells with a predominant V beta usage during the primary immune response to HIV. *Nature* **370**:463–467.
32. Rinaldo, C., X.-L. Huang, Z. Fan, M. Ding, L. Beltz, A. Logar, D. Panicali, G. Mazzara, J. Liebmann, M. Cottrill, et al. 1995. High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1-infected long-term nonprogressors. *J. Virol.* **69**:5838–5842.
33. Rosenberg, E. S., M. Altfeld, S. H. Poon, M. N. Phillips, B. M. Wilkes, R. L. Eldridge, G. K. Robbins, R. T. D'Aquila, P. J. Goulder, and B. D. Walker. 2000. Immune control of HIV-1 after early treatment of acute infection. *Nature* **407**:523–526.
34. Schacker, T., A. C. Collier, J. Hughes, T. Shea, and L. Corey. 1996. Clinical and epidemiologic features of primary HIV infection. *Ann. Intern. Med.* **125**:257–264.
35. Schacker, T. 1997. Primary HIV infection. Early diagnosis and treatment are critical to outcome. *Postgrad. Med.* **102**:143–146.
36. Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallion, J. Ghayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann. 1999. Control of viremia in simian immunodeficiency virus infection by CD8⁺ lymphocytes. *Science* **283**:857–860.
37. Srinivas, S. K., R. V. Srinivas, G. M. Anantharamaiah, R. W. Compans, and J. P. Segrest. 1993. Cytosolic domain of the human immunodeficiency virus envelope glycoproteins binds to calmodulin and inhibits calmodulin-regulated proteins. *J. Biol. Chem.* **268**:22895–22899.
38. Yang, O. O., and B. D. Walker. 1997. CD8⁺ cells in human immunodeficiency virus type I pathogenesis: cytolytic and noncytolytic inhibition of viral replication. *Adv. Immunol.* **66**:273–311.
39. Yang, O. O., S. A. Kalams, A. Trocha, H. Cao, A. Luster, R. P. Johnson, and B. D. Walker. 1997. Suppression of human immunodeficiency virus type 1 replication by CD8⁺ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms. *J. Virol.* **71**:3120–3128.