T Cell Receptor Usage and Fine Specificity of Human Immunodeficiency Virus 1–specific Cytotoxic T Lymphocyte Clones: Analysis of Quasispecies Recognition Reveals a Dominant Response Directed against a Minor In Vivo Variant

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Summary

Numerous virus-specific, class I–restricted cytotoxic T lymphocyte (CTL) epitopes have been identified, yet little information is available regarding the specificity of the CTL response in persons of the same human histocompatibility leukocyte antigen (HLA) type. In this study, the human immunodeficiency virus (HIV) 1 envelope-specific CTL response was evaluated in five HLA-B14–positive persons. CTL responses specific for a previously described nine–amino acid epitope in gp41 (aa 584-592, ERYLKDQQL) could be identified in all subjects, and CTL clones specific for this epitope could be isolated from four persons. Despite heterogeneous T cell receptor usage, the fine specificity of the clones was similar, as defined by recognition of alanine-substituted peptides as well as peptides representing natural HIV-1 sequence variants. Correlation with in vivo virus sequences revealed that the dominant species in two of the subjects represented poorly recognized variants, with a K→Q substitution at amino acid 588, whereas no variants were observed in the other two subjects. Although clonal type–specific responses to these dominant variants could be identified, the magnitude of these responses remained small, and the dominant CTL response was directed at the minor in vivo variant. These studies indicate that despite similar epitope-specific immunologic pressure in persons of the same HLA type, the in vivo quasispecies may differ, and that the major in vivo immune response to a given CTL epitope can be directed at a minor variant.

HIV-1 infection elicits a profound HLA class I–restricted, virus-specific CTL response in some persons, and this CTL response appears to correlate with maintenance of the asymptomatic state (1–3). These CD8$^+$ CTL recognize endogenously processed viral antigen on the surface of infected cells through a specific interaction of the TCR with the HLA–antigen complex on the target cell surface. The TCR is a heterodimer consisting of α and β chains that are formed by the rearrangement of noncontiguous V, D, and J regions. The addition of nongermline-encoded nucleotides at the junctions of these rearranged segments increases receptor diversity, and allows for an enormous repertoire of distinct TCR able to recognize a wide range of peptide–HLA combinations.

Studies of the TCR usage by class I–restricted CTL specific for viral epitopes have attempted to assess the structure–function relationship of the TCR and to define the extent of TCR diversity in the host–response to infection. Previous studies in humans have concentrated on the response to an acute infection, influenza A, and have described limited TCR usage against an HLA-A2 matrix epitope (amino acids [aa][1] 57-68) (4, 5) and an HLA-B27–restricted nucleoprotein epitope (aa 383-391) (6). These studies suggest there may be constraints on TCR usage for recognition of defined epitopes. Limited studies have ana-

[1]*Abbreviations used in this paper:* aa, amino acid; B-LCL, EBV-transformed B-lymphoblastoid cell line.

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lyzed the TCR usage in chronic persistent viral infections (7–9). The ability of distinct CTL clones specific for the same epitope to recognize sequence variation within the cognate epitope has not been defined, nor have these fine specificities been related to variants that arise in vivo.

We have previously demonstrated that a dominant HIV-1–specific CTL response to a B14-restricted epitope in gp41 (ERYLKDQQL, aa 584-592) could be due to an oligoclonal expansion of CTL that can persist for at least 5 yr (7, and our unpublished data). To characterize the response to this epitope in persons with diverse clinical courses of disease, five HLA-B14–positive subjects were analyzed for the ability to recognize this epitope. CTL clones were established from four of these subjects. The fine specificity of these CTL clones was determined by evaluating lysis of target cells incubated with peptides containing alanine substitutions, as well as peptides representing known HIV-1 variants. Despite a relatively heterogeneous TCR usage, our results indicate a striking similarity in the ability of different CTL clones to recognize sequence variants. However, the dominant detectable quasispecies in vivo varies markedly among subjects, and in some persons the dominant quasispecies is targeted by a minority CTL population.

Materials and Methods

Subjects. Subjects 15160 and 18030 are part of the San Francisco men's health study (10). At the time of isolation of CTL clones from these subjects, 15160 was infected with HIV-1 15 yr and was asymptomatic with a CD4 count of 1,213/mm^3. Subject 18030 was infected for ~6 yr and had a CD4 count of 692. Subject LWF is an infected laboratory worker who has been followed for several years and who had a CD4 count of 284. Subject 010-115i had been infected at least 5 yr and was asymptomatic with a CD4 count of 819. Subject 010-115i previously has been shown to have significant envelope-specific CTL activity (7, 11). All subjects gave written informed consent for these studies.

Cell Lines. EBV-transformed B lymphoblastoid cell lines (B-LCL) were established and maintained in RPMI 1640 medium containing 20% (vol/vol) heat-inactivated FCS (both from Sigma Chemical Co., St. Louis, MO) and supplemented with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), and Hepes (10 mM) as described previously (12, 13). All B-LCL were free of mycoplasma infection by standard culture techniques.

HLA typing. HLA typing was performed by the San Francisco Department of Public Health for subjects 15160 and 18030, by Dr. Dean Mann (Immunogenetics Section, Laboratory of Viral Carcinogenesis, National Cancer Institute, Bethesda, MD) for LWF, and by the Massachusetts General Hospital Tissue Typing Laboratory for subject 010-115i by standard serological techniques. The class I HLA types of these subjects are 010-115i: A2,28; B14,52; Cw8; 15160: A3,32; B14,44; 18030: A2,33; B14,27; Cw1; and LWF: A2,11; B14,35.

HIV-1 Sequencing. HIV-1 envelope sequences within the gp41 epitope were determined by a nested PCR technique as described in (3).

Synthetic Peptides. The nine–amino acid peptide gp41/584-592 and all peptides representing HIV-1 variants or alanine substitutions were synthesized as COOH-terminal acids on a Synergy peptide synthesizer (model 432A Applied Biosystems, Foster City, CA). The amino acid numbering is according to the PV22 sequence.

Isolation of HIV-1 Envelope-specific CTL Clones. CTL clones from subjects 15160, 18030, and 010-115i were isolated as previously described (7). Briefly, PBMC obtained by separation on Ficoll–sodium diatrizoate (Histopaque 1077; Sigma Chemical Co.) were cloned at limiting dilution with irradiated allogeneic feeder cells, R10 with 100 U/ml rIL-2, and PHA at 0.25 μg/ml (Nurex Diagnostics Inc., Atlanta, GA). Clones were screened for CTL activity against autologous targets infected with recombinant vaccinia virus expressing the HIV-1 envelope glycoprotein of the BH8 isolate of HIV-1 or against autologous B-LCL pulsed with peptide gp41/584-592. The CTL clones LWF A20, LWF C8, and 18030 G14 were derived by incubating PBMC with autologous B-LCL pulsed with gp41/584-592 for 10 d. These cells were then cloned at limiting dilution and screened for the ability to lyse Vac-Env–infected autologous B-LCL and peptide-pulsed autologous B-LCL. With the exception of clone 18030 G14, all active clones in this paper were subcloned at 10, 3, 1, and 0.3 cells per well in the presence of irradiated feeder cells and PHA. Subclones from plates that exhibited growth of <30% of seeded wells were expanded and subjected to TCR analysis.

Cytotoxicity Assay. Target cells consisted of B-LCL infected with vaccinia–HIV-1 expression vectors, vaccinia control, or B-LCL incubated with the indicated concentrations of the designated peptides for 60 min during ^{51}Cr labeling. Cytolytic activity was determined in a standard ^{51}Cr-release assay using U-bottomed microtiter plates containing 10^4 targets per well (14).

Limiting Dilution Assays of CTL Precursors. Precursor frequencies of HIV-1 epitope–specific CTL were estimated by performing limiting dilutions on freshly isolated PBMC followed by in vitro stimulation with autologous irradiated PBMC pulsed with peptides. PBMC were cultured at 250–16,000 lymphocytes per well in 24 replicate wells of 96-well microtiter plates. Stimulator cells were prepared by incubating autologous PBMC with 1 μg/ml of each peptide for 1 h. These cells were then washed twice with RPMI 1640 and irradiated. To each well of the precursor assay plate was added 5×10^4 γ-irradiated PBMC from a HIV-1–seronegative donor and 2.5×10^4 autologous peptide–pulsed, γ-irradiated PBMC. After 10–14 d, wells were split and assayed for cytotoxicity on ^{51}Cr-labeled autologous B-LCL incubated with the relevant peptides, or B-LCL without peptide. The fraction of nonresponding wells was the number of wells in which ^{51}Cr release did not exceed the mean plus three standard deviations of the average spontaneous release of the 24 control wells (15, 16). Activated cell frequency was estimated by the maximum likelihood method (1, 17, 18).

PCR Amplification of TCR-α and -β Sequences. The TCR usage of CTL was determined by methods previously described (7). Total RNA was isolated from 5×10^6 cells with Ultraspec (Cinna/Biotecx Laboratories International Inc., Friendswood, TX) and was converted into first strand cDNA with a constant region α or β primer, and avian myeloblastosis reverse transcriptase according to the manufacturer's specifications (Promega Corp., Madison, WI). Anchored PCR was performed on all CTL clones except 15160 DC4. A poly G tail was added with TdT (GIBCO BRL, Gaithersburg, MD). A heminested PCR was performed with previously described primers and reaction conditions (7). The resulting products were cloned into the pAMP1 vector using the Cloneamp system (GIBCO BRL) and sequenced after alkaline hydrolysis (19). The TCR usage of CTL clone 15160 DC4 was determined by amplifying the cDNA with a panel of TCR Vα

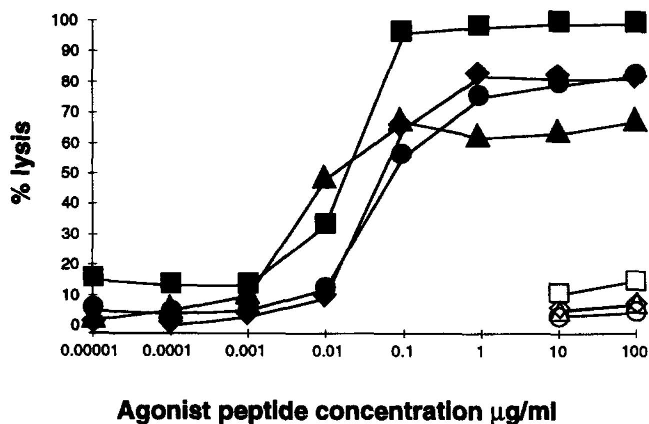

Figure 1. CTL recognition of index peptide gp41/584-592 titrated on autologous B-LCL. (*Empty symbols*) Autologous B-LCL pulsed with an irrelevant control peptide; (*filled symbols*) autologous B-LCL pulsed with index peptide gp41/584-592. (□, ■) 115 M21; (△, ▲) LWF C8; (◇, ◆) 18030 B31; and (○, ●) 15160 DC4.

and Vβ primers. In each case, only a single variable region gene was present and the PCR product was sequenced directly as described (7).

Results

Our initial finding that subject 010-115i had a vigorous response to the relatively conserved gp41 epitope ERYLKDQQL (aa 584-592) (7, 11) prompted us to evaluate whether CTL responses of identical specificity from other HLA-B14–positive subjects could be detected. CTL clones from four HLA-B14 subjects were isolated, tested against panels of peptides representing natural HIV-1 sequence variants as well as a series of alanine-substituted peptides, and subjected to TCR analysis. The CTL clones in this report had equivalent recognition of the index peptide as determined by serial log dilutions of peptide gp41/584-592 on autologous B-LCL, all requiring a peptide concentration between 100 and 10 ng/ml for target cell sensitization to achieve 50% maximal lysis of target cells (Fig. 1). A CTL line of identical specificity had been isolated from a fifth subject (20) but could not be successfully cloned in vitro for these analyses. The ability of other HLA-B14 subjects to recognize this epitope has also been reported (21, and Lieberman, J., personal communication).

TCR Gene Usage of gp41/584-592–specific CTL Clones. An examination of the TCR usage of these CTL clones was undertaken in order to assess the repertoire of TCR able to recognize this epitope. Eight of the nine CTL clones (lines) in this report were subcloned at limiting dilution; CTL clone 18030 G14 did not propagate well enough to allow subcloning. For each of the TCR sequences derived by anchored PCR, at least eight TCR-α and -β chains were sequenced. Evaluation of TCR-α sequences revealed considerable heterogeneity among the eight clones (Fig. 2 *A*). Two in-frame TCR-α chains were present in clones LWF A20 and LWF C8, a situation that is thought to occur in up to one third of α/β T cells (22). Both these CTL clones were derived from PBMC incubated with B-LCL pulsed with the gp41/584-592 peptide. The TCR-α chains of these CTL clones were identical at the nucleotide level. Both of the CTL clones from subject 010-115i had identical TCR-α chains at the nucleotide level, as has been previously described for all clones isolated from this subject (7). Although clone 18030 D6 shared Jα usage with 18030 B31, the N region sequences of these clones were distinct. In addition, a CD8-positive cell line 18030 G14, which was specific for the B14-restricted epitope but was not clonal, also contained a TCR-α nucleotide sequence identical to that of 18030 D6, indicating that the minority sequence (2 out of 11 molecular clones sequenced) was responsible for the observed lysis. In terms of Jα usage, less heterogeneity was observed. Four of the CTL clones utilized Jα 9.3 and a fifth clone utilized the related sequence Jα 9.12 (Fig. 2 *A*). Four of the clones contained an arginine residue in the N region of the TCR-α chain that may contribute to binding one of the negatively charged residues in this CTL epitope.

The TCR-β sequences were likewise determined for all of the clones (Fig. 2 *B*). Four clones from two subjects (010-115i and 18030) utilized Vβ4 and all of these clones utilized Jβ1.2 sequences as well. Although two β sequences were evident in the 18030 G14 cell line, TCR sequences identical to the minority sequences present in the 18030 G14 cell line were exclusively present in a CTL clone (18030 D6) isolated from this subject 11 mo later, indicating that these are the sequences responsible for CTL recognition of this epitope in this cell line. The clones from two other subjects utilized Vβl and Vβ12, respectively, and one of these clones also used Jβ1.2.

The similarity among the CTL from subjects 010-115i and 18030 extends across the Dβ region of the TCR-β chains with V and K residues in the first two positions of the Dβ sequences of three of the four CTL clones from these subjects, and a D residue in the third Dβ position in three of these CTL. In contrast to the observed similarities in TCR usage by subjects 010-115i and 18030, TCR usage by clones from subjects LWF and 15160 were quite heterogeneous. However, despite this heterogeneity in TCR usage, the index peptide was recognized to the same extent (Fig. 1), indicating that a wide range of TCR is able to recognize a particular peptide epitope, and that the observed differences in TCR usage do not appear to result in large differences in the amount of peptide required for recognition, even in persons with very different clinical stages of infection.

gp41/584-592–specific CTL Clones from Four Different Subjects Show Differences in Recognition of Alanine-substituted Peptides. Titrations of peptides with sequential alanine substitutions uncovered differences in the fine specificity of these CTL clones. Peptides were incubated with autologous B-LCL at concentrations ranging from 100 μg/ml to 10 pg/ml; the peptide concentration resulting in 50% maximal lysis was graphed (Fig. 3). Substitutions at positions

a

CTL alpha TCR

Clone	Vα	N	Jα	#/total #
115 M21	Vα14 F C A ttc tgt gct	 Y R T tat agg acg	Jα 9.3 S G G S N Y K L T F G K G T L agt gga ggt agc aac tat aaa ctg aca ttt gga aaa gga act ctc	10/10
115 E15	Vα14 F C A ttc tgt gct	 Y R T tat cgg acg	Jα 9.3 S G G S N Y K L T F G K G T L agt gga ggt agc aac tat aaa ctg aca ttt gga aaa gga act ctc	12/12
18030 B31	Vα6 F C A ttc tgt gca	 M R N atg aga aat	Jα 9.3 S G G S N Y K L T F G K G T L agt gga ggt agc aac tat aaa ctg aca ttt gga aaa gga act ctc	9/9
18030 D6	Vα3.1 F C A ttc tgt gct	 T G H acg ggt cat	Jα 9.3 S G G S N Y K L T F G K G T L agt gga ggt agc aac tat aaa ctg aca ttt gga aaa gga act ctc	10/10
18030 G14	Vα3.1 F C A ttc tgt gct	 T G H acg ggt cat	Jα 9.3 S G G S N Y K L T F G K G T L agt gga ggt agc aac tat aaa ctg aca ttt gga aaa gga act ctc	2/12
15160 DC4	Vα22 C A L tgt gct ctg	 R V K T aga gtc aaa act	Jα9.12 G A N S K L T F G K G I T gga gcc aat agt aag ctg aca ttt gga aaa gga ata act	*
LWF A20, C8	Vα23 L C A ctc tgt gct	 V F Y N N N D M R gtg ttt tac aat aac aat gac atg cgc	Jα1.7 F G A G T R ttt gga gca ggg acc aga	8/13
	Vα24 I C V atc tgt gtg	 V R G gtg aga ggc	Jα 17.3 Q F Y F G T G T cag ttc tat ttt ggg aca ggg aca	5/13

b

CTL beta TCR

Clone	Vβ	N - Dβ - N	Jβ	#/total #
115 M21	Vβ4 S agc	 V K D G A gtg aag gac ggg gct	Jβ 1.2 G Y T F G S G T R L T V V ggc tac acc ttc ggt tcg ggg acc agg tta acc gtt gta	12/12
115 E15	Vβ4 S agc	 V E D W G G A S gtt gaa gat tgg ggc gga gcg agc	Jβ 2.1 S Y N E Q F F G P G T R L tcc tac aat gag cag ttc ttc ggg cca ggg aca cgg ctc	10/10
18030 B31	Vβ4 S agc	 V K V T S Y gtt aag gtc acc tcc tat	Jβ 1.2 G Y T F G S G T R L T V V ggc tac acc ttc ggt tcg ggg acc agg tta acc gtt gta	11/11
18030 D6	Vβ4 S agc	 V K D S S Y gtt aag gac agt tcc tat	Jβ 1.2 G Y T F G S G T R L T V V ggc tac acc ttc ggt tcg ggg acc agg tta acc gtt gta	11/11
18030 G14	Vβ4 S agc	 V K D S S Y gtt aag gac agt tcc tat	Jβ 1.2 G Y T F G S G T R L T V V ggc tac acc ttc ggt tcg ggg acc agg tta acc gtt gta	3/11
15160 DC4	Vβ1 A gcc	 S G P G Q G E D agc ggc ccc gga cag ggt gag gac	Jβ 1.2 Y G Y T F G S G T R L T V tat ggc tac acc ttc ggt tcg ggg acc agg tta acc gtt	*
LWF A20, C8	Vβ12.3 S agt	 G S R A S ggt agt agg gca agc	Jβ 2.7 E Q Y F G P G T R L T V gag cag tac ttc ggg ccg ggc acc agg ctc acg gtc	10/10

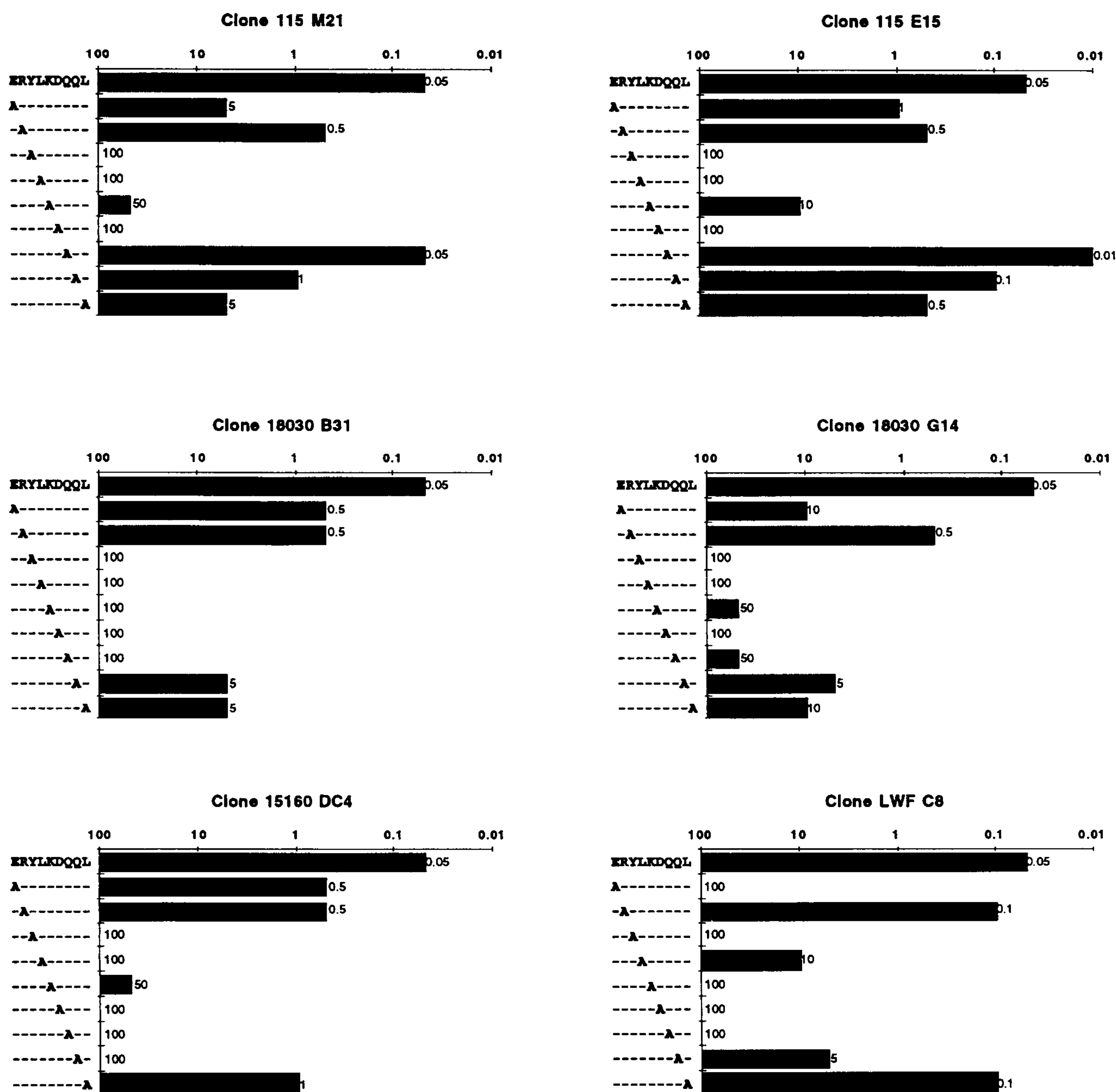

Figure 3. Titrations of gp41/584-592 alanine-substituted peptides. Autologous B-LCL were incubated with decreasing concentrations of each peptide. CTL clones were added at E/T ratios ranging from 3:1 to 10:1. The concentration of peptide sufficient for 50% maximal lysis is shown along the horizontal axis. Maximal lysis ranged from 50 to 100%. Spontaneous release values for all assays were <25%.

P3-P6 were poorly tolerated, shifting the amount of peptide required for half-maximal lysis by ≥2 log. The substitutions at P3 (Y586A) and P6 (D589A) were not appreciably recognized by any of the CTL clones. The lack of recognition of peptide Y586A is consistent with recent data showing P3 to be an important anchor residue for the HLA-B14 molecule (23) and is likely to reflect diminished HLA binding. For a few substituted peptides, recognition varied markedly among the clones. For example, a P1 substitution had effects ranging from complete abrogation of recognition (LWF C8) to a 1-log shift in observed lysis (15160 DC4).

Figure 2. TCR-α and -β sequences of HLA-B14–restricted, 588K-specific CTL clones. (*) Sequences were derived after amplification with a panel of TCR Vα and Vβ primers followed by direct sequencing of PCR products as described in (7). (*A*) TCR-α sequences of CTL clones. Nomenclature of TCR-α sequences is according to reference 34. (*B*) TCR-β sequences of CTL clones. At least eight and eight TCR-α and -β transcripts were sequenced for each CTL clone. These sequence data are available from EMBL/Genbank/DDBJ under accession numbers Z29579, Z29580, Z29581, Z29582, Z29614, U51442, U51443, U51444, U51445, U50555, U50556, U50404, U50405, and U50073.

The effect of an alanine substitution at a TCR contact site is well demonstrated in the case of an alanine substitution at P7 (Q590A). The concentration of this peptide that yields 50% maximal lysis is nearly the same as that of the index peptide for CTL clones 115 M21 and 115 E15. However, the other CTL clones show extremely poor recognition of this peptide, even at a concentration of 100 μg/ml. To ensure this differential degree of lysis was in fact due to a difference in TCR affinity, rather than due to a variant HLA allele present in this subject, titrations of this peptide were performed on the B-LCL from the other three subjects and yielded equivalent levels of lysis by CTL clone 115 M21 at each of the peptide concentrations (data not shown). This confirmed the ability of B-LCL from all these subjects to present this peptide for CTL-mediated lysis.

Recognition of HIV-1 Variants by gp41/584-592–specific CTL. To evaluate the role of the TCR in determining the ability of CTL to cope with HIV-1 sequence variation, the gp41-specific CTL clones were tested for their ability to recognize peptide sequences corresponding to known HIV-1 variants representing diverse HIV-1 clades. As can be seen in Fig. 4, substitutions at P5 were poorly tolerated. The nonconservative substitutions K588G and K588Q were poorly recognized by all CTL clones tested. Three CTL clones were able to recognize a peptide representing the SF2 isolate that contained a conservative K588R substitution. However, despite the conservative substitution, this peptide was poorly recognized by CTL clones from subjects 15160 and 18030. Not all conservative amino acid substitutions allowed for continued recognition. A conservative D→E

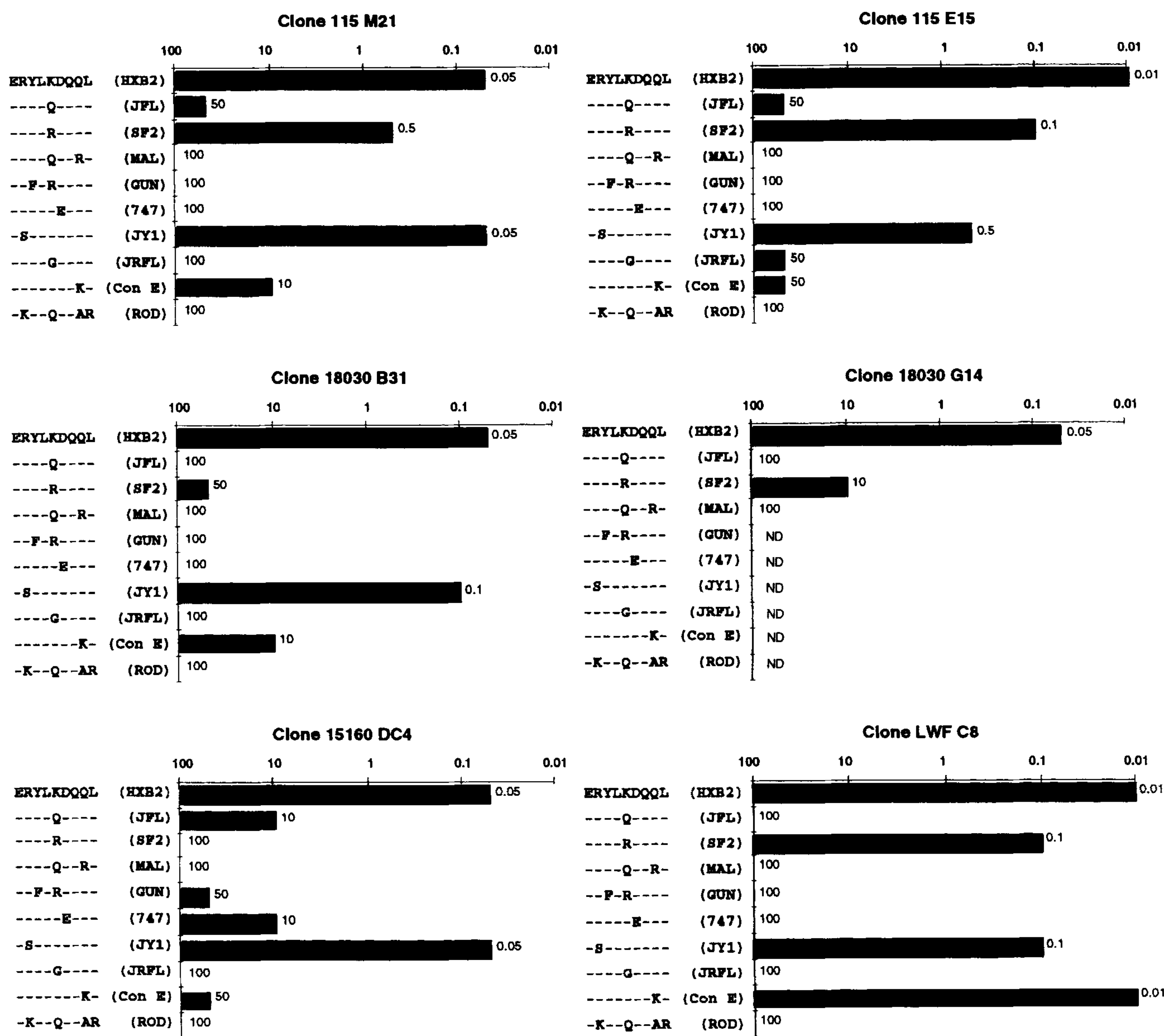

Figure 4. Peptide titrations of variant HIV-1 peptides. Autologous B-LCL target cells were incubated with peptides representative of HIV-1 clades A-E.

Table 1. *Analysis of HIV-1 Genotype within gp41/584-592 in Four HLA-B14 Subjects*

Subject	Date	Percent HIV-1 sequence prevalence*			
		ERYLKDQQL	(%)	----Q----	(%)
010-115i	3/91	0	(0)	11	(100)
	8/91	4	(33)	8	(67)
	4/92	1	(10)	9	(90)
	4/93	3	(25)	9	(75)
	11/93	4	(18)	18	(82)
15160	2/94	10	(100)	0	(0)
18030	3/94	0	(0)	9	(100)
	11/94	0	(0)	12	(100)
LWF	12/92	9	(100)	0	(0)

*3 × 10^6 PBMC at the indicated time points were lysed and subjected to nested PCR as described (3).

substitution at P6 (a presumed TCR contact site; 23) was poorly recognized by all six CTL clones. A clear case for diminished TCR binding can be made for the consensus E sequence (Con E) which has a Q→K substitution at P8 (Q591K). Five of the six CTL clones tested had limited ability to recognize this peptide. However, CTL clone LWF C8 recognized this peptide to the same level as peptide gp41/584-592. This clone was also able to recognize the consensus E peptide when titrated on the B-LCL of the other four subjects, again indicating that differences in the HLA-B14 alleles of these subjects did not account for the difference in CTL recognition (data not shown).

In Two Subjects the Predominant HIV-1 Genotype Is Not Recognized by the Majority of Their Isolated CTL Clones. To assess the relationship between the fine specificity of these CTL clones and in vivo sequence variation, the region of the HIV-1 genome containing this CTL epitope was sequenced in each of these subjects. A limiting dilution, nested PCR technique was used to amplify HIV-1 gp41 sequences from each of these subjects at the time points the CTL clones were isolated (for subject 18030, the first HIV-1 sequences were determined at a time point 4 mo after CTL clone 18030 B31 was isolated). In the four subjects, only two different sequences were identified. In two subjects, the dominant in vivo species was identical to the HXB2 sequence in this region, and in the other two subjects (010-115i and 18030) the dominant in vivo genotype was the K588Q variant (a glutamine substitution at P5, corresponding to the JFL variant; Table 1). The majority of CTL clones from these subjects did not recognize this variant at levels <10 μg/ml (Fig. 4). Only one CTL clone

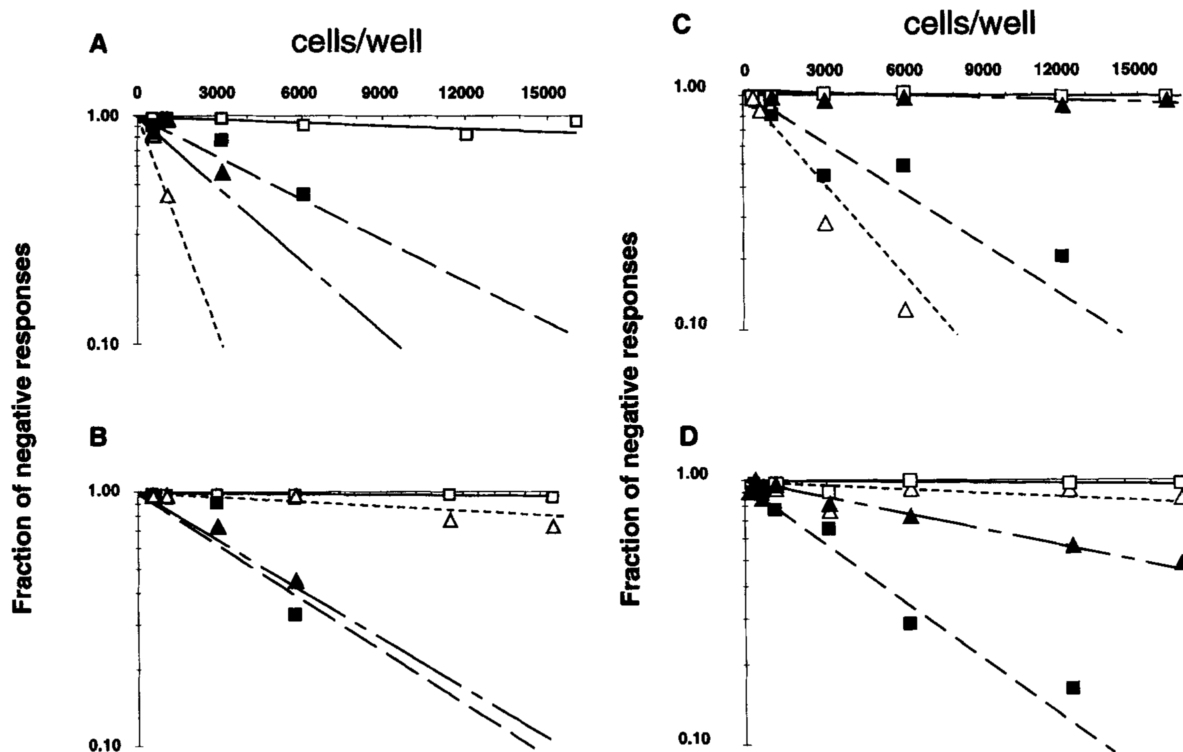

Figure 5. CTL precursor frequency analysis of PBMC from subjects 010-115i and 18030. Autologous PBMC from each subject were incubated with a combination of peptides 588K and p17/77-85 or K588Q and p17/77-85 (SLYNTVATL), irradiated, and used as stimulator cells in a CTL precursor assay as described in Materials and Methods. (□) B-LCL, (■) p17/77-85, (△) 588K, and (▲) K588Q. (*A*) PBMC from subject 010-115i stimulated with 588K and p17/77-85. (*B*) PBMC from subject 010-115i stimulated with K588Q and p17/77-85. (*C*) PBMC from subject 18030 stimulated with 588K and p17/77-85. (*D*) PBMC from subject 18030 stimulated with K588Q and p17/77-85.

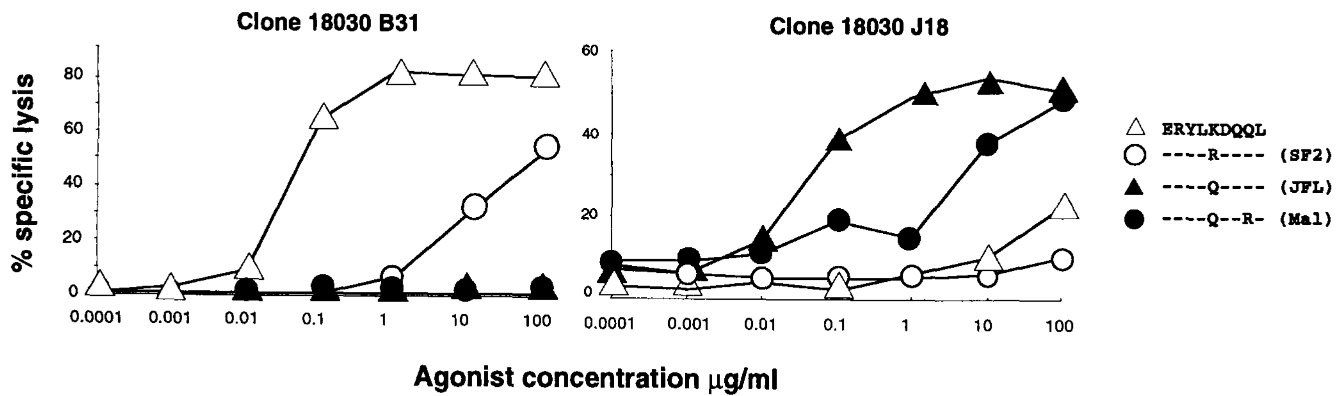

Figure 6. Peptide titrations of gp41/584-592 variants. CTL clones from subject 18030 are tested for their ability to recognize peptides representing HIV-1 variants. (△) 588K, (○) K588R, (▲) K588Q, and (●) K588Q, Q591R.

(15160 DC4) recognized this variant in addition to the 588K peptide, yet this recognition did not extend below a concentration of 1 μg/ml.

The Precursor Frequency of CTL Clones Can Be Dominated by CTL that Recognize a Minor In Vivo HIV-1 Genotype. In the two subjects whose CTL clones did not recognize the dominant autologous HIV-1 genotype (K588Q), CTL precursor analysis was performed to assess the degree of the CTL response against gp41/584-592 (588K) and variant (K588Q) peptide. For each subject, two parallel CTL precursor assays were performed. One assay was stimulated with fresh autologous PBMC incubated with the 588K and p17/77-85 peptides and the other was stimulated with fresh autologous PBMC incubated with K588Q and p17/77-85 peptides. p17/77–85 is an HLA-A2–restricted CTL epitope recognized by CTL from each subject, and served as a positive control in each assay. The parallel assays from each subject were tested on the same day against the same CTL targets, either autologous B-LCL alone or B-LCL incubated with p17/77-85, 588K, or K588Q at 1 μg/ml. Split-well analysis was used to determine whether there were two populations of CTL responsible for lysis of these targets. For subject 010-115i, the precursor frequency against p17/77-85 was similar in each assay (185 vs. 155/10^6 PBMC; Fig. 5). As expected, PBMC stimulated with 588K show a higher degree of recognition of the index 588K peptide than of K588Q (620 vs. 224/10^6 PBMC). Likewise, PBMC specifically stimulated with the variant peptide (K588Q) show a higher precursor frequency against the variant than against 588K (141 vs. 14), yet this is lower than the precursor frequency than can be generated against the index 588K peptide. A similar result was obtained for subject 18030. The precursor frequency against the control p17 peptide was similar in both cases (163 vs. 177/10^6 PBMC) yet the maximum frequency against the 588K peptide was 291/10^6 and against the K588Q peptide it was 48/10^6. In each case there is a four to six-fold higher precursor frequency against the subdominant in vivo HIV-1 genotype.

The existence of CTL able to preferentially recognize the variant peptide was confirmed by cloning. Split-well analysis made it possible to select wells from the precursor assay plates with especially high activity directed against the variant peptide. Cells from these wells were cloned at limiting dilution in the presence of PHA, irradiated allogeneic feeder cells, and rIL-2. Three CTL clones isolated from subject 18030 were preferentially able to recognize the K588Q epitope. A peptide titration from one of these CTL clones is shown in Fig. 6. This experiment demonstrates that the 50% maximal lysis of the K588Q variant by CTL clone 18030 J18 is the same as that of the 588K epitope by CTL clone 18030 B31. It also demonstrates that there is no cross-reactivity of the CTL clones for the two peptides. The TCR of this CTL clone is distinct from the previously isolated CTL that recognize the 588K peptide and utilizes the Vα7, Jα15.3, Vβ6, and Jβ1.5 gene segments, providing further evidence of the marked heterogeneity in TCR able to recognize the B14-restricted gp41 epitope (Fig. 7).

Discussion

In this study we demonstrate that CTL with heterogeneous TCR are able to recognize an epitope in gp41 that is restricted by the HLA-B14 molecule, and that this epitope

Vα7	N	Jα 15.3	#/total #
C A	V R A P T	S G G S Y I P T F G R G T S L	
TGT GCT	GTG AGG GCC CCA ACA	TCA GGA GGA AGC TAC ATA CCT ACA TTT GGA AGA GGA ACC AGC CTT	9/9
Vβ6	N-Dβ-N	Jβ 1.5	
S S	P R T G T G	N Q P Q H F G D G T R L S I L	
AGC AGC	CCT CGG ACA GGT ACG GGC	AAT CAG CCC CAG CAT TTT GGT GAT GGG ACT CGA CTC TCC ATC CTA	12/12

Figure 7. TCR-α and -β sequences of CTL clone 18030 J18 (EMBL/Genbank/DDBJ accession numbers U51446 and U51447).

is frequently recognized by HIV-1–infected subjects with this haplotype. We also show that the dominant in vivo quasispecies can vary markedly among persons of the same HLA haplotype. In two of the individuals studied in this report the dominant CTL response is directed against a HIV-1 variant that makes up the minority of the in vivo sequences. The ability of one of these subjects to recognize the major in vivo variant was confirmed by isolation of a CTL clone that preferentially recognized the dominant in vivo HIV-1 variant. However, despite the presence of CTL responses to the major in vivo variant, this response does not become dominant.

Few studies have evaluated the TCR usage of CTL clones directed against the same viral epitope in different individuals, and none has correlated this with the in vivo viral quasispecies. Most of the prior work on TCR usage of virus-specific, class I–restricted CTL has concentrated on the CTL response to influenza A, an acute viral infection. Two studies have focused on the CTL response to the HLA-A2–restricted matrix epitope (58-66) of influenza A, the only HLA-A2–restricted epitope described in this virus (4, 5). Lehner et. al. (5) determined that ~85% of CTL clones specific for this epitope utilize Vβ17. A study of CTL specific for a HLA B27–restricted influenza nucleoprotein epitope (383-391) found that five of eight of these CTL clones utilized the Vβ7 gene (6).

The only studies that have addressed CTL TCR usage in chronic viral infections have focused on HIV-1 and EBV infections. In a recent study (8) an extremely restricted pattern of TCR usage has been described against a HLA B8–restricted EBV epitope. Six CTL clones from five subjects were isolated and subjected to TCR analysis. Identical TCR protein sequences were used by CTL from each of four healthy unrelated subjects and a clone from a fifth subject differed by only two amino acids. It has been proposed that the extremely restricted TCR usage against the EBV epitope may be the consequence of its being a chronic viral infection that has evolved with its host over the history of human existence, and as a consequence, the narrowed TCR gene usage may be an adaptation of the host to this virus (8).

In this study, common TCR elements were found in five of nine of the CTL clones (lines) specific for the B14 peptide epitope in HIV-1 gp41. However, other heterogeneous TCR from two other individuals were still able to recognize this CTL epitope to the same degree. The isolation of a CTL clone (18030 D6) with TCR-α and -β sequences identical to those of a CTL clone isolated from the same subject 11 mo earlier provide another example of the persistence of these CTL in the circulation (7, 9). This finding also demonstrates that caution must be used before one assumes that a dominant TCR usage in a peptide-stimulated line allows one to attribute the TCR usage to the CTL clone responsible for the epitope specificity. The TCR sequences of CTL line 18030 G14 were derived after two rounds of in vitro peptide stimulation, and this line maintained excellent recognition of peptide-sensitized target cells at low E/T ratios, yet the TCR sequences of the epitope-specific CTL clone were in the minority (Fig. 2).

In two of the subjects described in this paper, the majority of in vivo HIV-1 sequences are not recognized by clones isolated from each of these patients. The sequences in this report were derived from proviral DNA. Since these sequences remained stable over 8 (subject 18030) and 32 mo (subject 010-115i), they are likely to also reflect plasma RNA sequences (24). A precursor frequency analysis in each case demonstrated a lower frequency of CTL specific for this dominant HIV-1 sequence and split-well analysis revealed the presence of a distinct CTL population in each case that was able to recognize the variant peptide. The isolation of a CTL clone from subject 18030 (J18) that recognizes the dominant in vivo sequence variant and that has a completely unique TCR implies that the relative lack of CTL precursors directed against this variant is not due to a "hole" in the TCR repertoire and also confirms the ability of this peptide to bind to the HLA-B14 molecule. This would speak against lack of HLA binding as a reason for the lower level of CTL precursors. This CTL clone can lyse target cells at the same peptide concentrations as CTL clones that recognize the gp41/584-592 peptide, thus the reasons for the diminished precursor frequency are unclear. We cannot rule out the possibility that the amino acid substitution within this epitope, or a substitution in one of the flanking regions of this HIV-1 variant, has an adverse effect on antigen presentation (25–27) resulting in the lower precursor frequency. Other investigators (28, 29) have shown that a dominant immunization with an alloantigen was able to inhibit CTL responses to a second alloantigen; a similar mechanism could potentially prevent effective CTL-mediated immunity to HIV-1 variants that arise over the course of infection. If a deficiency in antigen presentation is responsible for an ineffective CTL response against a dominant variant, efforts to augment existing CTL responses through a peptide-based immunotherapeutic agent designed to increase CTL activity against HIV-1 variants might bypass this mechanism of immune escape.

We also investigated the potential role of TCR antagonism in the two individuals with nonrecognized variants. Although there was evidence of antagonism by the K588Q peptide, this effect was only evident at relatively high peptide concentrations (>10 μg, data not shown) and is in contrast to recent reports (30, 31) showing potent antagonistic effects of peptides at extremely low concentrations. The significance of CTL antagonism in vivo remains unclear.

Because of the small number of subjects evaluated, we cannot correlate TCR usage or patterns of fine specificity with disease progression, but it is interesting that such a diverse repertoire of TCR is able to recognize this epitope. To our knowledge this type of study, assessing the fine specificity of CTL clones by titrating peptides representing both natural sequence variation and alanine substitutions, and attempting to correlate this fine specificity with the relevant TCR sequences has not been performed for a class I–restricted CTL epitope. A similar study (32) describing CD4-restricted myelin basic protein–specific CTL has been published, however no titrations were performed and rela-

tively high peptide concentrations were used. These types of studies may prove more useful for evaluating CTL restricted by HLA molecules for which the crystal structure is known, thereby helping to determine the ternary structure of the TCR–peptide–HLA complex. The significance of 1- or 2-log differences in the recognition of a particular HIV-1 variant by a particular clone is also uncertain. Although it is reasonable to assume that higher affinity TCR would be more effective at suppressing viral replication in vivo, this remains unproven and experiments on the recognition of acutely infected cells by HIV-1 variants containing point mutations representing natural sequence variation are needed.

The findings that subjects with a shared HLA allele can frequently recognize the same HIV-1 epitope and that CTL with differing fine specificities and heterogeneous TCR usage can result from natural infection have important implications for vaccine development. If subjects with more common HLA alleles target similar HIV-1 epitopes one could hypothesize that a peptide-based vaccine designed to elicit CTL responses against these HIV-1 epitopes and their common variants could benefit a large segment of the population. It is conjectural at this time whether HIV-1–antigenic variation within CTL epitopes plays a role in HIV-1 disease progression. A model of HIV-1–antigenic variation suggests that a narrowly directed CTL response directed against a conserved epitope may be more effective at containing HIV-1 replication than a diverse response against multiple epitopes (33). A central postulate of this model is that CTL-mediated suppression of a HIV-1 variant can lead to outgrowth of a nonrecognized HIV-1 mutant. The mutant then predominates until a new CTL response is generated; meanwhile the original CTL response wanes. Presumably, these shifting CTL responses are the cause of the HIV-1–antigenic oscillations observed in one of the two subjects in that report. We have shown in two of our subjects that a dominant CTL response can persist against a variant that makes up a minority of in vivo sequences, and in these cases no antigenic oscillation is seen over the time period studied. If this sustained CTL response is responsible for the continued suppression of the minority HIV-1 variant, one could hypothesize that enhancing the CTL response against the dominant variant would further suppress HIV-1 replication. Furthermore, having a diverse repertoire of CTL able to recognize naturally occurring variants in place before infection, or very soon after infection is established, would possibly be more effective than the situation in natural infection where mutations may occur within CTL epitopes after substantial damage to the immune system has taken place. In cases where virus variants generate less vigorous CTL responses, immunotherapeutic regimens designed to augment these responses may prove beneficial.

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