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The HIV-1 HLA-A2-SLYNTVATL Is a Help-Independent CTL Epitope[1]

June Kan-Mitchell,[2]* Brygida Bisikirska,* Flossie Wong-Staal,[†] Keri L. Schaubert,* Melissa Bajcz,* and Michal Bereta*

The CTL response to the HLA-A*0201-restricted, HIV-1 p17 Gag_{77-85} epitope (SLYNTVATL; SL9) has been extensively studied in patients. Although this reactivity is exceptionally prominent in chronically infected patients and inversely correlated to viral load, SL9-specific CTLs (SL9-CTLs) are rarely detected in acute infection. To explore the cellular basis for this unusual manifestation, SL9-CTLs primed ex vivo from naive circulating $CD8^+$ T cells of healthy, seronegative donors were generated and characterized. SL9 appeared to differ from other well-studied A*0201-restricted epitopes in several significant respects. In contrast to published reports for influenza and melanoma peptides and the HIV gag IV9 epitope studied here in parallel, SL9-CTLs were primed by immature but not mature autologous dendritic cells. Highly activated SL9-CTLs produce sufficient autocrine mediators to sustain clonal expansion and CTL differentiation for months without $CD4^+$ T cells or exogenous IL-2. Moreover, SL9-CTLs were sensitive to paracrine IL-2-induced apoptosis. IL-2 independence and sensitivity to paracrine IL-2 were also characteristic of SL9-CTLs immunized by dendritic cells transduced by a nonreplicating lentiviral vector encoding full-length Gag. In vitro-primed SL9-CTLs resembled those derived from patients in degeneracy of recognition and functional avidities for both SL9 and its natural mutations. Together, these data show that SL9 is a highly immunogenic, help-independent HIV epitope. The scarcity of SL9-CTLs in acute infection may result from cytokine-induced apoptosis with the intense activation of the innate immunity. In contrast, SL9-CTLs that constitutively produce autocrine help would predominate during CD4-diminished chronic infection. *The Journal of Immunology,* 2004, 172: 5249–5261.

It is clear that the HIV-1-specific CTL response plays a central role in controlling HIV-1 replication (1). A temporal association was shown for the initial control of viremia in acute AIDS virus infection with the emergence of virus-specific CTLs (2, 3). A strong negative association between CTL frequency and viral load was also demonstrated in chronic infection (4). Strong virus-specific CTL activity was detected in long-term nonprogressing infections (5, 6) and associated with highly exposed but uninfected individuals (7). In vitro studies showed virus-specific CTL to lyse virus-infected cells as well as to suppress viral replication (8, 9). In vivo containment of viral replication by $CD8^+$ CTLs was directly demonstrated in the SIV-infected Rhesus macaque model of AIDS (10). Despite clear evidence that CTL are vital in achieving control of HIV, they are inadequate in the majority of patients who inexorably decline to AIDS. Recent studies suggest qualitative differences among CTLs with different specificities in patients (11–14).

The HIV-1 Gag protein is preferentially targeted by HIV-1-specific CTLs, followed by Env, Nef, and RT during acute and chronic infection (14, 15). A total of five HLA-A2-restricted Gag epitopes have been defined to date, although patients do not appear to target all of them simultaneously (16). The human immune response to the HLA-A*0201-restricted Gag_{77-85} SLYNTVATL (SL9)[3] epitope is by far the most studied. Using SL9-tetramers, a strong negative association was shown between levels of SL9-CTLs and viral load in A*0201-positive adults with chronic infection (4), which suggests a role in restricting viral replication. This epitope is also targeted by 75% of A*0201-positive adults with chronic infection (4, 17–19), although SL9 reactivity was not found in patients with acute infections (20).

The prominence of the SL9-CTL response during the asymptomatic phase of chronic infection may be related to the abundance of Gag gene products relative to other viral proteins (15). The SL9 peptide also appears to undergo processing distinct from other A*0201-restricted subdominant RT epitopes (21) or naturally processed Nef peptides presented by A2 and B7 Ags (22) in that its presentation is not dependent on proteosomes sensitive to inhibition by lactacystin (21). Although differences in Ag processing and generation may contribute to patterns of CTL recognition in vivo (23–25), it is not immediately obvious how processing requirements might contribute to the unusual pattern of SL9-specific CTL responses.

The failure of SL9-CTLs to control initial viremia has led to the suggestion that this epitope, at least the native consensus sequence, is a poor choice for vaccine design (20). On the other hand, SL9 is recognized by the most prevalent HLA class I allele worldwide (26), an important attribute in terms of the large potential for patient coverage. Moreover, the majority of $A2^+$ individuals appear

[3] Abbreviations used in this paper: SL9, HIV-1 p17 Gag_{77-85} epitope (SLYNTVATL); DC, dendritic cell; tetramer, tetrameric HLA-A*0201-peptide complex; MFI, mean fluorescence intensity; pMHCI, peptide-MHC class I complex; AICD, activation-induced cell death.

to have a high-affinity T cell repertoire for this epitope (20). SL9 is also efficiently processed and presented by infected cells (27) and is located in a region of the viral genome that must be conserved to maintain viral fitness (18, 28). Recently, greater numbers of SL9-CTLs were found in gut-associated lymphoid tissue relative to peripheral blood, suggesting that these cells are also a major component of an active mucosal HIV-specific CD8$^+$ response (29), another important consideration in vaccine design as most HIV transmission occurs via mucosal surfaces.

To better understand why SL9 may be differentially targeted over the course of infection, we have undertaken considerable effort to generate and characterize SL9-specific CTLs primed ex vivo from naive CD8$^+$ T cells of seronegative healthy donors, to avoid the extraordinary complexities introduced by host-virus dynamics. We hypothesized that these Ag-driven, short-term SL9-CTL cultures might simulate the early CTL response in patients. For the most part, other studies of SL9 reactivities have relied on CTL cultures or clones expanded from chronically infected patients, a time when virus-specific T cell immunity is thought to be undergoing clonal exhaustion or replicative senescence (30–32). Most SL9-CTLs from patients were unstable cultures rescued by nonspecific stimulation with an anti-CD3 mAb. This Ab provides a powerful proliferative signal by cross-linkage of TCR that is qualitatively and quantitatively distinct from TCR triggering by peptide ligands (33). In this study, we describe novel conditions required for priming and Ag-specific expansion of SL9-CTLs from circulating naive CD8$^+$ precursors derived from A*0201 seronegative healthy donors. Although these are finite cultures, large numbers (10^9) of tetramer-binding cells can be generated from 50-ml heparinized blood samples. In vitro immunized SL9-CTLs from several donors were defined with respect to 1) the oligoclonality of the response, 2) degeneracy of recognition for the native epitope and its mutations, and 3) CD8 dependence for tetramer binding and cytotoxicity. The data indicate that SL9 is a CD4 help-independent epitope and provide new insight into the paradoxical clinical manifestation of the SL9 CTL immunity in HIV infection.

Materials and Methods

HLA typing

One hundred milliliters of heparinized blood were obtained from healthy HLA-A*0201 volunteers at weekly intervals over a period of 5 wk. HLA class I typing and A2 subtyping were performed by sequence-specific primer PCR by the Immunogenetics (HLA) Laboratory at the Detroit Medical Center (Detroit, MI). The study was approved by the institutional review board, and all subjects gave written informed consent.

Generation of dendritic cells (DCs) from adherence-purified monocytes

PBMC from fresh heparinized blood were isolated by Ficoll-Hypaque density gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden). Monocytes were isolated by adherence to plastic for 2 h in serum-free HEPES-buffered RPMI 1640 (Mediatech, Herndon, VA). Adherent cells were cultured in RPMI 1640 medium containing 2 mM L-glutamine, 100 U/ml each of penicillin and streptomycin, 1 mM sodium pyruvate, 1× MEM nonessential amino acids (Life Technologies, Rockville, MD), and 10% autologous serum (complete medium) supplemented with 1000 U/ml GM-CSF (Immunex, Seattle, WA) and 1000 U/ml IL-4 (R&D Systems, Minneapolis, MN). After 3 days, half of the medium was replaced with an equal volume of fresh medium containing cytokines. DCs used after 7 days in culture were considered as immature DCs (34). Maturation of DCs was achieved according to previously described methods (35, 36). In brief, immature DCs were incubated for overnight with 1 μg/ml LPS (E. coli serotype 026:B6; Sigma-Aldrich, St. Louis, MO).

The process of incubating immature DCs with 10 μg/ml SL9 or other peptides for 18 h did not induce maturation, as determined by lack of morphological changes or increase expression of CD86 and CD54. Furthermore, peptides were not toxic to cultures of immature and mature DCs as determined by staining with propidium iodide or trypan blue.

Primary in vitro immunization and expansion of CD8$^+$ T lymphocytes

DCs were pulsed in suspension with a peptide twice, first for 2 h at 37°C at 1–20 μM in serum-free RPMI 1640, and then for 1.5 h at room temperature with the same peptide in the presence of 3 μg/ml β$_2$-microglobulin and 1% human albumin. DCs were irradiated at 4000 cGy before admixing with immunobead-purified CD8$^+$ T cells (Dynal, Oslo, Norway) at the T cell:DC ratio of 5:1 in 48-well cluster plates. Cells were cultured in complete medium with 10 ng/ml IL-7 (Genzyme, Cambridge, MA) and restimulated every 7–10 days with autologous monocytes pulsed with peptides as described above. CTLs were cloned by limiting dilutions in U-bottom 96-well cluster plates in the presence of 30 ng/ml anti-CD3 mAb (Orthoclone OKT3; Ortho Biotech, Raritan, NJ) and 10^4/well irradiated PBMC used as feeder cells. IL-2 (20 U/ml; Chiron, Emeryville, CA) and IL-4 (2.5 ng/ml) were added the next day and every 3 days thereafter. T cells were restimulated every week with the anti-CD3 mAb in the presence of feeder cells.

In some experiments, monocyte-derived DCs were transduced by spinduction with the HIV-1 ΔEN vector (37). CD8$^+$ T lymphocytes were primed by HIV-1 ΔEN-transduced DCs at the DC:T cell ratio of 1:50 and cultured as above. Cells were restimulated weekly with irradiated HIV-1 ΔEN-transduced DCs.

Flow cytometry

Flow cytometry was performed on a FACSCalibur, and data were analyzed with CellQuest software (BD Biosciences, San Jose, CA). Unless indicated otherwise, staining was performed according to the manufacturer's recommendation. Lymphocytes and DCs were assessed for expression of phenotyping markers by using fluorescent Abs specific for CD3, CD4, CD8 (B9.11; Immunotech-Beckman Coulter, Marseille, France), CD14, CD28, CD40, CD54, CD80, CD83, and HLA-DR (BD Biosciences). The anti-HLA-A, B, C, and anti-HLA-A2.1 mAbs were generated from the hybridoma cell lines W6/32 and BB7.2 (American Type Culture Collection, Manassas, VA), respectively.

Staining and sorting of peptide-specific CTL with tetrameric HLA-A*0201-peptide complexes

Tetrameric HLA-A*0201-SL9 and -IV9 complexes (tetramers) were prepared by the National Institutes of Health/National Institute of Allergy and Infectious Diseases Tetramer Facility (Atlanta, GA). Cultured T cells were washed and resuspended in cold staining buffer containing PBS, 0.2% BSA, and 0.02% sodium azide. They were incubated with allophycocyanin-conjugated MHC peptide-tetramers at the final concentration of 1 μg/ml for 25 min on ice, washed twice with staining buffer, fixed with PBS containing 1% paraformaldehyde, and analyzed by flow cytometry. In some experiments, cells were preincubated with 100 ng/ml PE-anti-CD8 mAb for 30 min at 4°C and washed before staining with the tetramers.

To sort for tetramer-binding cells, T cells were stained with tetramers in sterile buffer without sodium azide using the FACSVantage (BD Biosciences). Cells were resuspended in RPMI 1640 with 10% FBS in the presence of 30 ng/ml anti-CD3 mAb and irradiated PBMC as feeder cells. IL-2 (20 U/ml; Chiron) were added the next day and every 3 days thereafter.

Staining for perforin

Intracellular staining for perforin was performed at room temperature on cells fixed for 10 min with 4% paraformaldehyde in PBS. After washing twice with staining buffer, the cells were permeabilized with 0.1% saponin (Sigma-Aldrich), 0.2% BSA, and 0.02% sodium azide in PBS for 15 min. The cells were incubated with FITC-conjugated mouse anti-human perforin mAb (BD PharMingen, San Diego, CA) for 30 min at 4°C in the dark. Cells were washed twice with permeabilization buffer and resuspended in staining buffer before flow analysis. Isotype-matched irrelevant FITC-conjugated mouse mAbs were used as controls.

Cytokine detection by ELISA

OptEIA Sets (BD PharMingen) were used to measure the concentrations of IL-2, IL-4, and IFN-γ in supernatants of CTLs restimulated for 24 h with peptide-pulsed T2 cells (the human TAP-deficient lymphoblast T-B hybridoma) (American Type Culture Collection). T2 were pulsed with peptides at concentrations ranging from 10^{-15} to 10^{-5} M and used at the T cell:T2 cell ratio of 1:10. The ranges of sensitivity for IL-2, IL-4, and IFN-γ are 7.5–400, 10–1000, and 5–300 pg/ml, respectively.

Cytotoxicity assay

T2 cells were labeled with sodium ^{51}Cr-labeled chromate (NEN, Boston, MA) and pulsed with peptides for 1 h at 37°C. After washing, the T2 cells were admixed with T cells at different E:T ratios in 96-well round-bottom plates. After an incubation period of 4 h, supernatants were harvested and mixed with scintillation fluid (Optiphase SuperMix; PerkinElmer-Wallac, Gaithersburg, MD) for counting in a MicroBeta counter (PerkinElmer-Wallac). T2 cells not pulsed with peptides were used as negative controls. Virus-specific cytotoxicity was assessed with JA2/R7Hyg cells as targets, an HIV-1-infected HLA-A*0201-positive Jurkat cell line (National Institutes of Health AIDS Research and Reference Reagent Program, Rockville, MD). Specific percent lysis was calculated using the following formula: $((cpm_{experimental} - cpm_{spontaneous})/(cpm_{total} - cpm_{spontaneous})) \times 100$. EC_{50} values, defined as the peptide concentration causing half-maximal cytotoxicity, were determined by curve fit with GraphPad Prism (GraphPad Software, San Diego, CA).

SLYNTVATL (SL9), ILKEPVHGV (IV9), and peptides selected based on previously published sequences of naturally occurring mutants of SL9 (SLYNTIAVL (6I8V), SLYNTIATL (6I), SLFNTVATL (3F), SLFNTVAVL (3F8V), SLSNTVATL (3S), SLFNTIAVL (3F6I8V), SLLNTVATL (3L), SLFNAVATL (3F5A), SLYNAIAVL (5A6I8V), SVYNTVATL (2V), and SLYNTVAVL (8V) (38)) were synthesized by Multiple Peptide Systems (San Diego, CA). The purity and identity of each peptide was verified by an electrospray mass spectrometer interfaced with a liquid chromatography system. Lyophilized peptides were dissolved in DMSO (50 mg/ml), aliquoted, and stored at −80°C.

Virus-specific cytotoxicity was also assessed with the JA2/R7Hyg cells as targets (National Institutes of Health AIDS Research and Reference Reagent Program). When cultured in hygromycin (250 µg/ml), these cells naturally process and present the SL9 epitope and are recognized and lysed by SL9-specific CTLs derived from patients (39).

Statistical analysis

Differences in the expression of surface markers on immature DC between productive and nonproductive donors were compared using Student's *t* test. A value of $p < 0.05$ was considered statistically significant.

Results

Cytokine requirements for in vitro priming and expansion of SL9-specific CTLs

Naive CD8$^+$ T cells from healthy seronegative donors were purified to >98% homogeneity by immunobead selection. Parallel cultures of CD8$^+$ T cells were primed by autologous immature DCs pulsed with SL9 or IV9 peptides and restimulated weekly thereafter with autologous monocytes presenting the same peptides. Cultures were supplemented weekly with different cytokines. The combination of IL-7, IL-2, and IL-10, previously described for a variety of tumor peptide-specific CTLs (40), was optimal for the generation of IV9-specific CTLs, as shown in Fig. 1*B*. In contrast, SL9-specific cytotoxicity was detected only in the culture supplemented with IL-7 alone (Fig. 1*A*). Addition of IL-2 (5–50 U/ml) and/or IL-10 (10 ng/ml) during first two restimulations resulted in consistent and complete loss of SL9-specific cytotoxicity. Fig. 1*C* showed the progressive increase of SL9-specific cytotoxicity with culture at the E:T ratio of 5:1, which correlated with increasing percentages of SL9-tetramer binding cells (bracketed numbers). Between days 20 and 42, the percentage of tetramer-stained T cells rose from 0.8 to 40%, with a concomitant increase of SL9-specific cytotoxicity (Fig. 1*D*). In contrast, only 1% of the T cells in a parallel culture primed to the IV9 peptide were stained by the IV9-tetramer (Table I). At the end of the seventh restimulation of 10^6 CD8$^+$ naive T cells, the number of SL9-CTLs was ~10-fold more than that of IV9-CTLs (3.5×10^7 as compared with 3.6×10^6). From ~5×10^6 circulating CD8$^+$ T cells, it is possible to expand 10^9 tetramer-binding SL9-CTLs before the culture became nonviable.

Most SL9-CTL cultures contained between 5 and 50% SL9 tetramer-binding T cells by the end of 8 wk. It was also possible to sort for tetramer-binding cells and isolate essentially homogeneous

tetramer-binding T cell cultures. Fig. 2*A* shows an unsorted culture containing 57% SL9-tetramer binding cells. Fig. 2, *B* and *C*, shows cultures expanded from sorted tetramer-binding cells from two donors (Wsu02 and Wsu03). These contained 89 and 91% tetramer-positive CD8$^+$ T cells, respectively. The CTL cultures were specific for SL9 (Fig. 2, *D–F*), preferentially lysing T2 cells pulsed with SL9 but not an irrelevant peptide at four different concentrations (1–1000 ng/ml) at the E:T ratio of 5:1. Nonspecific lysis associated with unsorted cells (Fig. 2*D*) was not evident in sorted cultures (*E* and *F*), showing that tetramer-binding cells were highly specific for the immunizing peptide.

Secretion of cytokines by the SL9- and the IV9-specific T cell cultures (day 57 in culture) was determined by enzyme immunoassay after restimulating 10^4 T cells with 10^5 T2 cells pulsed with the appropriate peptide in a total volume of 0.2 ml for 24 h. SL9-CTLs secreted copious amounts of IFN-γ ($\gg$500 pg/ml) and IL-2 (>500 pg/ml). Constitutive production of large quantities of IL-2 (~900 IU/10^6 T cells) explains why supplementation of exogenous IL-2 was not necessary. In contrast, the IV9-specific culture produced less IFN-γ (31 pg/ml) and minimal IL-2 (<7 pg/ml). This quantity of autocrine IL-2 was apparently insufficient to sustain proliferation. Both CTL cultures secreted background levels of IL-4 (10 pg/ml). Thus, CTLs specific for these two HIV peptides exhibited the type 1 lymphokine profile.
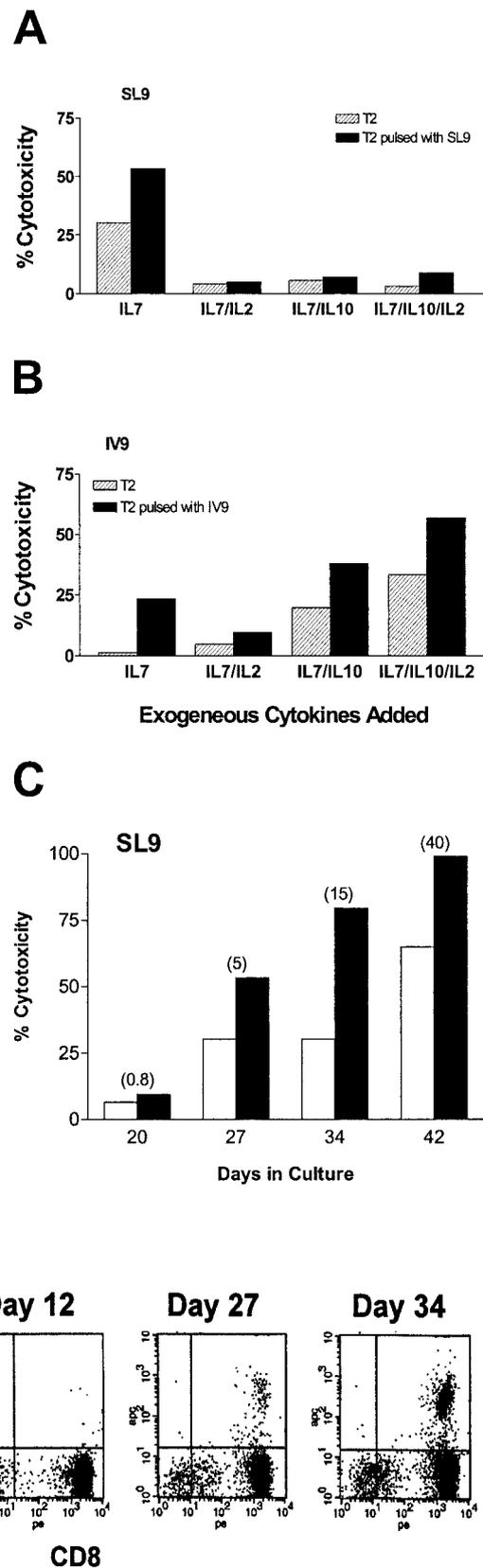
To show that the SL9-CTLs were capable of recognizing naturally processed viral peptides, they were tested against JA2/R7Hyg, a cell line considered to be chronically infected by HIV-1 (39). SL9-CTLs lysed between 20 and 53% of JA2/R7Hyg cells at E:T ratios of between 5:1 and 40:1. Addition of the anti-HLA class I mAb W6/32 (100 µg/ml) blocked lysis by half at all E:T ratios, showing that recognition was HLA class I restricted. Lysis of T2 cells pulsed with SL9 and T2 cells alone were also assessed simultaneously. Specific lysis of SL9-pulsed T2 cells was 30–50%, whereas nonspecific lysis of T2 cells was <10%. These results show that our in vitro-primed SL9-CTLs, characterized by their strong reactivity against synthetic peptide-treated T2 cells, also recognized natural SL9 peptides presented at low cell surface density.

The deleterious effect of exogenous IL-2 was confirmed with 10 sets of parallel cultures from five separate donors. Furthermore, none of the 20 SL9-CTL cultures established required supplementation with exogenous IL-2 for proliferation.

Vβ gene usage by SL9-specific CTLs

The virus-specific CD8$^+$ T cell response in acute primary HIV infection is frequently oligoclonal, as determined by the preferential usage of TCR Vβ genes (41, 42). In this study, we measured the usage of Vβ families by CTL cultures to a single HIV epitope, to study the breadth of the CD8$^+$ T cell repertoire at the earliest stages of priming and expansion (day 35) that was possible to monitor.

Using mAbs to 21 TCR Vβ chains representing ~70% of all Vβ genes, the relative usage of Vβ families in short-term SL9-specific CTL cultures was compared with fresh circulating CD8$^+$ T cells or to a polyclonal CD8$^+$ T cell culture from the same individual expanded in parallel with an anti-CD3 mAb and IL-2. A 2-fold augmentation of a Vβ family in question is the minimal to be considered significant (41). Table II shows a restricted Vβ repertoire in the Wsu06 SL9-CTL culture, with an increase for only one Vβ family (Vβ7; 4.2-fold) when compared with fresh circulating CD8$^+$ T cells purified from the same individual. Three independent SL9-CTL cultures (SL9-CTL1, -2, and -3) were generated from another donor over a course of a year. All of these contained

A

SL9

B

IV9

Exogeneous Cytokines Added

C

Days in Culture

D

Day 12   Day 27   Day 34   Day 42

Tetramer

CD8

FIGURE 1. Different requirements for exogenous cytokines for the generation of SL9- and IV9-CTLs. *A* and *B*, Cytotoxicity was determined on day 30 at an E:T ratio of 5:1 by the chromium release assay. All cultures were generated in the presence of IL-7 (10 ng/ml). IL-2 (20 U/ml) and/or IL-10 (10 ng/ml) were added to the cultures indicated after first restimulation. *C*, Increasing specific cytotoxicity of the SL9-CTLs expanded with only IL-7 and in the absence of the other cytokines over a 6-wk period is shown (■). Nonspecific lysis of T2 cells is indicated by open bars (□). The percentage of tetramer-positive cells is indicated by the number in parentheses for the various days. *D*, Progressive increase in SL9-tetramer$^+$CD8$^+$ T cells in culture is shown.

high percentages of Vβ5.1 and -8.1, indicating a consistent preferential usage of these Vβ genes by this individual. To determine whether other Vβ clonotypes were also represented among the SL9-tetramer-binding cells, SL9-tetramer-binding T cells were sorted from SL9-CTL2 and expanded with an anti-CD3 mAb.

Vβ8.1$^+$ T cells were drastically reduced by this process. The sorted culture, which was >80% tetramer$^+$, contained four other clonotypes in addition to the dominant parental Vβ5.1$^+$ and Vβ17$^+$ T cells. These were Vβ5.2$^+$, Vβ11$^+$, Vβ12$^+$, and Vβ13.1$^+$ T cells present at 5.5, 4.7, 7.1, and 10.5%, respectively,

TABLE I. *Number of CD8⁺ T cells and percentage of tetramer-binding cells in SL9- and IV9-specific CTL cultures after 42 days[a]*

Peptide Specificity	Cytokine(s) Added	
	IL-7/IL-2/IL-10	IL-7
SL9	0.8[b] (ND)	88 (40%[c])
IV9	36 (1)	1.8 (ND)

[a] Four parallel cultures, stimulated with SL9 or IV9 in the presence of two cytokine conditions (IL-7, IL-2, and IL-10, or IL-7 alone) were established from 1.5×10^6 purified CD8⁺ T cells from the same individual.
[b] T cell number $\times 10^6$
[c] Percentage of tetramer/peptide-binding cells.

of the total cells in culture. Together, they comprised almost 30% of SL9-specific T cells. This finding illustrates that the SL9 TCR repertoire was quite broad, involving 8 of the 21 Vβ chains tested, broader than predicted from the unsorted parental culture.

Rapid loss of SL9-specific T cells with addition of paracrine IL-2

To study the effect of paracrine IL-2 on SL9-CTLs, the cells were washed and then seeded into replicate wells containing fresh medium in the absence or presence of 100 U/ml rIL-2. After 24 h, both cultures were stained with SL9-tetramer, anti-CD8 mAb and annexin V. The percentage of SL9-tetramer⁺CD8⁺annexin V⁺ T cells was 12.2% in the well containing IL-2, more than twice (4.9%) that in the control culture. These cells constitutively expressed a low level of Fas, but little or no Fas ligand, which was not affected after exposure to exogenous IL-2. Thus, IL-2 negatively regulated SL9-specific T cells by inducing apoptosis, possibly via a Fas-independent apoptotic program. Other investigators have also reported that IL-2 can trigger the death of mature human T cells through several apoptotic programs (43).

To determine whether some clonotypes may be more sensitive to paracrine IL-2, the effect of a 24-h incubation with 100 U/ml IL-2 on Vβ gene usage by Wsu03 SL9-CTL3 cells was analyzed (Table II). Four Vβ genes accounted for ~50% of the cells in culture: Vβ5.1⁺ (17.6%), Vβ5.2⁺ (7.7%), Vβ12⁺ (5.5%), and Vβ13.1⁺ (17.5%). Vβ5.1⁺ and Vβ13.1⁺ T cells were the most sensitive to IL-2: >50% disappeared after exposure to paracrine IL-2. A 20% reduction was noted for Vβ5.2⁺ and Vβ12⁺ T cells. Two minor populations (Vβ8.1⁺ at 2.6% and Vβ11⁺ at 4.2%) were minimally diminished. Although all clonotypes were downregulated, there appeared to be a correlation between activation or proliferative ability and sensitivity to IL-2-induced cell death.

Immature but not mature DCs prime CD8⁺ T cells with SL9

Several recent studies have compared the ability of different populations of APCs to prime epitope-specific CD8⁺ T cell responses in vitro (44, 45). In particular, maturation of DCs was considered critical for priming CTLs in vitro (46). One issue not addressed is whether this requirement is universal for all peptides, which may have very different intrinsic immunogenicity. In this study, the ability of naive CD8⁺ T cells to be immunized by immature or mature DCs pulsed with the SL9 or IV9 peptide was compared. Table III demonstrates that the mature DCs, generated by exposing immature DCs to LPS, expressed higher levels of CD40, CD54, CD80, CD83, CD86, and HLA-DR. Fig. 3 shows parallel CD8⁺ T cell cultures primed by immature and mature DCs with SL9 (*A* and *B*) and parallel cultures from a different donor primed with IV9 (*C* and *D*). SL9-tetramer⁺ T cells were detected only when primed with immature DCs (6% after 26 days in culture). In contrast, IV9-tetramer-binding cells were detected in the culture primed
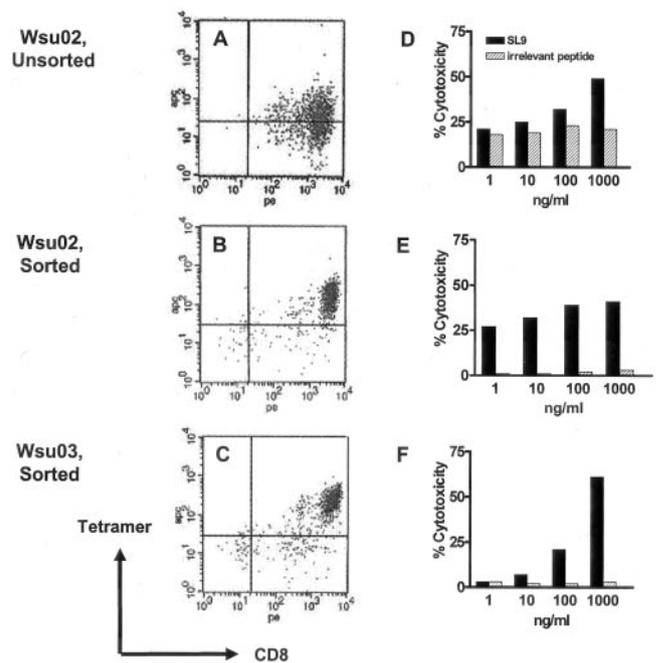

FIGURE 2. Isolation of essentially homogeneous SL9 tetramer-binding T cell lines. *A*, A SL9-CTL culture containing 57% tetramer⁺CD8⁺ T cells from donor Wsu02 is shown. *B* and *C*, Cultures expanded by anti-CD3 mAb and IL-2 from sorted tetramer-binding cells from donors Wsu02 and Wsu03 are shown. *D–F*, SL9-specific lysis of T2 cells at E:T ratio of 5:1 at four different concentrations of the peptide is shown.

with mature DCs (27% by day 19). The inability of mature DCs to prime SL9-CTLs has been confirmed using parallel cultures on four separate occasions using naive T cells from three donors. In contrast, we have successfully generated five CTL lines to IV9 with mature DCs.

To date, SL9-specific T cells have been consistently isolated from five of eight A*0201⁺ donors. In contrast, they could not be generated from the other three individuals, despite repeated attempts. Because there were no detectable differences in the expression of CD40, CD54, CD80, CD83, CD86, and HLA-DR between immature DC cultures generated from productive or nonproductive donors (data not shown), the inability to prime SL9 from some individuals suggests a defective T cell repertoire for this peptide.

SL9-specific T cells primed by DCs transduced by a multiply deleted pseudotyped HIV-1 vector (HIV-1ΔEn) are also sensitive to paracrine IL-2

To determine whether SL9-specific T cells could be generated in vitro by naturally processed and presented peptides, naive CD8⁺ T cells from one donor (Wsu05) were primed and restimulated three times with autologous DCs transduced with the multiply deleted HIV-1ΔEn vector. IL-7 (10 ng/ml) was added on the day of priming and at each restimulation. The day 28 culture consisted of 95% CD8⁺ T cells (Fig. 4*A*, *left panel*), of which 23% were Vβ3⁺ (*right panel*) and the remainder expressed TCR with Vβ chains not recognized by the other 20 mAbs in our panel. Forty-six percent of the CD8 T cells were stained by the SL9 tetramer, a third of which (16%) were Vβ3 expressing. To determine whether these SL9-specific T cells were also negatively affected by paracrine IL-2, this cytokine (20 IU/ml) was added to the culture for two consecutive restimulations. Fig. 4*B* compares IL-2-treated T cells with a parallel culture after staining with tetramer and mAbs to CD8 and

Table II. *Preferential Vβ gene usage by SL9-specific CD8+T cell cultures derived from donors Wsu06 and Wsu03[a]*

Donor/CD8+T Cells	Percentage of T Cells Stained by the mAb to Vβ Chain (Fold over Control T Cells)																				
	1	2	3	5.1	5.2	5.3	7	8.1	9	11	12	13.1	13.6	14	16	17	18	20	21.3	22	23
Wsu06																					
Fresh, purified	2.3	4.5	0.6	2.3	0.3	0.7	2.3	1.8	0.5	0.3	1.0	0.6	1.9	2.5	0.5	3.1	0.1	0.7	2.1	1.5	1.6
SL9-CTL (day 35)	2.9	3.8	0.7	2.8	0.2	0.9	**9.7** (4.2)[b]	1.8	1.2	0.4	2.0	1.4	1.7	4.6	1.4	2.8	0.2	1.2	2.4	2.3	1.3
Wsu03																					
Fresh, purified	2.7	2.2	0.9	1	0.5	0.3	3.4	1.2	0.5	0.2	0.5	0.6	7.1	0.2	0.4	1.1	0.2	1.1	8.5	0.8	1.2
SL9-CTL1 (day 35)	4.3	3.2	1.9	**12.7** (12.7)[b]	0.2	0.3	2.2	**6.8** (6.7)	0	0.2	1.1	1.2	1.5	**1.9** (8)	0.6	**2.4** (2.2)	0	1.2	3.9	1.9	0.6
Anti-CD3 expanded	1.2	0.9	1.2	1.2	0.6	1.3	4.2	1.0	2.3	1.1	2.0	2.5	0.9	1.9	2.6	0.9	1.6	1.7	1.2	1.5	0.8
SL9-CTL2 (day 35)	1.6	1.5	2.2	**12.7** (10.6)[c]	0.3	0.9	0.7	**5.5** (5.5)	0.1	1.0	2.4	1.9	0.2	**8.0** (4.2)	1.5	**2.1** (2.3)	0	1.1	0.5	2.5	0.5
SL9-CTL2 sorted	1.6	0.4	0.6	**9.9** (8.3)	**5.5** (9.2)	0.9	0.6	1.4	0.1	**4.7** (4.3)	**7.1** (3.6)	**10.5** (4.2)	0.1	2.3	2.4	**5.7** (6.3)	0.1	1.9	0.4	0.9	0
SL9-CTL3 (day 35)	4.2	1.4	0.8	17.6	7.7		5.6	2.6		4.2	5.5	17.5	1.6	0.9	3.6	1.9					0.4
SL9-CTL3 24 h post-100 U/ml IL-2	3.7 (0.9)[d]	0.9 (0.6)	0.5 (0.6)	8 (0.5)	6.1 (0.8)		4.4 (0.8)	0.3 (0.11)		2.1 (0.5)	4.3 (0.8)	7.1 (0.4)	1.2 (0.8)	0.9 (1)	2.5 (0.7)	4 (2)					0.6 (1.5)

[a] Boldface type indicates a 2-fold or greater augmentation of a Vβ gene as compared to a control parallel T cell culture.

[b] Fold increase compared to fresh (uncultured) purified peripheral blood CD8+T cells from the same donor within a week.

[c] Fold increase compared to a parallel culture of polyclonal CD8+ T cells stimulated by 30 ng/ml anti-CD3 mAb in the presence of 30 U/ml IL-2.

[d] Fold change compared to a parallel culture of SL9-CTLs without IL-2.

Vβ3. Two weeks of IL-2 provoked a selective and dramatic loss of tetramer+Vβ3+CD8+ T cells (from 38 to 2%) (Fig. 4B). However, tetramer+Vβ3− tetramer-binding T cells were largely unaffected, remaining at ~26–28% of the total cell population. Thus, these data substantiated our previous observation that different SL9-specific Vβ clonotypes are differentially sensitive to the deleterious effects of IL-2.

Functional avidity of SL9-specific T cell cultures and clones

CTLs with higher avidity, as measured in vitro, had greater in vivo antiviral activity when adoptively transferred (47). In this study, functional avidity of CTLs was measured operationally as the negative logarithm of the pulsed peptide concentration that resulted in 50% maximal T2 cell lysis. With one exception (CL-288), the functional avidities of three CTL lines (Wsu01, Wsu02, and Wsu03) and four CTL clones from two of the three donors were in the range of 10^{-8}–10^{-9} M (Fig. 5A). These values are consistent with those reported for SL9-specific CTLs from patients (17, 38).

CL-288 cloned T cells were approximately five logs more sensitive to the SL9 peptide at 4×10^{-15} M.

Functional avidities based on IFN-γ release were also determined for CL-287 and CL-288 cloned T cells. CL-288 T cells were more responsive to soluble peptide (1.2×10^{-9} M) than those of CL-287 (3.7×10^{-8} M), thus indicating a concordance between cytokine release and cytotoxicity. The high-avid CD-288 T cells also produced a substantially greater quantity of IFN-γ (2.5 ng/24 h/10^4 cells) than those of CL-287 (0.2 ng/24 h/10^4 cells) after a 24-h stimulation period with peptide-pulsed T2 cells. The intensity of staining for intracellular perforin was similar for both clones (~25% cells stained with mean fluorescence intensity (MFI) of 34–48). Overall, in vitro-primed and -expanded SL9-specific T cells were highly differentiated CTLs with all associated effector functions.

The CD8 coreceptor enhances recognition by the TCR by binding to the MHC class I molecule. In this study, we compared the ability of the two clones to bind tetramer in the presence of

Table III. *CD phenotypes of immature and mature DCs from productive donors*

	Immature		Mature (LPS)	
Donor/CD Markers	Percentage stained	MFI	Percentage stained	MFI
Wsu02				
CD40	14	40	16	76
CD54	96	283	99	3880
CD80	36	53	81	245
CD83	1	47	65	131
CD86	60	70	82	114
HLA-DR	98	453	99	837
Wsu06				
CD40	3	33	36	33
CD80	11	29	91	65
CD83	0.5	21	31	32
CD86	37	293	67	469
HLA-DR	98	176	99	144
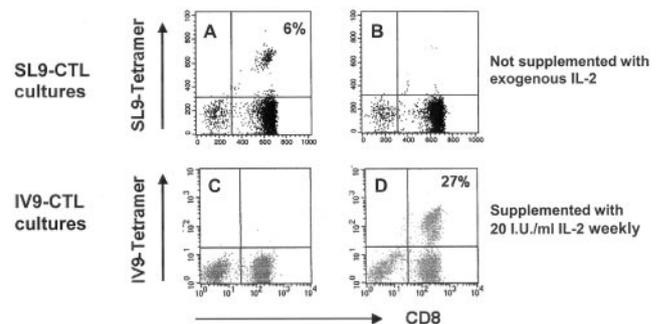
FIGURE 3. Tetramer-binding CD8+ T cells in cultures primed and expanded with SL9 or IV9. *A* and *B*, Parallel cultures primed with immature and mature DCs pulsed with SL9 and expanded for 26 days are shown. *C* and *D*, Parallel cultures from another donor primed with immature and mature DCs pulsed with IV9 and cultured for 19 days are shown. Tetramer-binding cells were found in T cell cultures stimulated with immature DCs pulsed with SL9 (6%) (*A*) and mature DCs pulsed with IV9 (27%) (*D*).
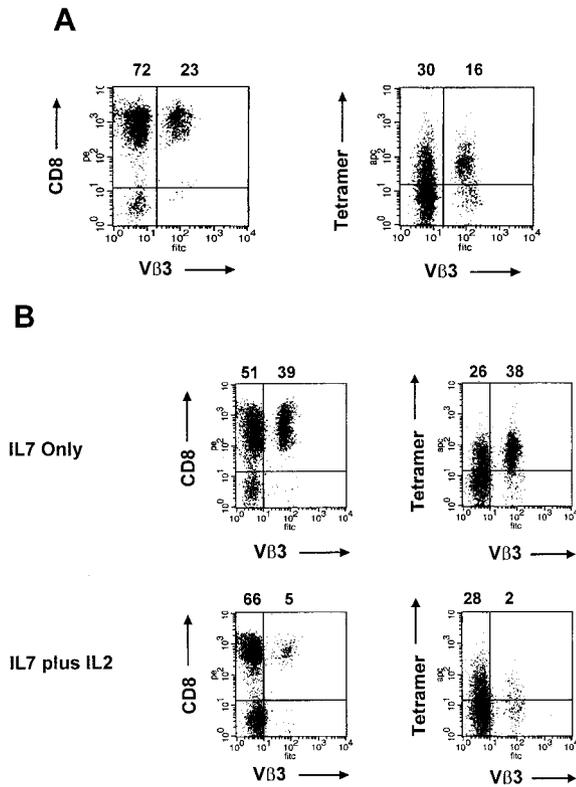
FIGURE 4. Differential sensitivity of SL9-specific T cells primed by transduced DCs encoding Gag to paracrine IL-2. *A,* SL9-tetramer$^+$ CD8$^+$Vβ3$^+$ T cells represent 30% of all SL9-tetramer$^+$CD8$^+$ T cells in a day 28 culture from donor Wsu05. *B,* Selective loss of SL9-tetramer$^+$ CD8$^+$Vβ3$^+$ T cells after two consecutive weekly exposures to paracrine IL-2 is shown.

increasing concentrations of the blocking anti-CD8 Ab B9.11. T cells were preincubated with the Ab for 30 min at 4°C before addition of the SL9 tetramer. Fig. 5*B* shows that tetramer binding by low-avid CL-287 T cells was readily blocked by the lowest concentration (10 ng/ml) of the anti-CD8 Ab, with maximal inhibition (94%) at 200 ng/ml. In comparison, binding of the tetramer to high-avidity CL-288 T cells was minimally affected (13% inhibition) even at much higher Ab concentrations (400 ng/ml), which inhibited cytotoxicity. These data show that the contribution of the CD8 molecule to stabilizing tetramers varied with the affinity of the T cell clones. Moreover, it was possible to dissociate the role of CD8 as glue for tetramer binding from its involvement as a coreceptor in activating cytotoxicity for some T cells with high TCR affinity.

Because binding of the tetramer to the T cells is dynamic rather than static, i.e., the multimeric class I–peptide binding sites engage and disengage with TCRs over time, the kinetics of dissociation of prebound tetramers to the two clones and the parental SL9-specific CTL line were compared over a 4-h period. Cells were first stained with the SL9-tetramer and then incubated at 4°C without (Fig. 5*C*) or with (*D*) 200 ng/ml B9.11 mAb. At various times, cells were washed and fixed for flow cytometry. Tetramer staining appeared to be stable for the CTL culture and clones for the entire incubation period. However the addition of B9.11 reduced tetramer binding to the less avid CL-287 T cells by 90% within the first 5 min. Similarly, a slower but complete displacement was also observed for the parental CTL culture. In contrast, minimal dissociation was observed for the high-avidity CTL clone. These results confirmed results from the blocking studies, indicating that tetramer binding to high-avidity T cells is less dependent on stabilization by CD8.
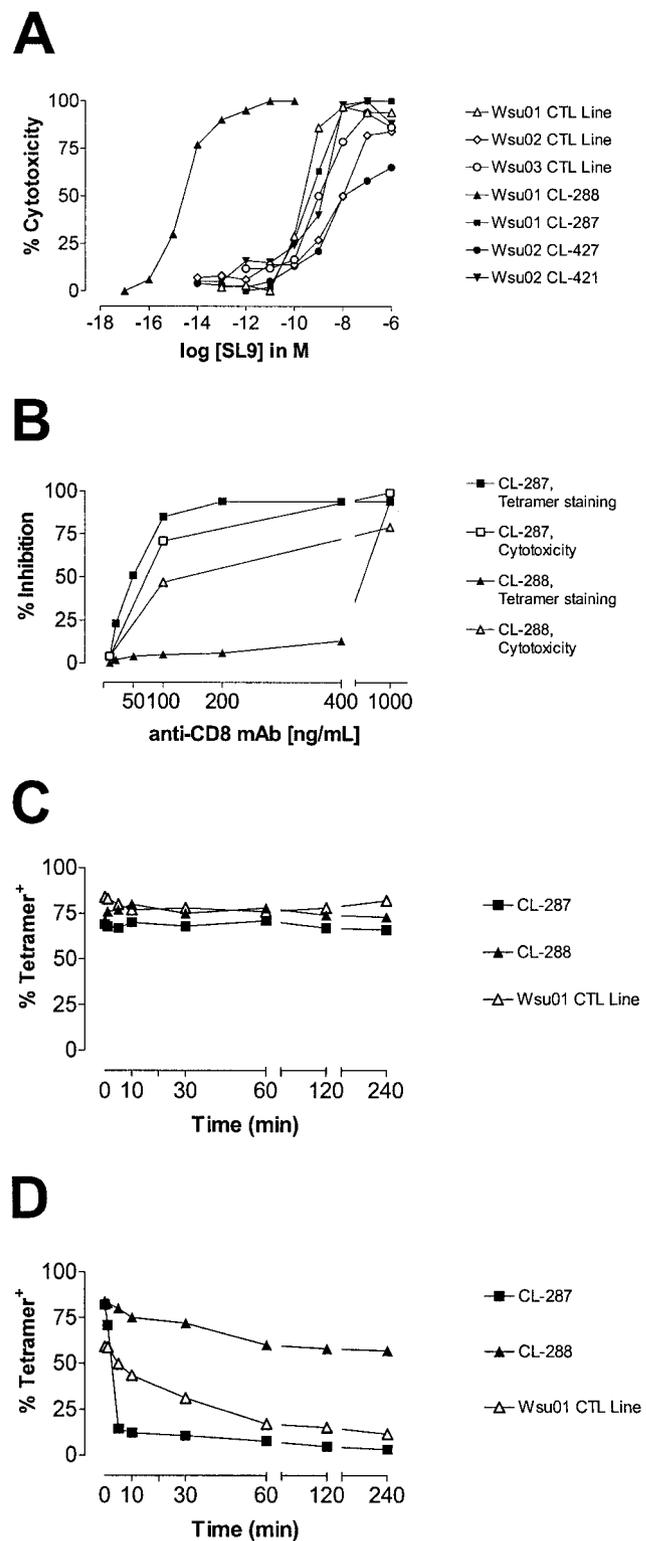
FIGURE 5. Characteristics of SL9-specific CTL clones and cultures. *A,* The functional avidities SL9-CTL cultures from three donors and clones isolated from two cultures are shown. The E:T ratios for the T cell cultures were 10:1, and that for the clones was 1:1. *B,* Compares the sensitivity of tetramer binding and cytotoxicity of 2 SL9-CTL clones to the blocking effects of an anti-CD8 mAb (B9.11) at several concentrations. *C,* Tetramer binding was stable at 4°C for the clones and a CTL line. *D,* Compares the kinetics of dissociation of SL9 tetramers from the clones and the CTL line with the addition of 200 ng/ml B9.11 blocking Ab.
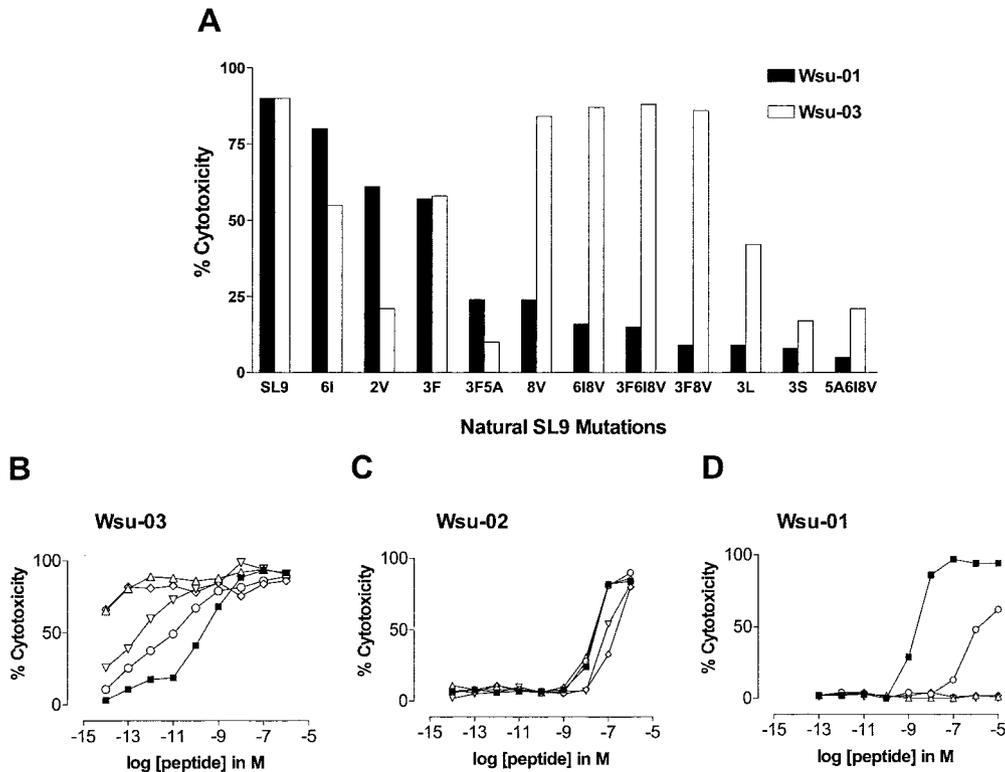
FIGURE 6. Distinctive recognition patterns for 11 natural variants of SL9 by cultures of SL9-CTLs from two donors. *A*, Shown is the percent cytotoxicity to T2 cells pulsed with SL9 and its variants at the E:T ratio of 10:1 for SL9-CTLs from donors Wsu-01 and Wsu-03. *B–D*, Shown are the functional avidities of CTL cultures from three donors to SL9 (■—■), 8V (○—○), 6I8V (△—△), 3F8V (▽—▽), and 3F6I8V (◇—◇).

There was no detectable difference between the two T cell clones in their expression of $\alpha\beta$-TCR, CD8, or CD28 molecules, as determined by flow cytometry.

SL9-CTLs primed in vitro recognize naturally occurring mutations

A single TCR can interact with a broad range of different peptide ligands presented by MHC molecules (48). In this study, we compared the ability of de novo-primed CTL cultures from two individuals to recognize and lyse T2 cells pulsed with 11 naturally occurring mutations of the SL9 epitope (18, 38) (Fig. 6*A*). The CTL culture from Wsu-01 was capable of lysing three variants (6I, 2V, and 3F) in addition to the native SL9 peptide. In contrast, CTLs from Wsu-03 recognized at least six variants (6I, 3F, 8V, 6I8V, 3F6I8V, and 3F8V), some as efficiently as the SL9 native sequence. Thus, SL9-specific T cell lines were capable of recognizing several SL9 variants, but with different efficiency. Moreover, CTLs from different donors can have very distinctive functional fingerprints of TCR recognition.
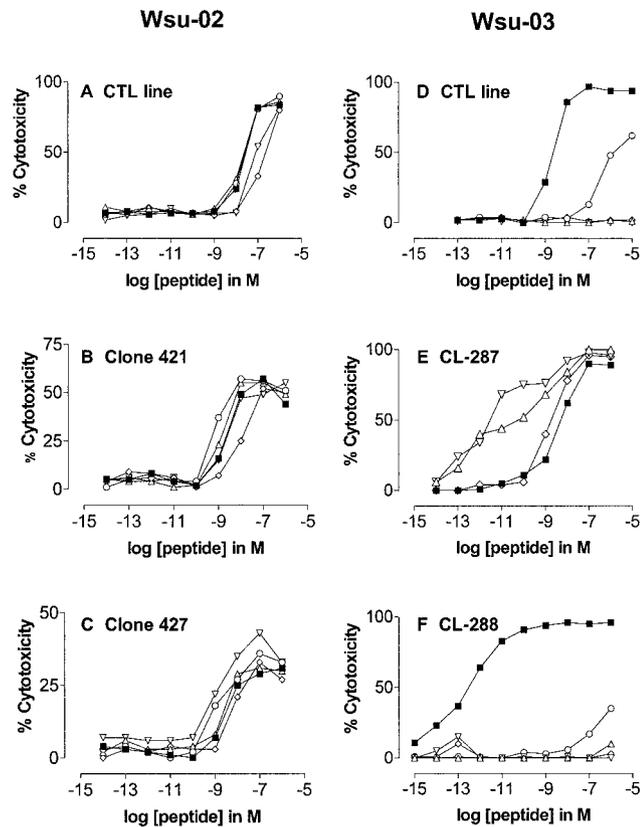
The functional avidities of three CTL cultures from different donors were determined for SL9 and four mutations (8V, 6I8V, 3F8V, and 3F6I8V). The EC_{50} values in nanomolar concentration

of the three CTL cultures for the native SL9 peptide were comparable, ranging from 0.73 to 52 (Table IV). The EC_{50} of the Wsu-03 CTL culture from sorted tetramer-binding cells (Wsu-03 sorted) was lower than its parental culture, possibly due to enrichment of more avid CTLs. Fig. 6*B* shows that Wsu-03 CTLs recognized all four variant peptides better than SL9 itself. The EC_{50} values for the variants were up to 4 logs lower than that determined for the SL9 peptide (Table IV). In contrast, Wsu-02 CTLs recognized two variants (8V and 6I8V) as well as SL9, with essentially identical EC_{50} values (Table IV). Two peptides, 3F8V and 3F6I8V, were not as well recognized, as evident by their higher EC_{50} values (Fig. 6*C*, Table IV). Lastly, Wsu-01 CTLs were highly specific for SL9, barely reactive to 8V, and completely nonresponsive to three other peptides (Fig. 6*D*). The results of the functional fingerprinting and functional avidity analyses demonstrate that SL9-CTL responses, at the population level, can be very different among donors.

Fig. 7 compares the avidities to SL9 and its mutations between representative clones and their parental CTL cultures. Clones 421 and 427 had avidities to SL9 and variant peptides in the same range as their parental line, Wsu-02. In contrast, whereas the Wsu-03 culture was highly specific for SL9, its clones (CL-287

Table IV. *Range of EC_{50} values in nanomolar concentration (95% confidence intervals) for SL9 and four natural variants of CTL cultures or clones from three donors*

Peptide	Wsu-01-Sorted	Wsu-02	Wsu-03	Wsu-03-Sorted	Wsu-02 Clone 421	Wsu-02 Clone 427	Wsu-03 CL-287	Wsu-03 CL-288
SL9	1.3–3.2	12.8–52	0.73–1.6	0.09–0.5	0.6–7.7	1.7–7.1	2.6–6.9	0.0001–0.0006
8V	294–584	17–40.6	0.038–0.207	0.003–0.056	0.3–1.5	0.5–2.8	ND	77–219
6I8V	ND	12.8–37.6	0.00006–0.0003	0.000009–0.0002	0.6–3.2	1.1–6.9	0.02–2.9	ND
3F8V	ND	166.6–408.6	0.00007–0.0002	~0.0000007	1.4–5.6	0.4–3.0	0.0007–0.01	ND
3F6I8V	ND	44.6–155.1	0.0004–0.071	0.0003–0.005	5.9–3.0	1.9–17.6	1.2–2.2	ND

FIGURE 7. Functional avidities of CTL cultures and their representative clones. *A*, *B*, and *C*, The CTL line and two clones from donor Wsu-02, respectively, against SL9 (■—■) and its variants, 8V (○—○), 6I8V (△—△), 3F8V (▽—▽), and 3F6I8V (◇—◇). *D*, *E*, and *F*, Those from donor Wsu-03.

FIGURE 8. Correlation between functional avidities for the variant peptides and sensitivity of cytotoxicity to blocking with an anti-CD8 Ab of SL9-CTLs. *A–C*, Shown are the functional avidities of SL9 (■—■) and its variants, 8V (○—○), 6I8V (△—△), 3F8V (▽—▽), and 3F6I8V (◇—◇), for the Wsu-03 CTLs and its clones, CL-287 and CL-288, respectively. *D–F*, The percent inhibition of cytotoxicity by different concentrations of the anti-CD8 Ab is shown.

and CL-288) were very different from each other. CL-287 was more degenerate, recognizing 3F6I8V and SL9 equally but with greater avidity for 3F8V and 6I8V. In contrast, the CL-288 CTL clone resembled the parental line in terms of a limited degeneracy, recognizing with only the native SL9 but not the three variant epitopes. In addition, the EC_{50} for SL9 was extraordinarily high, in the 10^{-13} M range. Thus, the CTL response to SL9 may differ among individuals in terms of TCR degeneracy and at least for one donor, limited degeneracy was associated with greater affinity for the natural peptide.

The interaction between CD8 and the peptide-MHC class I complex (pMHCI) contributes to the activation of human antiviral CTLs, and this contribution may be more significant when binding between TCR and MHC-peptide complex is weak (49). Fig. 8 compares functional avidities of the Wsu-03 CTL line and clones for SL9 and its variant peptides and the inhibition by the anti-CD8 Ab of the lysis of T2 cells pulsed with these peptides. Although cytotoxicity mediated by CTL line and clones, CL-287 and 427, was progressively blocked by increasing concentrations of anti-CD8 mAb (Fig. 8, *D–F*), maximal inhibition depended on the antigenicity of the peptide under study. This inverse correlation is more prominent with the cloned T cells. In the case of CL-287 T cells, blocking was most effective for SL9 (Fig. 8*E*), followed by 3F6I8V, 6I8V, and 3F8V. Blocking was minimally effective for the last peptide, 3F8V, for which CL-287 had the highest avidity (Fig. 8*B*). Essentially identical observations were obtained for clone 427 (Fig. 8, *C* and *F*). Thus, when a CTL clone has a strong avidity for a particular peptide, it does not have to depend on CD8 as much to mediate lysis. Recently, it was reported that that human

anti-CD8 blocking Abs block T cell activation by interfering with the TCR/pMHCI interaction and not, as previously assumed, the pMHCI/CD8 interaction (49). Our data are consistent with this phenomenon.

Discussion

There is compelling correlative evidence that HIV-specific CTLs play a major role in inhibiting HIV replication and determining the rate of disease progression (3, 50). However, despite their robust antiviral immune responses, the majority of patients progress to AIDS. Powerful new assays (such as tetramers and ELISPOT assays) have led to delineation of CTL epitopes and their HLA restriction elements throughout the entire HIV genome and permitted detailed analyses of the breadth and strength of these responses in patients (14). Comprehensive epitope analysis of HIV-specific cellular immune responses against the entire expressed HIV genome, however, failed to reveal definite correlations with plasma viral load (14). Functional impairments in a high proportion of HIV-specific CTLs have also been described (51–53). It appears that other intrinsic immunological characteristics beyond the breadth and magnitude of the virus-specific T cell responses may also be important for viral restriction.

Despite the pivotal role of CTLs in the adaptive immunity to HIV, other pathogens, and tumors, the optimal conditions for priming and/or expansion of Ag-specific CTLs in vitro remain elusive. Published peptide-based methods using T cells from peripheral blood as precursors have produced relatively short-lived CTL cultures (<1 mo) exhibiting modest Ag-specific reactivities (45, 46, 54–56). Although details of the procedures vary among laboratories, CTLs specific for a very limited set of peptides had been

consistently generated (46). These are the A*0201-restricted epitopes of the Flu matrix protein, hepatitis B virus nucleoprotein, and several tumor-associated Ags (40). Induction of primary CD8$^+$ CTLs from healthy HIV-negative blood donors has been reported for only a few HIV peptides (45, 56), and these cultures had been generally transient and poorly characterized. In the past, investigators have attempted to prime CTLs to SL9 ex vivo without much success (57).

CTL priming in vivo is initiated by specialized APCs such as DCs. Much attention has been focused on how the state of activation or maturity of DCs defines the outcome of T cell responses (58–60). Immature DCs produced from monocytes by culture in IL-4 and GM-CSF do not have obvious in vivo counterparts. Although generally considered to be poor initiators of immunity, they have been extensively tested as DC-based vaccines to activate CTL responses. Concerns have been raised that such immature DCs may actually be tolerogenic in vivo (60, 61). Maturation of these cells in vitro is effected by exposure to Toll-like receptor stimuli, CD40 ligand, or inflammatory cytokines (62), and may be required for successful ex vivo priming of peptide-specific T cells (44) and expansion of influenza A-specific memory T cells (63). Our results show that SL9-CTLs were primed exclusively by immature but not mature DCs. In contrast, under similar priming conditions, IV9 (Fig. 3) and other A*0201-restricted peptides (HIV Gag p24$_{19–27}$, tyrosinase$_{368–376}$, Melan-A/MART1$_{27–35}$, and MUC-1$_{12–20}$) were either preferentially or exclusively presented by mature DCs (data not shown). The minimal requirements for APCs to stimulate unprimed CD8$^+$ T cells are the expression of HLA class I Ag and costimulatory molecules (CD80 and ICAM-1) (64). Immature DCs express these structures and therefore have the necessary components. Stimulation of naive CD8$^+$ T cells by APCs involves signal 1 from the TCR recognition of pMHCI, and signal 2 from contact with costimulatory molecules. The two signals are synergistic but are most influenced by the affinity and concentration of the peptide (64). Because saturating concentrations of immunizing peptides were used, our data suggest that SL9, but not the other epitopes, may provide a qualitatively stronger TCR signal to compensate for lower costimulation afforded by immature DCs (63). Alternatively, the SL9 precursor CTL frequency of the naive T cell repertoire may be greater than those for the other epitopes. A high precursor frequency can facilitate CTL expansion after cross-priming, particularly in the absence of CD4$^+$ T cells (65). Mature DCs may excessively stimulate SL9-T cells, thus failing to induce this reactivity. CTL cross-priming is influenced by multiple factors (66), including, as here, the intrinsic immunogenicity of the epitope.

In this study, we have characterized the in vitro-primed SL9 CD8$^+$ CTLs in some detail. In terms of the degeneracy of TCR recognition, i.e., cross-reactivity to natural SL9 escape mutations and functional avidities for the native epitope, ex vivo-primed SL9-CTLs were very similar to those derived from patients (21). Restricted Vβ staining suggests a minor drift of the SL9 TCR repertoire with continuous culture, as has been suggested for T cells to an EBV epitope during the first year of infection (67). Many SL9-CTL cultures established at different times from the same donor favored the same dominant Vβ sequences, suggesting a stable naive T cell precursor frequency. However, analysis of an essentially homogeneous tetramer-binding culture revealed a broad TCR repertoire, involving 8 of the 21 Vβ genes studied for this individual. Sequence analysis of V(D)J regions of TCR has revealed greater receptor diversity than that predicted by Ab staining (67). Thus, the early human SL9-CTL response appears to be very broad.

CD4$^+$ T cells are thought to provide help for CTL responses in two ways, by conditioning APCs to enhance their ability to prime naive CD8$^+$ T cells and thus initiating CTL responses, and by producing growth or survival factors such as IL-2. However, there are circumstances under which CD8$^+$ T cell responses can occur in the absence of CD4$^+$ T cells in vivo and in vitro (68, 69). Naive CD8$^+$ TCR transgenic T cells activated in vitro by engagement of the TCR with peptide/MHC tetramers (70) or with Ag and costimulation (71) can produce autocrine IL-2 to sustain their own proliferation and differentiation into cytolytic effectors. In some strains of TCR transgenic mice, such helper-independent CD8 expansions were transient and required a source of exogenous IL-2, presumably from CD4 cells, for a sustained CTL response (71, 72). Recently, it was shown that the nature of the peptide Ag determines whether the in vivo CTL response requires extraneous help (73). In this study, we showed that SL9-CTLs can undergo sustained clonal expansion and CTL differentiation for months in the absence of CD4$^+$ T cells and exogenous IL-2. Addition of IL-7, which is produced by bone marrow stromal cells but not T cells, was absolutely necessary. This cytokine has important effects at several checkpoints of a CD8$^+$ T cell response to promote proliferation and survival, particularly of memory cells (74). Taken together, the conditions for effective in vitro priming and expansion of SL9-CTLs differ in significant details from the typical protocols used for CTLs specific to other viral or tumor epitopes (44, 75) and suggest that SL9 has unique immunogenic properties.

Helper cell-independent CTL responses typically occur under nonlimiting conditions, such as high precursor frequency of Ag-specific CD8$^+$ T cells or activation with a high-affinity Ag (73, 76). A high precursor frequency for SL9-specific T cells is suggested by quantitative analyses in HIV-infected patients. Not only is this reactivity extraordinarily prevalent among chronically infected individuals (75%) (4, 17, 18, 19), the circulating frequencies of the precursor CTLs are also very high. In one study, the numbers of SL9 precursor CTLs were at least 1–2 logs greater than two other A2-restricted specificities (IV9, RT, aa 476–484, and VL9, RT, aa 346–354) monitored simultaneously in the same patients (18). In contrast, the binding affinity of a peptide epitope for MHC class I is also a key factor in determining helper independence (73). Peptides with high affinities, with IC$_{50}$ $\leq$ 50 nM, were thought to induce CD4$^+$-independent CTLs, whereas those with low binding affinity (IC$_{50}$ > 500 nM) were rarely immunogenic. The binding of SL9 to A*0201 is relatively weak and falls between these limits (290 nM; Epimmune, San Diego, CA), and thus, binding to the class I molecule alone is inadequate to explain why this peptide is uniquely helper independent. Because T cell responsiveness to an epitope is also dependent on the affinity of the MHC-peptide complex for the TCR (77), the robust in vitro SL9-CTL response suggests a high-affinity human T cell repertoire for this peptide.

IL-2, a logical candidate for an autocrine growth factor, is found at elevated levels in the spent supernatants of SL9-CTL cultures, although other cytokines, including TNF-α and IFN-γ, were also detected. Interestingly, a recent report shows that autocrine and paracrine IL-2 exerted different effects in the in vivo CD8$^+$ T cell response to a virus infection (78). Specifically, expansion of antiviral CD8$^+$ T cells in secondary lymphoid tissues was paracrine IL-2 independent, whereas autocrine IL-2 is important for continued expansion within nonlymphoid tissues. The ability of SL9-CTLs to produce autocrine IL-2 may explain why this specificity is enriched in the CD4$^+$ depleted, gut-associated lymphoid tissues, whereas CMV-specific T cells are reduced in chronically HIV-infected individuals (29).

Primarily produced by activated T cells, IL-2 has diverse but incompletely defined functions in the generation and maintenance of CD8$^+$ T cell immunity (76, 79). Although autocrine-IL-2 initiates a CTL response, IL-2 also paradoxically prepares CD8$^+$ Ag-specific lymphocytes for activation-induced cell death (AICD) (80). Even small quantities (5 IU/ml) of exogenous IL-2 caused rapid reduction of SL9-CTLs, possibly via a Fas-independent mechanism. We also found it necessary to replace the spent medium regularly for the purpose of reducing levels of cytokines. The more activated the T cells, the greater was their susceptibility to IL-2-induced apoptosis (81, 82). This appeared to be the case for dominant Vβ3$^+$ SL9-CTLs primed by the transduced DCs, which were disproportionately lost after exposure to paracrine IL-2. Similarly, the dominant Vβ clonotypes in Wsu03 SL9-CTL3, Vβ 5.1 and Vβ 13.1, were also preferentially lost after a 24-h incubation to 100U/ml IL-2. This unique sensitivity of SL9-CTLs to IL-2-mediated AICD may explain why others have not been successful at priming SL9-CTLs in vitro, because IL-2 is invariably added as a growth factor to T cell cultures.

SL9 appears to provide unique structural constraints at the TCR level, among others studied, to induce helper-independent primary and secondary CTL responses. The sensitivity of SL9-CTLs to cytokine-induced cell death may explain why this reactivity is rare during the innate response to acute infection, which is associated with a massive release of inflammatory cytokines. Because the high antigenic load associated with viral infection can drive high-affinity T cells into apoptosis (82, 83), high viral titers would also select against highly activated SL9-T cells and increase the frequency of lower-affinity cells with other specificities. In contrast, the ability to produce autocrine mediators to sustain proliferation may explain the predominance of SL9-CTLs in the circulation as well as in gut-associated lymphoid tissues during chronic HIV infection, when CD4 helper activity is diminished.

The pathogenesis of HIV infection and AIDS is associated with a dynamic equilibrium between T cell activation, proliferation, and cytokine-modulated cell death (84). The response to a single HIV-1 epitope is not predictive of the entire response mediated by an infected person, because many proteins are recognized and virus-specific T cell responses broaden with continuous exposure (15). SL9 illustrates how the aberrant immunogenicity of a single immunodominant epitope may contribute to the complexity and perhaps ineffectiveness of the HIV CTL response. We are unaware of other help-independent epitopes or their prevalence in the HIV sequence, but the highly abundant A*0201-restricted immunodominant CTL epitope of measles virus nonstructural C protein is another example of such an epitope. Despite strong IFN-γ CD8$^+$ T cell responses in acute measles, no memory responses were detected after recovery (85).

Our studies demonstrate that SL9 produces a poor CTL response, one that appears to be overstimulated and sensitive to destruction by apoptosis. Moreover, SL9-CTLs may lack the ability to differentiate into memory cells: help-independent CTL responses have been shown to be defective in secondary encounters with Ags (86, 87). Because an immunodominant epitope can inhibit T cell expansion against other epitopes during an immune response (88), the anti-SL9 response may actually be deleterious to the development of a protective antiviral CTL immunity.

Native viral proteins may not be optimal vaccines (89), although most currently available HIV vaccines are based on the natural form of the pathogen, leading groups to explore purposeful alterations to increase potency (89–91). Proof-of-principle studies have validated that epitope enhancement can improve immunogenicity and the quality of an antiviral immune response (88–92). Modifications of the antigenic peptides can result in significant changes

in T cell activation (92) and AICD (44). In contrast to previous assumptions (93), we propose that less immunogenic but help-dependent peptide variants of SL9 will be better in vivo immunogens, because they will provoke memory responses. SL9 variants might be incorporated into multiepitopic vaccines or rationally modified for vaccines containing HIV Gag. The availability of consistently generated, highly homogeneous SL9-specific CTL cultures would significantly facilitate this effort.

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