HIV-specific Cytotoxic T Cells from Long-Term Survivors Select a Unique T Cell Receptor

Tao Dong,[1] Guillaume Stewart-Jones,[2] Nan Chen,[1] Philippa Easterbrook,[3] Xiaoning Xu,[1] Laura Papagno,[1] Victor Appay,[1] Michael Weekes,[1] Chris Conlon,[4] Celsa Spina,[6] Susan Little,[6] Gavin Screaton,[1] Anton van der Merwe,[5] Douglas D. Richman,[6] Andrew J. McMichael,[1] E. Yvonne Jones,[2] and Sarah L. Rowland-Jones[1]

[1]*Medical Research Council Human Immunology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford, OX3 9DS, UK*

[2]*Division of Structural Biology, Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford OX3 7BN, UK*

[3]*Department of HIV/GUM, The Guy's, Kings' and St Thomas' School of Medicine, Weston Education Centre, London, SE5 9RS, UK*

[4]*Nuffield Department of Medicine, John Radcliffe Hospital, Oxford, OX3 9DU, UK*

[5]*Sir William Dunn School of Pathology, Oxford University, Oxford, OX1 3RE, UK*

[6]*San Diego VA Research Center for AIDS and HIV Infection, University of California San Diego, La Jolla, CA 92093*

Abstract

HIV-specific cytotoxic T lymphocytes (CTL) are important in controlling HIV replication, but the magnitude of the CTL response does not predict clinical outcome. In four donors with delayed disease progression we identified Vβ13.2 T cell receptors (TCRs) with very similar and unusually long β-chain complementarity determining region 3 (CDR3) regions in CTL specific for the immunodominant human histocompatibility leukocyte antigens (HLA)-B8–restricted human immunodeficiency virus-1 (HIV-1) nef epitope, FLKEKGGL (FL8). CTL expressing Vβ13.2 TCRs tolerate naturally arising viral variants in the FL8 epitope that escape recognition by other CTL. In addition, they expand efficiently in vitro and are resistant to apoptosis, in contrast to FL8–specific CTL using other TCRs. Selection of Vβ13.2 TCRs by some patients early in the FL8-specific CTL response may be linked with better clinical outcome.

Key words: HIV • cytotoxic T lymphocytes (CTL) • T cell receptor • cross–reactivity • apoptosis

Introduction

There is considerable diversity in the rate of HIV–1 disease progression, with some infected people progressing rapidly to AIDS whereas others remain asymptomatic and maintain normal CD4^{+} T cell counts for a decade or more. Several mechanisms have been linked with delayed HIV-1 disease progression: some long-term nonprogressors (LTNPs) are infected with attenuated strains of HIV-1 (1) and others have genetic polymorphisms in their chemokine receptor and MHC genes associated with good prognosis (2). However, in most cases there is no single mechanism that accounts for delayed disease progression (for review see reference 3). LTNPs exhibit strong cellular immune responses to the virus, both CD4^{+} (4) and CD8^{+} T cell responses (5, 6), but it has not proved possible to distinguish LTNPs from progressors simply by the magnitude of their CTL response (7). Similarly, a comprehensive study of CD8^{+} T cell responses generated against all HIV-1 proteins has shown no correlation between either the magnitude or the breadth of the response and plasma viral load (8). This is paradoxical when there is good evidence that CTLs are the major force controlling

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Address correspondence to Sarah L. Rowland-Jones, MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DS UK. Phone: 44-1865-222316; Fax: 44-1865-222502; email: sarah.rowland-jones@ndm.ox.ac.uk

Abbreviations used in this paper: ART, antiretroviral therapy; CDR3, complementarity determining region 3; LTNPs, long-term nonprogressors; SIV, simian immunodeficiency virus; Tunel, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling.

HIV replication from early infection (for review see reference 9). Qualitative aspects of CTL activity such as their specificity and ability to respond to naturally occurring HIV variants, functional aspects such as perforin content, cytokine and chemokine secretion pattern, and the proliferative ability of individual CTL clones may also play a part in mediating viral control. Studies of antigen-specific T cells using peptide–HLA class I tetrameric complexes ("tetramers") to look at both quantitative and qualitative aspects of CTL function in samples taken directly ex vivo, have shown that the majority of HIV-specific CTL secrete antiviral cytokines and chemokines (10), but this ability may decline with disease progression (11). HIV-specific CTL are in an unusual intermediate state of differentiation, expressing CD27 but lacking CD28, which distinguishes them from other virus–specific CD8$^+$ populations studied to date (12). This differentiation phenotype is linked with low perforin expression and reduced cytolytic ability (10), which has led to speculation that functional impairment of HIV-specific CTL underlies the ultimate failure of the infected person to control viral replication (13). These observations raise the possibility that qualitative aspects of the CTL response may play a part in spontaneous control of HIV replication in some infected people.

A critical component of the virus-specific CD8$^+$ T cell response is the selection of CTL-bearing TCRs that efficiently recognize the infected target cell. In most individuals with HLA–A2, infection with influenza A virus stimulates expansions of T cells expressing TCRs with remarkably similar sequences (14): the recent crystallization of one such TCR–HLA–peptide complex has revealed how the TCR fits over the exposed peptide main chain, and the complex is stablized by insertion of a conserved Arginine residue from the β chain complementarity determining region 3 (CDR3) into a notch between the bound peptide and the α helix of HLA–A2 (15). Similarly, most people with HLA–B8 and EBV infection select a conserved TCR in the response to the EBNA-3A antigen (16). The structure of this TCR complexed with HLA–B8 and the EBV peptide has shown how efficiently the conserved TCR interacts with its HLA–peptide target, forming a network of interactions between the TCR and both peptide and HLA molecule and engendering conformational changes in the CDR loops (17). Previous studies of the TCR repertoire in the HIV-specific CTL response have shown oligoclonal TCR usage (18) but no common receptors in unrelated individuals (19, 20). Here we describe selection of a distinctive Vβ13.2 TCR with remarkable β chain conservation in CTL expansions that recognize an immunodominant epitope in HIV-1 nef from four HIV-infected individuals with HLA–B8 and delayed disease progression. In contrast to the immunodominant TCRs described in influenza A and EBV infection, not all donors with HLA–B8 and HIV-1 infection who make nef-specific responses select this TCR. The apparent link with delayed disease progression suggests that this TCR confers particular advantages to the host. We show that CTL using this TCR

are more likely to proliferate in vitro and resist apoptosis than CTL specific for the same epitope from the same or other donors using other TCRs. Common "escape" variants in this epitope are frequently selected under CTL pressure, often early in infection (21): however Vβ13.2-bearing CTL recognize these variants as efficiently as the index sequence (in contrast to B8 nef-specific T cells expressing other TCRs) and escape variants have not been selected in donors with the Vβ13.2 TCR. The Vβ13.2 TCR is shown to have a moderately high affinity for HLA–B8 complexed with either the index or most common escape variant sequence. Taken together these data suggest that this unique TCR is expressed on CTL that are particularly well-equipped to control HIV replication and that this could be linked with good clinical outcome in individuals that have generated a response that includes the Vβ13.2 TCR.

Materials and Methods

Patient Samples and HLA Typing. Samples were taken from members of a well-characterized cohort, established in 1995, of 165 long-term HIV-1–infected volunteers attending clinics in London who had been enrolled into a nested case-control study of the biological and behavioral correlates of nonprogression in HIV-1 infection (22). Of the original cohort: 46 subjects were defined as LTNPs based on a stable CD4$^+$ T cell count >500 cells/μl at 8 yr of infection and 92 as slow progressors based on a CD4$^+$ T cell count <500 cells/μl at 8 yr of infection. The majority (95%) of patients were white homosexual or bisexual men. We studied four donors with HLA–B8 who had been shown to respond to the FL8 epitope in previous studies (7): their clinical characteristics are shown in Table I. At the time of analysis, donors 065 and 200 met the definition of LTNPs and donors 005 and 046 were slow progressors. In addition, samples were analyzed from a rapid progressor (defined as progressing to a CD4$^+$ T cell count <200 mm^3 in 5 yr or less after seroconversion) from the London cohort and two further patients attending an HIV clinic in Oxford, both of whom were hemophiliac men with CDC stage IV disease. Donors with primary HIV-1 infection were adult subjects with a symptomatic viral illness after high risk sexual exposure to HIV who had been referred to the San Diego AIDS Treatment Center for evaluation. Acute HIV infection was defined by detectable plasma HIV RNA and nonreactive HIV antibody test or indeterminate Western blot assay. Upon diagnosis of acute HIV-1 infection, the majority of the subjects were treated with antiretroviral therapy (ART). Human subjects approval for these studies was given by the following bodies: The Central Oxford Regional Ethics Committee, the Ethics Review Committee of King's College Hospital, and The University of California, San Diego, Institutional Review Board. PBMCs were separated from heparinized blood and either used fresh or cryopreserved for subsequent studies. Molecular HLA typing was performed using the Amplification Refractory Mutation system with sequence-specific primers as described previously (23).

Synthesis of Peptide–HLA Tetramers. The HLA molecule heavy chain cDNAs were modified by substitution of the transmembrane and cytosolic regions with a sequence encoding the BirA biotinylation enzyme recognition site, as described previously (24). These modified HLA heavy chains, and β2-microglobulin, were synthesized in a prokaryotic expression system (pET; R&D Systems), purified from bacterial inclusion bodies,

solubilized in the presence of denaturant (4 M urea), and allowed to refold with the relevant peptide by dilution. Refolded monomeric complexes were purified by FPLC and biotinylated using BirA (Avidity), and then combined with PE-labeled streptavidin (Sigma-Aldrich) at a 4:1 molar ratio to form tetrameric HLA–peptide complexes ("tetramers"). The tetramers used in these studies were as follows: HLA–A*0201-SLYNTVATL (A2 gag p17), HLA–A*0201-ILKEPVHGV (A2 pol), B8-FLKEKGGL (B8 nef), B8-FLKEQGGL (B8nef Q5), and B8-DIYKRWII (B8 gag p24) complexes, with HLA–A*0201-NLVPMVATV (A2 CMV) as a control.

Antigens and Antibodies. Peptides were synthesized by FMOC chemistry, and corresponded to previously defined and optimized CTL epitopes. Anti-CD8 (Peridinin chlorophyll protein) antibodies were purchased from Becton Dickinson. A panel of Vβ antibodies was made available as part of the TCR mAB workshop by Dr. Margaret Callan (Weatherall Institute of Molecular Medicine). The hybridoma for the Vβ13.2 antibody was a kind gift from Dr. Philippa Marrack (University of Colorado, Health Sciences Center, Denver, CO).

Cell Surface and Intracellular Staining. Cell surface and intracellular stainings were performed on fresh or carefully thawed cryopreserved PBMCs. Titrated tetramers (PE conjugated) were added for 15 min at 37°C, followed by addition of a panel of titrated unconjugated Vβ antibodies, after which rabbit anti–mouse FITC antibodies were added. The cells were then stimulated with antigenic peptides for 1 h at 37°C, then Brefeldin A was added and the cells were incubated overnight at 37°C. The next day the lymphocytes were washed in PBS, 0.5 mM EDTA, 1% BSA, fixed, and permeabilized in FACS Permeabilization buffer (Becton Dickinson). After washing, the cells were incubated with IFNγ-APC and CD8-Peridinin chlorophyll protein antibodies for 15 min at room temperature. Cells were then washed and stored in Cell Fix buffer (Becton Dickinson) at 4°C until flow cytometry analysis was performed. Samples were analyzed on a Becton Dickinson FACSCalibur.

Enzyme-linked Immunospot Assays. Synthetic peptides corresponding to previously defined and otpimized CTL epitopes were used at a concentration of 10 μM in IFN-γ enzyme-linked immunospot assays to define CD8$^+$ T cell responses directly ex vivo, as described previously (25). PHA was always included as a positive control.

Apoptosis Assays. We compared the susceptibility of different T cell PBMC populations to apoptosis in overnight culture using the Tunel (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling) assay that measures late apoptotic cells with DNA fragmentation (26). In brief, freshly obtained PBMC were stained with tetramer and CD8 (tri-color) followed by staining with TUNEL (FITC) according to the manufacturer's protocol (Roche).

Establishment of Clones. CTL clones were established from PBMCs by using appropriate antigen-specific tetramers in combination with MACS anti-PE microbeads (Miltenyi Biotec). In brief, tetramers were added to 3–5 million PBMCs, then incubated at 37°C for 20 min. The cells were then washed with cold buffer and resuspended in 40μl of anti-PE beads, mixed well and incubated for 15 min at 4–8°C. The cells were then washed and resuspended in 500 μl cold buffer, and magnetic separation was performed according to the manufacturer's protocol (Miltenyi Biotec). Sorted tetramer-positive cells were counted and CTL cloning was performed by using a standard protocol as described previously (27).

CTL Lysis Assays. CTL lysis assays were performed using standard ^{51}Chromium release assays. In brief, HLA-matched B cell lines were labeled with ^{51}Chromium for 1 h and then washed three times: target cells were then divided and pulsed with peptides at different concentrations. After another hour of incubation at 37°C, the peptide solution was then washed off and cells were counted and cocultured with CTL clones at appropriate effector to target (E/T) ratios in 96-well plates. The plates were incubated at 37°C for 4 h, supernatants were harvested, and then counted for radioactivity using a Beta-plate counter (WALLAC). Specific lysis was calculated from the formula: % lysis = (experimental counts − media control)/(detergent control − media control) × 100%.

To study the off-rate of the variant Q5 peptide, the assay was modified as follows: targets (B cell lines) were pulsed with 10 μM peptide and then washed twice and incubated for varying time periods, as shown. The cells were then harvested, labeled with Chromium, and washed before culturing with CTL clones.

T Cell Receptor Sequence Analysis. RNA was extracted using Tri-Reagent from CTL clones or from FACS-sorted PBMC after staining with the FL8 tetramer and subjected to an RT-PCR amplification technique with a switching mechanism at the 5′ end of the RNA transcripts (28). This was performed with C-region α primer: TCRAC: GTCCATAGACCTCATGTC-TAGCACAG and a C-region β primer: TCRBC: ATTCACC-CACCAGCTCAGCTCCACG.

Sequencing was performed with gel-purified PCR products using the dideoxy chain-termination method on a Megabase 1000 in the Wellcome Trust Centre for Human Genetics and in the MRC Human Immunology Unit sequencing facility.

Confirmation of TCR CDR3 Sequence from Tetramer-sorted Cells. FL8 tetramer–positive cells from donor 005 were sorted using anti-PE magnetic beads (Miltenyi Biotec). mRNA from sorted cells was extracted using a Microfast RNA isolation kit (Invitrogen) and cDNA was synthesized using a Smart PCR cDNA synthesis kit (CLONTECH Laboratories, Inc.). PCR was performed with a Vβ13.2-specific primer: TGGTGAGGGTA-CAACTGCC and a 3′ C-region primer Cβ: AGGCGGCT-GCTCAGGCAGTATCTGGAGTCA, with optimized cycles. The PCR products were then purified and put into TOPO TA cloning vectors (Invitrogen). Plasmid DNAs from 20 colonies containing inserts of the correct size were then sequenced in the MRC Human Immunology Unit sequencing facility.

TCR Affinity Measurement Using Surface Plasmon Resonance. Residues 1–201 and 1–247 from the α- and β-chains, respectively, were cloned into Pett22b$^+$ (Novagen), where the α-chain was in frame with the COOH-terminal vector his$_6$ tag. Residues α threonine 159 and β threonine 174 were mutated to cysteines and the protein expressed in recA$^-$ derivative BL21 (BLR) strain *Escherichia Coli* (Novagen) as inclusion bodies. Soluble TCR protein was refolded and purified as described (15) and diluted to 0.106, 0.313, 0.625, 1.25, 2.5, 5, 10, 20, 40, and 80 μM in Hepes-buffered saline (Biacore) and injected at a flow rate of 10 μl/min over Research Grade CM5 Sensor chip surfaces onto which ∼1,000 response units of B8-nef or B8nef-Q5 mutant proteins had been immobilized. Both pMHC class I proteins were enzymatically biotinylated on the COOH terminus and size purified on a Superdex 200 before immobilization on streptavidin-coupled flow cells. Simultaneous data collection from single TCR injections for both pMHCs allowed for direct comparison of binding parameters.

Results

The HLA–B8-restricted Response to HIV-1 nef Is Dominated by Vβ13.2 Usage in Some Donors. Samples from members of a well-characterized cohort of long-term HIV-1–

Table I. *Characteristics of Long-Term Surviving Study Patients*

Donor	Sample date	First positive HIV test	HLA type	CD4 count	Viral load (RNA copies/ ml using βDNA)	Percent of FL8-specific cells using Vβ13.2
				cell/mm³		
005	7.25.00	1.11.86	A*0201, A*2402, B*0801, B*5701, Cw*0602, Cw*0701, DRB*0701, DRB*1301	728	38,219	51
065	1.10.02	1.01.85	A*0101, A*0201, B*0801, B*5701, Cw*0602, Cw*0701, DRB*08032, DRB*15011	2,782	18,817	93
200	5.13.02	1987	A2, A*2402, B*0801, B15, Cw7, Cw12, DRB*1301, DRB*0301	569	1,980	96
046	6.23.02	4.08.87	A*0101, A*0201, B*0801, B*5701, Cw*0602, Cw*0701, DRB*08032, DRB*15011	236	365,000	56

infected volunteers, who had been enrolled into a nested case-control study of the biological and behavioral correlates of nonprogression in HIV-1 infection (29) were studied using HLA–peptide tetramers for immunodominant HIV-1 CTL epitopes, selected on the basis of their HLA type and results from enzyme-linked immunospot analysis of their CD8^{+} T cell responses to HIV-1 antigens (7; and unpublished data). Tetramer-staining populations were co-stained with a panel of TCR Vβ antibodies to determine T cell receptor usage of the dominant responses. In four donors with HLA–B8 (005, 046, 065, and 200) the response to the FL8 nef epitope (amino acids 90–97, FLKEKGGL) was dominated by Vβ13.2 usage (Table I), accounting for between 51 and 96% of the FL8-specific population. All four donors have remained healthy in the absence of ART for between 13 and 17 yr, but donor 046 had recently progressed to a CD4^{+} T cell count of below 300 mm³.

Sequence Similarities in the Vβ13.2 T Cell Receptors Used by Different Individuals in the B8 nef Response. CTL clones were generated from all four donors and all but one expressed TCR Vβ13.2. Vβ usage in the non-Vβ13.2 population was generally heterogeneous, but in donor 046 was dominated by usage of Vβ6: a single Vβ6-expressing clone was generated from this donor. The T cell receptor sequences of these clones were determined (Table II) and clones expressing identical Vβ13.2 TCRs were found in samples taken at time-points between 4 and 6 mo apart in donors 005 and 200. The CDR3 was defined as starting at position 95 of Vβ and finishing at the phenylalanine of the

motif FGXG within Jβ, as described previously (30). There was a surprising degree of homology in both the length and sequence of the β-chain CDR3 region of CTL clones from these unrelated donors. A striking feature was the length of the CDR3 region, which unusually incorporated the entire Jβ segment and was either 15 or 16 amino acids in length. Analysis of the α-chain sequences revealed no similar conservation. It proved very difficult to generate clones that did not use Vβ13.2, but the single Vβ6-expressing clone generated from donor 046 showed a shorter CDR3 loop, in keeping with the sequences of other TCRs, which have a median CDR3 length of 9.7 residues (31).

To confirm that clones generated from our donors were representative of the tetramer-reactive population in peripheral blood, FL8 tetramer-staining cells were separated from donor 005 PBMCs using magnetic beads. mRNA was extracted from the tetramer-sorted cells and the TCR CDR3 region was sequenced: sequence analysis of 20 of 20 cloned products showed an identical CDR3 sequence to those expressed by the CTL clones generated from this donor.

Vβ13.2 Usage for B8-restricted CTL May Be Selected in Primary HIV-1 Infection But Is Not Seen in Rapid Progressors. The B8 nef FL8 response is an immunodominant response in both acute primary HIV-1 infection (21) and during chronic infection (7): however, not all donors making this response selected a Vβ13.2 TCR with a similarly long CDR3 region. We examined three donors with advanced HIV-1 disease progression: one was a rapid progressor from the King's College Hospital cohort and two were donors

Table II. *Sequence Analysis of the CDR3 Regions of the T Cell Receptor α- and β-Chains of FL8-specific CTL Clones Derived from Four HIV-infected Long-Term–surviving Donors*

Donor	CDR3	Jβ	Vα	CDR3	Jα
005	CAS SYLPGQGDHYSNQPQH	Jβ1.5	Vα26.1	CIV RAPGRADMR	Jα44
	"	"	"	"	"
	"	"	"	"	"
065	CAS SFEAGQGFFSNQPQH	Jβ1.5	ND		
200	CAS SFEPGQGFYSNQPQH	Jβ1.5	Vα23	CAV PSGAGSYQLT	Jα28
	"	"	"	"	"
046	CAS SYEPGQVSHYSNQPQH	Jβ1.5	Vα2.1	CAV KASGSRLT	Jα58
046 (Vβ14)	CAS SALASLNEQF	Jβ2.1	Vα14.1	CAY RPPGTYKYI	Jα40

from the Oxford Clinic who remained asymptomatic but had progressed to a CD4^{+} T cell count of 200/mm³. All three donors generated a substantial population of FL8-specific cells measured using the B8-FL8 tetramer but no Vβ13.2^{+} responding cells (Fig. 1). We then examined cells from patients who had been referred to the San Diego AIDS Treatment Center and were defined as having primary HIV-1 infection on the basis of a symptomatic viral illness after high risk sexual exposure to HIV with detectable plasma HIV RNA and nonreactive HIV antibody test or indeterminate Western blot assay. 5 out of 10 HLA–B8[+] donors with primary HIV-1 infection who had FL8 tetramer responses showed a variable degree of Vβ13.2 usage (between 6 and 100%). However, when tetramer-staining cells were sorted and the TCR sequence was determined,
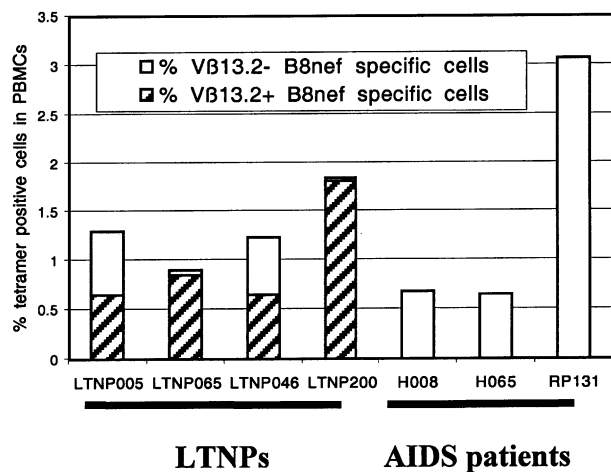
only one donor had selected a Vβ13.2 TCR with a similar CDR3 loop sequence to the four long-term survivors. Because most of these patients were given ART early in the course of HIV-1 infection it is not possible to correlate TCR usage with clinical outcome in this cohort: however, these data suggest that selection of a Vβ13.2 TCR with a homologous CDR3 region is an uncommon event.

HLA–B8-restricted CTL That Use Vβ13.2 Are Resistant to Apoptosis. There is increased susceptibility of antigen-specific CD8^{+} T cells to apoptosis in HIV-1 infection (32). We compared the extent of apoptosis of different T cell PBMC populations in overnight culture using the Tunel (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) assay that measures late apoptotic cells with DNA fragmentation (26). In general, HIV-specific CD8^{+} T cell populations were more vulnerable to apoptosis than CMV-specific cells, which showed very little evidence of cell death in this assay (Fig. 2 a). There was a marked difference in the degree of apoptosis in FL8-specific CD8^{+} T cells between cells expressing Vβ13.2 and those using other TCRs, with the non–Vβ13.2 population showing levels of cell death comparable to other HIV-specific CTL, although very little apoptosis was detected in the Vβ13.2 T cells. Using samples from donor 005, in whom the B8 nef-specific response contains both Vβ13.2 positive and negative cells, we found that the non–Vβ13.2 population (although representing almost 50% of the FL8-specific response in fresh blood) could no longer be detected when cryopreserved cells from the same time-point were thawed (Fig. 2 b). Similarly, when PBMCs from donor 005 were cultured overnight in R/10, only the Vβ13.2-expressing population could be detected the following day (unpublished data). This is consistent with the relative ease of generating Vβ13.2[+] FL8-specific CTL clones whereas we could only generate one clone using a non–Vβ13.2 TCR. Tetramer-staining T cell populations expressing the Vβ13.2 TCR showed significant up–regulation of the antiapoptotic molecule Bcl–2 (unpublished data). Taken together these data demonstrate a striking difference in susceptibility to apoptosis in CD8^{+} T cells specific for the same epitope but expressing different TCRs,

Figure 1. Vβ13.2 usage in the FL8-specific population predominates in HIV-infected donors with a good clinical outcome. Samples from donors with chronic HIV-1 infection who made an HLA–B8-restricted FL8-specific response were costained with the HLA–B8 FL8 tetramer and the TCR Vβ13.2 antibody. The bars show the proportion of CD8^{+} T cells staining with the B8–FL8 tetramer, divided into Vβ13.2 positive (shaded bars) and negative (unshaded) components. Four donors in the first part of the graph are untreated, asymptomatic long-term survivors, and the three donors on the left side of the figure are patients with progressive HIV-1 infection and a CD4^{+} T cell count <200 mm³.
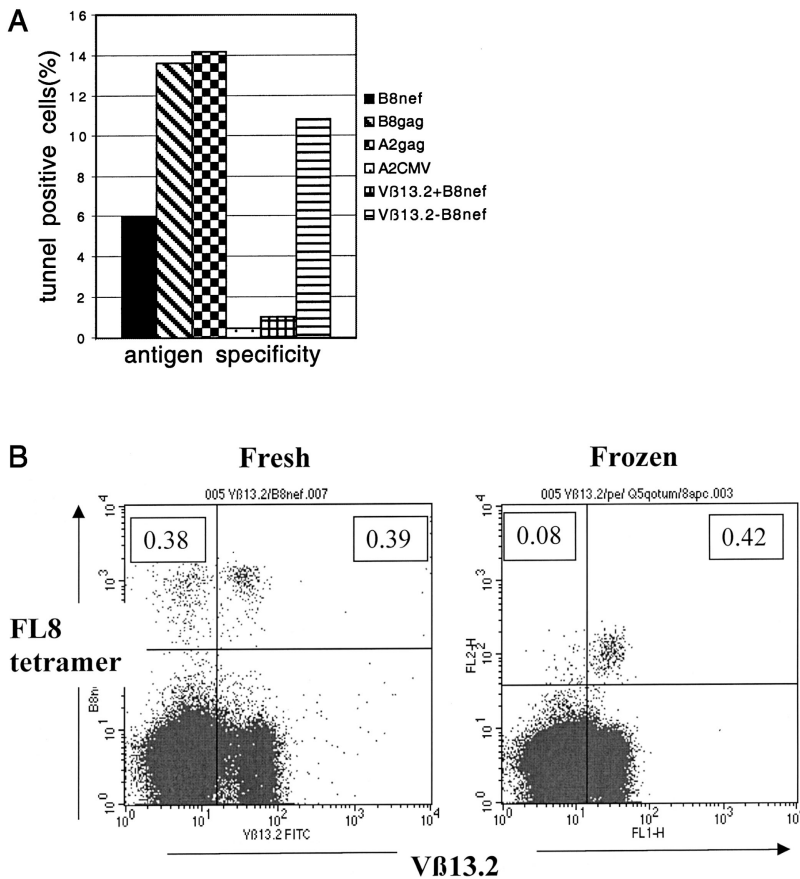
Figure 2. (A) Vβ13.2 usage by HIV-specific CD8⁺ T cells is associated with resistance to apoptosis. This figure shows the extent of Tunel positivity in different tetramer staining virus-specific populations as an indicator of early apoptosis in freshly isolated PBMC from donor 0005, which have been cultured overnight at 37°C. (B) In donors with mixed TCR usage for the FL8-specific response, the non-Vβ13.2 cells are lost on freeze–thawing. Two aliquots of PBMC taken at the same time from donor 005 are stained with the Vβ13.2 antibody (x axis) and the FL8–HLA–B8 tetramer (y axis). The left-hand panel shows staining from fresh cells whereas the right hand panel shows staining from a sample that had been cryopreserved and then thawed.

and suggests that Vβ13.2 usage is a marker for CTL that resist apoptosis and survive well under in vitro culture conditions.

Vβ13.2 Usage Permits Recognition of the Principal Escape Variant of the B8 nef Epitope. Several variants of the FL8 epitope are commonly found in the Los Alamos HIV sequence database with substitutions at the P5 anchor residue, from lysine to glutamine (Q5), threonine (T5), asparagine (N5), and methionine (M5). The Q5 sequence was reported to be amongst several epitope variants selected very early in primary HIV-1 infection in a donor initially infected with wild-type virus who made a dominant FL8 response and resulted in significantly reduced CTL recognition (21). We studied the ability of bulk T cell populations from donor 005 to recognize the Q5 escape variant. Freshly isolated Vβ13.2⁺ cells responded to both index and Q5 peptides in a FACS-based assay of IFN-γ secretion (Fig. 3 a). However, the non-Vβ13.2 population responded exclusively to the index epitope. This result was confirmed using an antibody against Vβ6, which stains all the remaining non-Vβ13.2 B8 nef tetramer–positive cells in donor 046. Only the non-Vβ6 population responded to the Q5 variant, whereas both Vβ6-positive and -negative populations secreted IFN-γ in response to the index peptide (unpublished data). We generated Vβ13.2-expressing CTL clones from all four donors and these were able to respond to variant peptides Q5, T5, N5, and M5: however, a single CTL clone from donor 046 that did not express the Vβ13.2 TCR recognized only the index variant (Fig. 3 b). Peptide titrations revealed significant differences between 046 clones with and without the Vβ13.2 TCR in their ability to recognize the most common Q5 variant (Fig. 3 c). Further studies using three additional non-Vβ13.2 FL8-specific CTL clones generated from some of the seroconverter donors showed recognition only of the index variant and not of peptides with substitutions (Q, T, and M) for lysine at position 5 (unpublished data).

To assess the stability of HLA–B8 in complex with the index and Q5 variants of the FL8 epitope we pulsed target cells with peptide, washed off excess peptide, and then assessed how long after pulsing the cells remained targets in conventional lysis assays. Although the loss of the Q5 variant from the target cells surface was more rapid than for the index peptide, the cells nevertheless retained the variant peptide for at least 4 h. The reduced stability of HLA–B8 with the Q5 variant is probably sufficient for it to escape recognition by low affinity CTL clones, but not so great as to prevent recognition by moderate and high affinity T cell receptors such as the Vβ13.2 TCR described here.

Taken together, these data show that the Vβ13.2 TCR has the ability to recognize the most common database variants that would otherwise escape the dominant FL8 CTL response. In the absence of a selective advantage for viral variants expressing mutations in the P5 position of the FL8
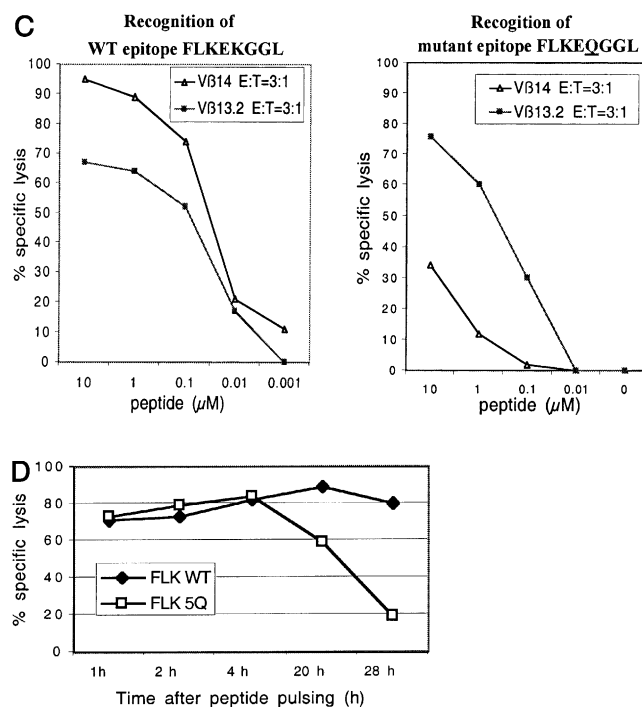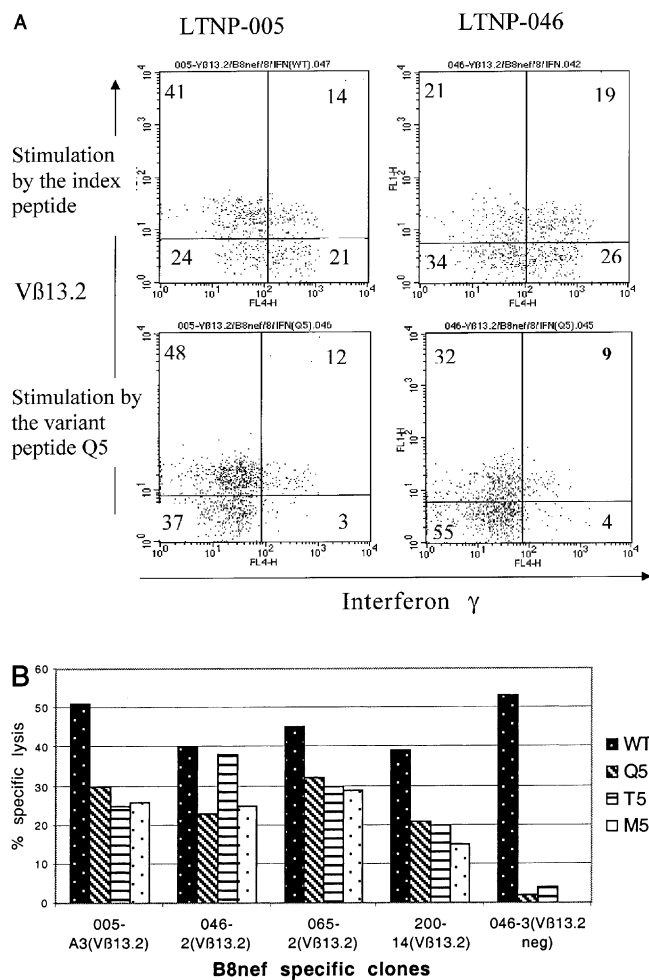
1552   Conserved HIV-specific T Cell Receptor

A

LTNP-005   LTNP-046

Stimulation by the index peptide

Vβ13.2

Stimulation by the variant peptide Q5

Interferon γ

B

C

Recognition of WT epitope FLKEKGGL

Recognition of mutant epitope FLKEQGGL

D

Figure 3. (A) Vβ13.2 usage is associated with cross–recognition of FL8 variants at P5 The FACS plot has been gated on FL8-specific CD8^{+} T cells from two donors (005 [left] and 046 [right]) with mixed populations of Vβ13.2 positive and negative FL8-specific cells, and shows intracytoplasmic staining for interferon γ (x axis) following 6 h of stimulation with the index FL8 peptide (top panels) and the Q5 variant peptide in relation to Vβ13.2 staining (y axis). The figures show the proportion of cells in each quadrant. (B) CTL clones expressing the Vβ13.2 TCR show broad cross-reactivity against FL8 peptide variants. A panel of FL8-specific CTL clones from long-term surviving donors 005, 046, 065, and 200 are tested for recognition of HLA–B8 matched [51]-chromium labeled target cells pulsed with either the index FL8 peptide or a panel of three variant peptides with naturally occurring substitutions at position 5 (Q5, T5, and M5) in a 4-h cytotoxicity assay. Four of the clones express Vβ13.2 and the fifth (046–3) does not. The clones are used at an effector to target ratio of 3:1 and the target cells have been pulsed with 0.25 μM peptide before use in the assay. (C) Peptide titrations show distinct patterns of recognition by CTL clones according to TCR usage CTL clones generated from donor 046, using either Vβ13.2 (square symbols) or a non-Vβ13.2 TCR (triangles), are tested for recognition of target cells pulsed with varying concentrations of either the index FL8 peptide or the Q5 variant. (D) Cells pulsed with the variant peptide make less stable targets for CTL lysis than do cells pulsed with the index peptide. Target cells expressing HLA–B8 were pulsed with either the index or the Q5 variant peptide for 1 h and then residual peptide was thoroughly washed off. The target cells were harvested at intervals as shown, labeled with chromium, and used in conventional CTL assays with an FL8-specific CTL clone.

epitope, we predicted that there would be limited sequence variation in this region in viral isolates from donors with the Vβ13.2 TCR. In keeping with this hypothesis, sequence analysis of proviral DNA in all four long-term surviving donors showed only the index sequence at the FL8 epitope.

The Vβ13.2 TCR Shows Moderately Strong Affinity for Both the Index and Q5 Variant of the FL8 Epitope. Recent biochemical and structural data for the immunodominant Vβ17 TCR (JM22) responding to an influenza matrix peptide presented by HLA–A2 have shown that this TCR has an affinity at the higher end of the range exhibited for TCR binding to peptide–HLA (15, 33). We used surface plasmon resonance analysis to determine the binding affinity of the Vβ13.2 TCR for HLA–B8 complexed with both the index FL8 peptide and the Q5 variant. Analysis of equilibrium-binding data by direct nonlinear curve fitting of the 1:1 Langmuir-binding equation and by Scatchard plots

indicated that the K_D values for the wild-type and Q5 mutant epitopes were 3.4 and 13.1 μM, respectively, at 25°C (Fig. 4). This demonstrates that the binding affinities of the Vβ13.2 TCR for both index and variant peptides are in the middle of the range of TCR-binding affinities reported for functional TCR–MHC class I recognition (34). Similar studies were undertaken using the Vβ6 TCR generated from donor 046, which showed a lower affinity for the index peptide (35 μM) and an affinity for HLA–B8 complexed with the Q5 variant peptide likely to be outside the range for T cell recognition (66 μM).

Discussion

We have identified oligoclonal populations of CD8^{+} T cells responding to an epitope in HIV-1 nef presented by HLA–B8 that are characterized by expression of a TCR
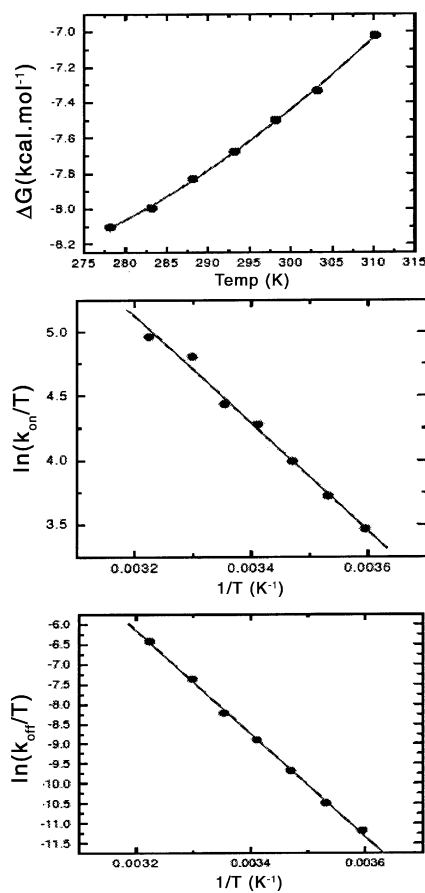
Figure 4. Surface plasmon resonance studies show that the Vβ13.2 TCR has a high affinity for both index and Q5 variant peptides. The binding affinity of the Vβ13.2 TCR for HLA–B8 complexed with the index FL8 peptide was determined by surface plasmon resonance at 25°C. Scatchard transformation of specific response units and analysis of the slope by linear regression indicated that the K_D value for the wild-type peptide was 5.5 μM.

β-chain consisting of Vβ13.2 and an unusually long CDR3 region in which the entire Jβ segment is represented. The sequence of the hypervariable CDR3 region shows a remarkable degree of conservation between unrelated donors. HIV-1 FL8-specific populations using this TCR are prominent in long-term survivors, but not in donors with progressive HIV-1 disease, which raises the possibility that these CTL have an enhanced ability to control HIV-1 replication in vivo. Two features of CTL expressing the Vβ13.2 TCR may contribute to better viral control. First, these T cells show a significant degree of resistance to apoptosis in vitro, making them more similar to CMV-specific CD8^{+} T cells than to other HIV-specific CTL populations that we have studied. If this property is also present in vivo then these cells may be particularly well equipped to kill HIV-infected cells without themselves succumbing to apoptosis (32). Second, cells expressing this TCR are able to respond equally well to both the index peptide and to commonly occurring potential escape variants at position 5 of the octamer epitope. This flexibility of recognition may be a function of the long β-chain CDR3 region

and could confer an advantage on the infected host by limiting the advantage of viral mutations in an immunodominant CTL epitope. This hypothesis is given weight by the absence of sequence variation in the FL8 epitope despite persistent and strong FL8 CTL responses in donors who have been infected with HIV-1 for between 13 and 17 yr.

The capacity of HIV to mutate in order to escape CTL recognition from the earliest stages of infection has been well described in both human studies (21, 35) and in the simian immunodeficiency virus (SIV)–infected macaque model (36). The ability of emerging viral variants to evade dominant CTL responses may pose a significant problem for vaccine strategies (37). Indeed the impact of HLA-associated selection pressure on viral evolution has recently been demonstrated at the population level (38), clearly demonstrating the potential of CTL selection to shape the emerging viral quasispecies. The immunodominant CTL response to the FL8 epitope has been detected shortly after seroconversion and was associated with the emergence of viral variants with coding changes in the epitope over the ensuing 6 mo (21). The majority of epitope variants had changes at position 5 of the epitope that were poorly recognized by the patient's own CTL. The findings of Price and colleagues suggest that although the FL8 epitope is highly immunogenic, the effectiveness of the response is limited by the ability of the virus to generate variants that escape the host CTL response. Our observation that only 1 out of 10 seroconverters who mounted an FL8 response selected a TCR with a homologous β chain to the Vβ13.2 TCR seen in the four long-term survivors suggests that only a minority of donors can mount a CTL response capable of recognizing FL8 epitope variants.

When epitope variation abrogates binding of the peptide to the HLA molecule, all T cell populations will be equally affected, but epitope variants that impact on the interaction between the TCR and the peptide-HLA complex may be tolerated by some TCRs and not by others. We have recently shown that CTL specific for an immunodominant gag epitope restricted by HLA–B57, an HLA molecule consistently shown to be associated with delayed HIV-1 disease progression, show a significant degree of tolerance to the common viral variants of this epitope (39), a phenomenon that may contribute to better viral control. The apparent association between expression of the Vβ13.2 TCR and delayed disease progression in this study could indicate that the capacity to generate a B8 nef response that includes this TCR facilitates viral control. Although P5 of the FL8 peptide is an anchor residue that mediates binding to the HLA–B8 molecule rather than being exposed for direct TCR recognition (40), it is likely that the substitution of lysine for other residues induces a conformational change at the surface of the peptide–HLA complex that could affect TCR recognition of the bound peptide. The structure of HLA–B8 complexed with another HIV-1 peptide, GGKKKYKL from gag p17, showed that substitutions at the P3 anchor position caused significant structural alterations at the surface of the peptide–HLA complex, whereas changes at P5 had more modest conformational impact

(40). Similarly, crystal structures of HLA B8 complexed to the index FL8 and Q5 variant peptides (unpublished data) again show that the peptide anchor side chain (lysine) at P5 points downward and is involved in stabilizing the peptide in the groove. The exposed peptide backbone conformation shows no significant differences between the index FL8 and Q5 variant, but glutamine acts less efficiently as an anchor residue, leading to increased mobility of the bound peptide. Our studies to determine the stability of the HLA–B8 complexed with either index or Q5 peptide by assessing how long peptide-pulsed cells remain CTL targets confirm reduced stability of the the complex with the variant peptide, but we propose that cells expressing the variant epitope could nevertheless be recognized by T cells expressing a sufficiently high affinity TCR.

The human T cell receptor α-and β-chains are derived through the selection and rearrangement of a large number of variable (V), diversity (D), and joining (J) gene segments: further complexity is conferred by the addition of N region nucleotide diversity in the junctional region. The hypervariable CDR3 region spans this junctional region and in a number of MHC–TCR crystal structures has been shown to interact directly with the peptide epitope held in the cleft of the MHC class I molecule. The length of the CDR3 region is relatively conserved in the human TCR repertoire with a mean of 9.7 residues (standard deviation 1.7) for both α and β chains (31), in contrast to the Vβ13.2 TCR, which has a CDR3 length of 16 amino acids. The CDR3 region of the β-chain predominantly contacts the COOH-terminal half of the peptide, so the advantage of this TCR may be the flexibility of the long CDR3 region, allowing it to tolerate viral mutations that alter the conformation of the epitope at position 5 of the peptide. The binding data for this TCR show that it has an affinity for HLA–B8 complexed with the index peptide in the middle of the range reported for other TCRs: thus it is not an unusually high affinity TCR but has similar properties to others studied to date. The affinity of this TCR for B8 and the Q5 peptide is lower, but still well within the range described for other TCRs. In contrast, the single non-Vβ13.2 TCR that we have been able to generate with specificity for the FL8 peptide shows a lower affinity for the B8 index peptide complex (despite the similar peptide titrations in conventional CTL assays) and an affinity for the mutant peptide that is almost certainly outside the range for T cell recognition. These data provide some explanation for the immunodominance of the Vβ13.2 TCR and the extent of cross-reactivity that it exhibits.

Why should expression of this TCR be associated with an enhanced ability to proliferate and a striking resistance to apoptosis compared with the majority of HIV-specific T cell populations? We noted that this functional phenotype was associated with the up-regulation of the antiapoptotic molecule Bcl-2. Although detailed crystallographic data of the HLA–B8 molecule complexed with the Vβ13.2 TCR are still awaited, it is plausible that the stability of the TCR–peptide–HLA complex formed by the Vβ13.2 TCR confers certain survival properties on the memory T cell population as a function of prolonged stimulation, as recently suggested by Gett and colleagues (41) .

The potential repertoire of different αβ T cells is thought to be between 10^{15} and 10^{20}, far exceeding the 10^{12} lymphocytes in the adult human. Random selection of a restricted T cell response in unrelated individuals is therefore highly unlikely and has not been described previously in HIV-1 infection. In primary HIV-1 infection, a narrow oligoclonal CD8^{+} response in acute infection is associated with more rapid disease progression, thought to be due to limited ability to respond to viral escape variants (18). Recent studies have highlighted the diversity of T cells that respond to the same epitope within and between individuals: analysis of TCR usage in the HLA–A2-restricted response to a dominant epitope in p17 gag suggested that the repertoire is selected for its ability to respond to emerging viral variants, with the response to a single epitope in one donor eliciting 15 distinct clonotypes (20). Other studies have shown oligoclonal TCR usage in tetramer-staining HIV-specific populations but no common features of TCR usage for HLA–A2 and B27-restricted HIV-specific responses between different individuals (19, 42). In chronic SIV infection, the immunodominant response to SIV gag restricted by Mamu-A*01 is oligoclonal with a preference for Vβ13 and conserved CDR3 usage (43): however, TCR usage can vary over time and a single dominant clone has not been identified (44).

Highly restricted TCR usage has been described in the human CTL responses to influenza A matrix in individuals with HLA–A2, which is dominated by Vβ17 and Vα10 usage (14), and in EBV infection, in which donors with HLA–B8 exhibit striking TCR conservation of both α and β chains in their response to an immunodominant epitope in EBNA 3A (16). Our findings in HIV-1 infection differ in several regards. First, selection for the Vβ13.2 TCR is confined to the β-chain with no restriction on α-chain usage: the advantage of the Vβ13.2 TCR may lie principally in its potential to recognize variants in the COOH-terminal part of the peptide. Second, highly conserved TCR usage is seen in most indivuals with HLA–A2 and CTL responses to influenza matrix, or HLA–B8 and CTL responses to EBV EBNA-3, whereas our studies in primary HIV-1 infection suggest that only a minority of infected people with a strong response to the FL8 epitope can select a Vβ13.2 TCR homologous to the TCRs from the four long-term survivors. Although the number of patients we have been able to study is relatively small, given the frequency of HLA–B8 (which is not enriched in LTNP cohorts), the four donors we identified with large populations of Vβ13.2 TCR B8 nef-specific CD8^{+} T cells have all shown significantly delayed disease progression, suggesting that the ability to select this TCR may be linked with enhanced viral control. Although further studies with larger numbers of patients are needed to provide support for this hypothesis, we propose that individuals who are able to mount an HLA–B8-restricted response that includes the Vβ13.2 TCR have a greater ability to control viral replication by limiting the advantage of potential CTL escape variants.

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