Cells were washed with fluorescence-activated cell sorter (FACS) buffer (PBS supplemented with 2% FCS and 0.02% NaN_3) and further stained with either fluorescein isothiocyanate (FITC)-labeled or Pacific Blue-labeled anti-CD8 T-cell antibodies (BD Pharmingen, San Jose, California, USA) or anti-TCR β-chain V gene 4-1 antibodies (TRBV4-1) (Beckman Coulter, Fullerton, California, USA) at 4°C for 30 min. Cells were then washed with FACS buffer and fixed by a 20-min incubation at reverse transcriptase in the dark in PBS containing 1% paraformaldehyde.

Flow cytometry was performed using a FACS Calibur (Beckton Dickinson, Franklin Lakes, New Jersey, USA) and FACS Aria (Beckton Dickinson). Flowjo ver. 6.4.7 (Tree Star, Ashland, Oregon, USA) was used for the analysis. For cell sorting, the cells were stained in R10 medium instead of FACS buffer, stained with propidium iodide to remove dead cells, and sorted without fixation using a FACS Aria.

T-cell receptor repertoire determination

Total RNA was extracted from sorted T cells using an RNeasy Micro Kit (Qiagen, Venlo, The Netherlands). We synthesized full-length cDNA by anchored RT-PCR using the super switching mechanism at 5'-end of the RNA transcript (SMART) PCR cDNA synthesis kit (TakaraBio, Otsu, Shiga, Japan), according to the manufacturer's protocol, with the switching mechanism at the 5′ end of RNA transcript. To amplify the variable, diversity, and joining regions of the TCR genes, the second PCR was done with the first primer and the reverse primers specific for the TCR α or β constant region: the 3′ T-cell receptor α chain constant region (TRAC) primer (5′-GGCAGACAGACTTGTCACTG GATTTAGAG-3′) or the 3′ T-cell receptor β chain constant region (TRBC) primer (5′-TGACCC CACTGTGCACCTC-3′), respectively. Reaction conditions in the second PCR were as follows: 94°C for 1 min; 25 extension cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min; and a final extension at 72°C for 7 min. Reaction products from the second PCR were purified with Wizard PCR preps DNA purification System (Promega, Madison, Wisconsin, USA) and subcloned into pGEM-T East vector (Promega).

DNA sequencing was performed using an ABI Prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, California) on a Perkin-Elmer ABI-377 sequencer. Designation of TCR genes follows the international immunogenetics (IMGT) nomenclature [19]. We defined the CDR3 region of the TCR β-chain as the region from aa 104 in T-cell receptor Vβ (TRBV) to aa 7 in TRBJ and the CDR3 region of the TCR α-chain as the region from aa 105 in TRAV to aa 11 in TRAJ.

Results

Nef138-10-specific response in HLA-A*2402+ patients

We analyzed PBMCs from seven A24-positive patients with chronic HIV-1 infection. All patients except one (S15) were naive to antiretroviral treatment. The median viral load was 6700 copies/ml (range, 120–24000 copies/ml), and the median CD4 T-cell count was 437 cells/μl (range, 278–807 cells/μl).

Using the plasma samples obtained closest to the following analyses, we confirmed that plasma viruses had stereotypically Nef138-10(2F) in all the patients analyzed (Fig. 1). Nef138-10-specific responses of $CD8^+$ T cells were analyzed by IFN-γ ELISPOT assay. Although the magnitude of specific response varied substantially among the samples, all showed a response to Nef138-10(wt) and Nef138-10(2F) (Fig. 1).

Tetramer dual-staining of Nef138-10-specific $CD8^+$ T cells

After 2-week culture in the presence of 10 nmol/l Nef138-10(wt) or Nef138-10(2F) peptides, PBMCs were stained with Nef138-10(wt)/A24-APC [Tet(wt)] or Nef138-10(2F)/A24-PE [Tet(2F)] or both. Using cultured cells from patient S15, we examined whether dual staining fractionated the Nef138-10-specific cells more precisely than single staining. Single stainings with Tet(wt) and Tet(2F) stained 9.8% (Fig. 2a) and 6.2% (Fig. 2b) of S15 $CD8^+$ T cells, respectively, after stimulation with Nef138-10(wt). In dual staining with the two tetramers, 7.2% of S15 $CD8^+$ T cells were in the Tet(wt)-positive/Tet(2F)-positive (dual-positive) fraction and 4.2% were in the Tet(wt)-positive/Tet(2F)-negative (wt-positive) fraction (Fig. 2c). Similarly, after Nef138-10(2F) stimulation, single staining of $CD8^+$ T cells from patient S15 with Tet(wt) and Tet(2F) stained 6.0 (Fig. 2d) and 6.1% of cells, respectively (Fig. 2e). With dual staining, 3.3% of S15 $CD8^+$ T cells stimulated with Nef138-10(2F) were dual-positive, and 2.2% were wt-positive.

These preliminary pilot experiments suggested that dual staining could fractionate dual-positive and wt-positive cells regardless of the peptide used for stimulation. Under the culture and staining conditions we used, the Tet(wt)-negative/Tet(2F)-positive (2F-positive) population was minimal. As we could fractionate dual-positive and wt-positive cells under these conditions, we inferred that the $CD8^+$ T cells in each fraction had different affinities for pMHC, that is, Tet(wt) or Tet(2F).

To examine the TCR repertoire in different fractions, we cultured PBMCs from seven patients and stained the cells with both Tet(wt) and Tet(2F) (Fig. 2g–m). In cells from patient O14, only Tet(wt)-positive cells were more than

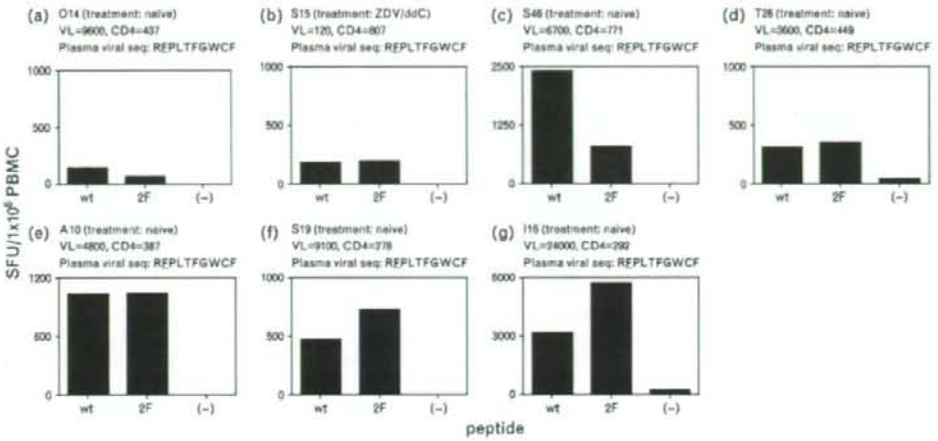

Fig. 1. Study patients and Nef138-10-specific responses. PBMCs from seven patients were stimulated with Nef138-10(wt) (RYPLTFGWCF), Nef138-10(2F) or mock. Spot forming units (SFUs)/1 × 10^6 PBMCs after stimulation are shown in subparts a–g. The viral load (VL) and CD4 T-cell count (CD4) of each patient are shown. All patients had HLA-A*2402 and their plasma viruses had Nef138-10(2F) mutation (RFPLTFGWCF).

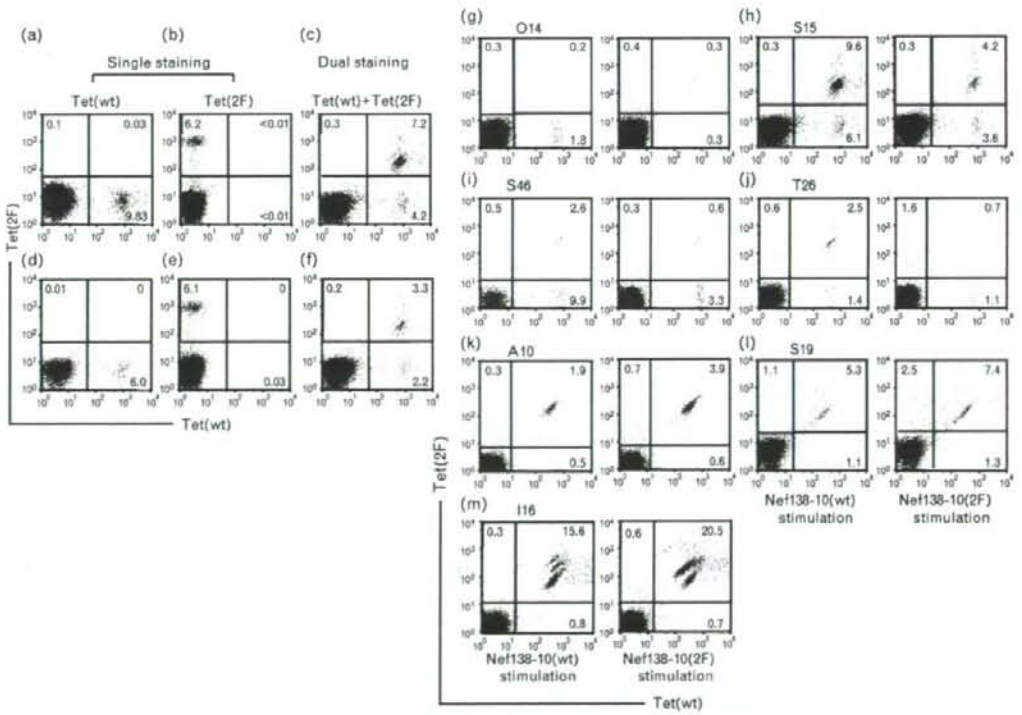

Fig. 2. Tetramer staining of peripheral blood mononuclear cells stimulated with Nef138-10(wt) or Nef138-10(2F). PBMCs from patient S15 were stimulated with Nef138-10(wt) (a, b, c) or Nef138-10(2F) peptides (d, e, f). Cells were stained with Nef138-10(wt)/A24-APC-tetramer [Tet(wt)] (a, d) or Nef138-10(2F)/A24-PE-tetramer [Tet(2F)] (b, e) or both (c, f) as described in 'Materials and Methods'. Cultured PBMCs from seven patients were stained with both Tet(wt) and Tet(2F) (g–m) including an S15 sample from a different culture (h). Dot plots are gated on $CD8^+$ T cells. Numbers refer to the percentages of gated cells in each quadrant.

1% of the population after Nef138-10(wt) stimulation (Fig. 2g). Nef138-10(2F) failed to induce specific $CD8^+$ T cells in cultures derived from patient O14. In cultures from patients S15, S46, and T26, both dual-positive and wt-positive $CD8^+$ T-cell populations were detected (Fig. 2h–j). Although Nef138-10-specific $CD8^+$ T cells were induced with both Nef138-10(wt) and Nef138-10(2F), the former induced higher expansion of cells derived from patients S15, S46, and T26. However, in cultures from patients in A10, S19, and I16, higher expansion of Nef138-10-specific $CD8^+$ T cells was induced with Nef138-10(2F) stimulation than with Nef138-10(wt) stimulation (Fig. 2k–m). In cultures derived from these three patients, the great majority of cells after stimulation were dual-positive.

Only in patient T26 was the 2F-positive population distinguished clearly from the dual-positive population, though the 2F-positive population showed lower fluorescent intensity (phycoerythrin) than did the dual-positive population (Fig. 2j). With a 1000-fold increase, from 10 nmol/l to 10 μmol/l, in concentration of Nef138-10(wt) and Nef138-10(2F) peptides used for stimulation, we observed expansion of 2F-positive $CD8^+$ T cells from other patients (data not shown).

T-cell receptor repertoire of wt-positive and dual-positive $CD8^+$ T-cell populations

Cell sorting showed substantial diversity among the study patients in the TCR repertoire of the wt-positive population of CD8 cells induced with Nef138-10(wt) (Fig. 3). The TCR repertoire also varied slightly according to the peptides used for stimulation. We observed 7.0 ± 2.2 different TCR β-chain clonotypes per individual after stimulation with Nef138-10(wt) peptides, compared with 6.3 ± 3.5 different clonotypes per individual after stimulation with Nef138-10(2F) peptides. Patients differed in whom β-chain V gene (TRBV) was most frequently used after Nef138-10(wt) stimulation. After Nef138-10(2F) stimulation, TRBV7-9 was the most common TCR β-chain clonotype seen in each of the three patients analyzed (patients S15, S46, and T26) (Fig. 4a).

In the dual-positive population, we observed only 3.3 ± 1.5 clonotypes per patient following Nef138-10(wt) stimulation and 1.8 ± 1.0 different clonotypes per patient following Nef138-10(2F) stimulation (Fig. 3). There were significantly fewer clonotypes in the dual-positive populations than in the wt-positive populations, regardless of stimulation conditions. Following Nef138-10(wt) stimulation, the mean number of clonotypes observed in the dual-positive population was 3.3 ± 1.6, compared with 7.0 ± 2.2 in the wt-positive population ($P = 0.01$, Mann–Whitney *U*-test). Following Nef138-10(2F) stimulation, the mean numbers of clonotypes in the dual-positive and wt-positive populations were 1.8 ± 1.0 and 6.5 ± 3.5, respectively ($P = 0.048$).

Notably, TRBV usage was highly restricted in the dual-positive population in all patients examined. TRBV4-1 was used in 84% of the analyzed clones, irrespective of stimulating peptides, and was the major TRBV in all clones except in those from patient I16 (Figs 3 and 4a). At the time of our analysis, the major TCR β-chain gene segments in patient I16 were TRBV15 and TRBV10-3; however, further studies using frozen PBMCs obtained from this patient 2 years earlier showed TRBV4-1/TRBJ2-7 to be the most frequently used clonotype (data not shown).

TCR β-chain joining gene (TRBJ) usage was more restricted in the dual-positive population compared with the wt-positive population, irrespective of the peptides used for stimulation (Fig. 3). Ninety-four percent of the dual-positive population in the analyzed clones used TRBJ2-7, whereas in the wt-positive population, TRBJ usage was more diverse (Fig. 4a). The CDR3 TCR β-chain region was also conserved in the dual-positive $CD8^+$ T-cell population. The length of the CDR3 region ranged from 12–16 amino acids in the dual-positive population, compared with 10–19 amino acids in the wt-positive population (Fig. 3). The CDR3 region had a length of 13 amino acids in 63% of the dual-positive population induced by Nef138-10(wt) stimulation and in 68% stimulated with Nef138-10(2F). More than 70% of the dual-positive clones using TRBV4-1/TRBJ2-7 conserved proline at the variable–diversity junction (fifth position of CDR3) and glycine and isoleucine at the diversity–joining junction. Surprisingly, the most frequent CDR3 amino acid sequence was identical in clones from three patients (S15, S46, and S19) (Fig. 4b). However, the nucleotide sequences were not identical, and distinctive clones were isolated from patients S15 and S46 (Fig. 4b), clearly indicating that the same TCR β-chain arose from different recombination events.

To examine the TCR α-chain diversity in a population with a highly restricted TCR β-chain repertoire, we analyzed TCR α-chain sequences in the dual-positive population after Nef138-10(2F) stimulation in PBMCs from patients S46, A10, and S19. In these three patients, the dual-positive population used TCR α-chain variable gene (TRAV) 8-3 and joining gene (TRAJ) 40 (Fig. 4c). The CDR3 region was highly conserved in length, ranging from 14 to 15 amino acids and clones using TRAV8-3/TRAJ40-conserved proline at the sixth position. Thus, the dual-positive $CD8^+$ T-cell population showed a highly restricted repertoire in both TCR α and β chains, including the CDR3 sequence.

T-cell receptor Vβ usage in the dual-positive $CD8^+$ T-cell population *in vivo*

The analyses described above showed that dual-positive $CD8^+$ T-cell populations expanded *in vitro* had a highly restricted TCR repertoire; that is, TRBV4-1/TRBJ2-7 and TRAV8-3/TRAJ40. We hypothesized that selection

wt-positive CD8+T cells

	Nef138-10(wt)-stimulation						Nef138-10(2F)-stimulation					
	TRBV	CDR3	TRBJ	Freq. (%)	No.Seq		TRBV	CDR3	TRBJ	Freq. (%)	No.Seq	
O14	27	CASS TGG EQF	2-1	(38)	104clones							
	11-2	CASS SDTGTGGLI NGYT	1-2	(35)								
	19	CASS IGAGTGT PTLY	1-3	(9)								
	5-5	CASS SEVL AEAF	1-1	(7)								
	6-1	CASS DIGRGV HEQY	2-7	(6)								
	6-1	CAS RIDGSSY NEQF	2-1	(2)								
	7-2	CASS LGGGGT DTQY	2-3	(1)								
	7-9	CASS LVEGTG WEQF	2-1	(1)								
	2	CA RRTSGRE TQYF	2-5	(1)								
S15	10-2	CASS ESIGGRDNN QPQH	1-5	(43)	23clones		7-9	CASS SYYRSG NTIY	1-3	(22)	27clones	
	28	CASSLSTGRGT YEQY	2-7	(26)			28	CASS LSTGRGT YEQY	2-7	(22)		
	7-9	CASS SYYRSG NTIY	1-3	(9)			10-2	CASS ESIGGRDNN QPQH	1-5	(19)		
	4-1	CASS QGWFAASV QPQH	1-5	(4)			6-5	CTSV PAVRSQRDRGQ ETQY	2-5	(7)		
	4-1	CASS PTAGI YEQY	2-7	(4)			5-4	CASS LTG ETQY	2-5	(7)		
	6-1	CASS DIGQGAI EQY	2-7	(4)			12-3	CASS LGQGE ETQY	2-5	(7)		
	30	CAW GNPGLNT GELF	2-2	(4)			10-1	CAKR AGGAT DTQY	2-3	(3)		
	11-2	CASS YDRS YEQY	2-7	(4)			6-1	CASS DIGQGA IEQY	2-7	(3)		
							7-9	CASS LDETGG YEQY	2-7	(3)		
							7-9	CASS ARAGTSGA GELF	2-2	(3)		
S46	5-5	CASS LEQFT DEQY	2-7	(32)	25clones		7-9	CASS LRDEVP ETQY	2-5	(58)	19clones	
	7-9	CASS LRDSVP ETQY	2-5	(24)			5-5	CASS LEQFTD EQY	2-7	(42)		
	7-9	CASS SWDTG ELF	2-2	(20)			25-1	CAS SASGQQP YEQY	2-7	(10)		
	6-1	CASS DAQTGTVLN YGYT	1-2	(8)								
	25-1	CAS SASGQQP YEQY	2-7	(8)								
	5-1	CASS LELST GELF	2-2	(4)								
	4-2	CASS PPGL NTQY	2-3	(4)								
T26	7-9	CASS LRDRVP STQY	2-5	(57)	30clones		7-9	CASS LRDRVP ETQY	2-5	(50)	30clones	
	6-1	CASS DPGQGD EAF	1-1	(23)			6-1	CASS DPGQGD EAF	1-1	(20)		
	21	CATW DMSSYN SPLH	1-6	(12)			6-1	CASS FRPGLAV TGELF	2-2	(10)		
	6-1	CASS FRPGLAV TGELF	2-2	(6)			7-8	CASS LIVQGW YEQY	2-7	(10)		
							21	CATW DMSSYN SPLH	1-6	(6)		
							27	CASS AGYN EQF	2-1	(3)		

Dual-positive CD8+T cells

	Nef138-10(wt)-stimulation						Nef138-10(2F)-stimulation					
	TRBV	CDR3	TRBJ	Freq. (%)	No.Seq		TRBV	CDR3	TRBJ	Freq. (%)	No.Seq	
S15	4-1	CASS GQ YEQY	2-7	(28)	29clones		4-1	CASS GQ YEQY	2-7	(53)	30clones	
	4-1	CASS QLA V YEQF	2-1	(28)			4-1	CASS QLA V YEQF	2-1	(40)		
	4-1	CASS RTSG S YEQY	2-7	(18)			4-1	CASS QLSG T YEQY	2-7	(7)		
	4-1	CASS TA YEQY	2-7	(14)								
	4-1	CASS TSG T YEQY	2-7	(10)								
	4-1	CASS QLSG T YEQY	2-7	(3)								
S46	4-1	CASS GQ YEQY	2-7	(92)	24clones		4-1	CASS GQ YEQY	2-7	(100)	26clones	
	5-1	CASS LELST GELF	2-2	(8)								
T26	4-1	CASS GT YEQY	2-7	(90)	31clones		4-1	CASS QTSG T YEQY	2-7	(100)	31clones	
	4-1	CASS QTSG T YEQY	2-7	(6)								
	20	CSAG RTS YEQY	2-7	(4)								
A10	4-1	CASS TQ YEQY	2-7	(98)	45clones		4-1	CASS TQ YEQY	2-7	(100)	50clones	
	24	CATS DPDREVE ETQY	2-5	(2)								
S19	4-1	CASS GQ YEQY	2-7	(90)	48clones		4-1	CASS GQ YEQY	2-7	(95)	55clones	
	5-4	CASS PGSNL YEQY	2-7	(6)			4-1	CASS NTT L YEQY	2-7	(5)		
	4-1	CASS NTT L YEQY	2-7	(4)								
I16	15	CATS RASG T YEQY	2-7	(68)	31clones		10-3	CAIS ESTGLAVF YEQY	2-7	(77)	30clones	
	10-3	CAIS ESTGLAVF YEQY	2-7	(20)			4-1	CASS QK YEQY	2-7	(16)		
	4-1	CASS QK YEQY	2-7	(10)			15	CATS RASG T YEQY	2-7	(7)		
	28	CASS LHKAGDG YGYT	1-2	(2)								

Fig. 3. T-cell receptor β-chain repertoire of Nef138-10/A24-tetramer wt-positive and dual-positive CD8$^+$ T-cell populations. CDR3 amino acid sequence, TRBV and TRBJ usage and relative frequency of Nef138-10-specific CD8$^+$ T cells stimulated with Nef138-10(wt) or Nef138-10(2F) are shown. Data from Tet(wt)+/Tet(2F)−(wt-positive) population are shown in the upper column, and data from Tet(wt)+/Tet(2F)+ (dual-positive) are shown in the lower column. Numbers in parentheses indicate frequency (%) of each clonotype. TCRs detected in both Nef138-10(wt) and Nef138-10(2F) stimulation in the same patient are highlighted with gray bars. The TCR clonotype conserved among patients is highlighted with yellow bars. The consensus amino acid sequences, occurring in more than 70% clones in TRBV4-1/TRBJ2-7, are indicated with red. The designation of TRBV and TRBJ follows Folch's nomenclature [19].

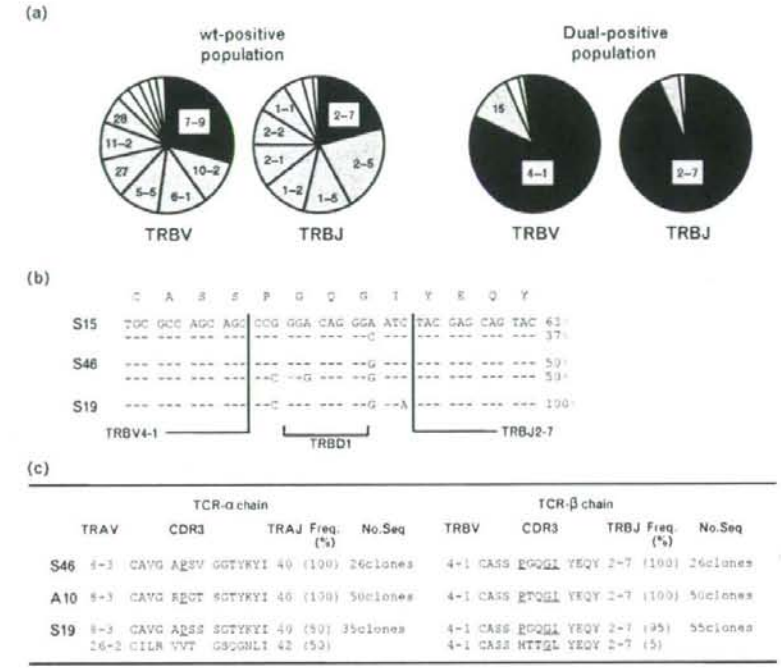

Fig. 4. Characteristics of T-cell receptor β-chain of Nef138-10/A24-tetramer wt-positive and dual-positive CD8$^+$ T-cell populations. Frequencies of TRBV and TRBJ gene usage in CD8$^+$ T cells stimulated with Nef138-10(wt) (Fig. 3) were calculated and shown in a graph. Tet(wt)+/Tet(2F)− (wt-positive)(left) and Tet(wt)+/Tet(2F)+ (dual-positive) (right) populations are shown. The most frequent genes are indicated by black shading, and genes found in more than 5% of the population are indicated by gray shading (a). Nucleotide sequence of CDR3-coding region (CASSPGQGIYEQY) in S15, S46, and S19 was aligned (b). CDR3 amino acid sequence, TRAV, TRAJ, TRBV, and TRBJ usage, and relative frequency of Tet(wt)+/Tet(2F)+ (dual-positive) population in S15, S46, and S19 are shown. Numbers in parenthesis indicate frequency (%) of each clonotype. The consensus amino acid sequences are underlined (c).

could be occurring during in-vitro stimulation by peptides. In order to examine whether the dual-positive population also used TRBV4-1 primarily *in vivo*, frozen PBMCs were thawed and stained with Tet(wt), Tet(2F), and anti-TRBV4-1 antibodies (Fig. 5). PBMCs from patients S15 and T26 had too few dual-positive cells to assess; however, in PBMCs from patients A10 and S19, more than 95% of the dual-positive CD8$^+$ T-cell population were TRBV4-1 positive.

Discussion

Error-prone reverse transcriptase creates the genetic diversity of HIV-1 and serves as an effective means for the virus to evade immune surveillance. Considering the plasticity and hypervariable nature of HIV-1, stereotypic amino acid substitution in the CTL epitope is an enigma that might provide clues to understanding the difficulties encountered in developing effective vaccines for HIV-1. One might predict that point mutations affecting the HIV-1 anchor residues would remove its ability to bind to an MHC heavy chain, resulting in a CTL epitope incapable of eliciting specific CTL response [4]. However, substituted CTL epitopes such as Nef138-10(2F) show the escape phenotype, yet bind to MHC molecules almost as efficiently as the wild-type epitope, with the interaction eliciting strong CD8$^+$ T-cell response [12,13,20].

In this study, we examined the TCR repertoire of CD8$^+$ T cells responding to wt [Nef138-10(wt)] and mutant [Nef138-10(2F)] epitopes. By tetramer dual-staining of A24-positive PBMCs stimulated *in vitro* from HIV-positive patients, we were able to examine two CD8-positive T-cell populations: wt-positive and dual-positive populations. In three patients, the CD8$^+$ T cells could be differentiated according to their relative affinity to Nef138-10(2F)/A24 tetramers (Figs 2 and 3); we designated as 'wt-positive' the population with lower affinity to Nef138-10(2F)/A24 tetramers. Regardless of

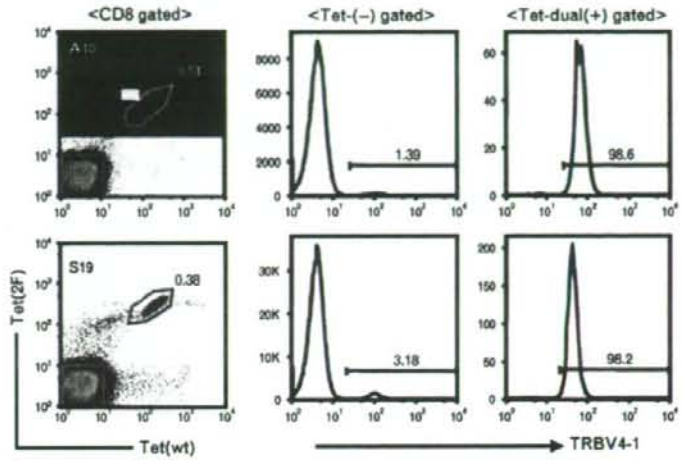

Fig. 5. TRBV4-1 expression in Nef138-10/A24-tetramer dual-positive population *in vivo*. Frozen PBMCs from two patients (A10, S19) were stained with Tet(wt) and Tet(2F), anti-CD8 (Pacific Blue), and anti-TRBV4-1(FITC). Tet(wt)+/Tet(2F)+ (dual-positive) and tetramer negative [Tet-(−)] populations are gated. Expression of TRBV4-1 in each population is shown in histogram. FITC, fluorescein isothiocyanate.

which peptide was used for stimulation, the TCR repertoire of the wt-positive population was similar in each patient.

An unexpected finding was that the dual-positive $CD8^+$ T-cell population grown *in vitro* had a highly restricted TCR repertoire compared with the wt-positive $CD8^+$ T-cell population, which showed substantial diversity. Furthermore, the dual-positive $CD8^+$ T-cell population *in vivo* also appeared to have a restricted CTL repertoire in which TRBV4-1 was the predominant clonotype.

Patient I16 appeared to be an exception; in the dual-positive $CD8^+$ T-cell population from this patient, TRBV15 and TRBV10-3 were the major TCR β-chain gene segments. At the time of the study, patient I16 had been followed as an HIV-infected patient in our clinic for at least 8 years, longer than any other study patient. Analysis using frozen PBMCs from patient I16 showed that TRBV4-1/TRBJ2-7 had been the most frequently used clonotype 2 years earlier (data not shown).

In this study, the 2F-positive population was distinguished clearly only in patient T26. Expansion of 2F-positive $CD8^+$ T cells was observed in PBMCs from some of the other patients only following stimulation with much higher peptide concentrations (data not shown). We speculate that 2F-positive $CD8^+$ T cells may require higher amounts of antigen for stimulation and proliferation.

One apparent limitation of our study is that we did not formally prove that the wt single-positive cells are not dual-specific for wt and unknown epitopes, including variant Nef138-10 other than Nef138-10(2F). Although sequencing results showed that the plasma viruses were all Nef138-10(2F) (Fig. 1), we could stimulate and expand $CD8^+$ T cells not only with Nef138-10(2F), but also with Nef138-10(wt) peptides. Our interest is not in the hidden additional specificity of the wt-positive population, but in their relative inability to bind the pMHC to which they must have been exposed *in vivo*. The dual-positive population with higher affinity to Nef138-10(2F)/A24 tetramers had a restricted TCR repertoire. Notably, the patients were viremic even in the presence of CTLs that had a higher affinity against the cognate 'mutant' CTL epitope, Nef138-10(2F)/A24, yet were still able to recognize the cognate 'wt' CTL epitope.

Our data suggest that mutation at the second residue (P2) in the HLA-A24-binding epitope influences T-cell recognition. P2 is the anchor residue of the HLA-A24-binding peptide and dips into the B pocket, forming a hydrogen bond with H70 in the HLA-A24 molecule [21]. A single amino acid substitution from Y to F at a secondary anchor position has been shown to modify the overall conformation of the HLA-A2-restricted HIV Gag-SL9 peptide [22]. However, our recent studies suggest that Y139F substitution in Nef138-10 does not cause a drastic change in pMHC structure (unpublished observation).

Dong *et al.* [23] reported the close correlation between Vβ13.2 usage and long-term nonprogression in four HLA-B8-positive patients. Vβ13.2-positive CTLs were widely reactive to possible escape mutants. In our study, the patients were randomly selected. The relationship between the restricted TCR repertoire and the patient's prognosis is not known, yet we do know that two of the

six treatment-naive patients had CD4 T-cell counts of less than 300 (data not shown). Thus, we infer that the six treatment-naive patients were not necessarily long-term nonprogressors.

The most plausible cause of the restricted TCR repertoire in our case may be lower expression of the Nef138-10(2F) epitope due to impaired or hyper-intracellular processing. We previously examined the killing activity of specific CTL clones against target cells expressing whole Nef protein with or without the 2F mutation (12). Specific killing activity directed against cells expressing Nef protein with the 2F mutation was lower than the activity against cells expressing the wt Nef protein, suggesting that hyper-processing or insufficient processing of Nef protein due to 2F mutation might result in a decrease in cell-surface expression of pMHC molecules. In this situation, CTL with higher avidity might be selected to cope with low antigen expression, resulting in a restricted TCR repertoire of the CTL population. However, the mechanisms enabling the maintenance of strong CTL responses against Nef138-10(2F) *in vitro* are not currently understood. Further studies are needed to show whether the introduction of the 2F mutation actually interferes with processing and leads to a decrease in pMHC molecules on the cell surface.

Previous studies have examined the kinetic association between the emergence of escape variants and the TCR repertoire in HIV-1/SIV infection [24–26]. In acute SIV infection, the emergence of escape viruses was associated with highly conserved TCR β-chain CDR3 motifs, but not with the presence of diverse clonotypic repertoires [24]. We observed viral escape in all patients in this study. We did not detect wild-type viruses even in patients without detectable wt-positive $CD8^+$ T cells. Dual-positive T cells may be enough to remove HIV-1 with the wild-type epitope. Molecular mimicry between wt-pMHC and mutant pMHC may elicit both wt-positive and dual-positive $CD8^+$ T-cell populations whether or not A24-positive patients are infected with wt-positive or mutant HIV-1. The more efficient removal of the wild-type viruses may result from better antigen presentation on the infected cells. Although further evidence is needed, we speculate that impairment of antigen presentation may give viruses with the Nef138-10(2F) mutation a selective advantage in A24-positive patients infected with HIV-1. Not only qualitative, but also quantitative analyses of epitope processing and presentation may shed light on the fundamental mechanism of immune evasion by HIV-1.

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E.M., A.K.-T., and J.-i.N. cloned and sequenced the TCRs. A.K.-T. also wrote the initial draft. M.T. did ELISPOT assays and established epitope-specific $CD8^+$ T-cell lines. T.O. and T.F. were responsible for patient care and contributed to the writing. Y.S. and G.F.G. are analyzing the structure of pMHC and TCRs described in this article and contributed to the discussion. A.I. is responsible for the entire study.

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Monoclonal Antibody and siRNAs for Topoisomerase I Suppress Telomerase Activity

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Telomerase, a ribonucleoprotein enzyme, is a cellular RNA-dependent DNA polymerase that serves to maintain the tandem arrays of telomeric repeats at the eukaryotic chromosomal ends. We previously reported that topoisomerase I dissociates HIV-1 reverse transcriptase from genomic RNAs, and binding of topoisomerase I to RNA template regulates cDNA synthesis. We also found that a monoclonal antibody (MAb) against topoisomerase I, designated as MAb 1, suppresses the reverse transcription efficiency using a detergent-disrupted HIV-1 virion. In this study, we describe how MAb 1 suppresses telomerase activity in cellular lysates. In addition, siRNAs of topoisomerase I has attenuated telomerase activity in culture cells. These results suggest that topoisomerase I is involved in telomerase activity, as well as HIV-1 reverse transcription.

Introduction

TELOMERES ARE SPECIALIZED STRUCTURES positioned at the ends of linear eukaryotic chromosomes, maintaining chromosome length and stability, and are elongated by telomerase.[1] The termini of telomeric DNA cannot be fully replicated by the conventional replication machinery. Telomerase consists of two essential molecular components, the telomerase RNA (TR) component, which includes a template for telomeric DNA, and telomerase reverse transcriptase (TERT), which mediates telomere synthesis.[2,3] It has been reported that adenosine triphosphate (ATP) enhances telomerase activity of *Saccharomyces cerevisiae*.[4]

We previously reported that topoisomerase I dissociates HIV-1 reverse transcriptase (RT) from genomic RNAs,[5] and that ATP regulates binding of topoisomerase I to RNA and enhanced cDNA synthesis of HIV-1 by dissociating from the topoisomerase I–RNA complex.[6,7] On the basis of these findings, we hypothesize that topoisomerase I regulates binding of TERT to TR and telomerase activity. We found that monoclonal antibodies for human topoisomerase I suppresses HIV-1 cDNA synthesis by an endogenous reverse transcription (ERT) assay using a detergent-disrupted HIV-1 virion.[8] Therefore, we investigated whether the anti-topoisomerase I monoclonal antibody and siRNA for topoisomerase I suppresses telomerase activity in cell lysates and culture cells, respectively.

Materials and Methods

Cell cultures

HeLa and 293T cells were cultured in DMEM medium (Gibco, Grand Island, NY), supplemented with 10% fatal calf serum, 4 mM glutamamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Sigma, St. Louis, MO).

Antibody and immunoblotting

The monoclonal antibody raised against purified topoisomerase I, MAb 1 has been described previously.[8] In order to obtain crude cellular extracts containing topoisomerase I, HeLa or 293T cells were washed, lysed by 1x sample loading buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), and sonicated. The lysed cellular extracts were heated for 10 min at 100°C, separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto Immobilon PVDF membranes (Milllipore, Billerica, MA). The membranes were blocked with phosphate-buffered saline (PBS) containing 2% skim milk and incubated with the MAb 1 (1 μg/mL) in PBS containing 0.2% Tween-20. After washing, the bound antibody was detected by goat anti-mouse IgG conjugated with horseradish peroxidase (1 : 5000; Amersham Pharmacia, Uppsala, Sweden) followed by chemiluminescent detection (ECL, GE Healthcare, Piscataway, NJ).

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Inhibition of topoisomerase I using siRNA

To inhibit topoisomerase I expression, we used two siRNAs, including topoisomerase I siRNA-80 and siRNA-117 which were purchased from Qiagen (Valencia, CA). The nucleotide sequences of topoisomerase I siRNA-80 were AAC ACA AAG AUC GAG AAC ACCtt and GGU GUU CUC GAU CUU UGU GUUtt. For topoisomerase I siRNA-117, the sequences were GAA GGA GAA GGA CCG GGA AAAtt and UUU UCC CGG UCC UUC UCC UUCtt. We also used control siRNA, miR16, of which sequences were AAA GUG CCU UAG CAG CAC GUAtt and UAC GUG CUG CUA AGG CAC UUUtt (lower case letters represent deoxyribonucleic acid). Two rounds of siRNA transfection into 293T cells were performed using lipofectamine 2000 (Invitrogen, Carlsbad, CA).

AU1 ▶

Telomere repeat amplification protocol assay

Telomerase activity was measured by a TRAPeze Telomerase Detection kit (Intergen, Purchase, NY) according to the manufacturer's protocol. The methodology is based on a modification of the method, described by Kim and Wu,[9] as telomeric repeat amplification protocol (TRAP). Briefly, 1.0×10^6 HeLa or 293T cells in logarithmic growth phase were lysed in 200 μL CHAPS, 3-[(3-cholamidopropyl)-dimethylamimonio]-1-propanesulfonate buffer (Intergen, Gaithersburg, MD). The lysates were centrifuged at 12,000 *g* at 4°C for 20 min. The supernatant was subjected to the TRAP assay. An aliquot of 1 μg of TS substrate primer(5'-AAT CCG TCG AGC AGA GTT-3') was end-labeled in 10 μL reaction mixture (10 μCi of [γ-32P] ATP [3000 Ci/mmol], 1 U of T4 polynucleotide kinase) and incubated for 10 min at 37°C and then 5 min at 95°C. TRAP reaction mixture (50 μL) is composed of 1x TRAP buffer (63 mM KCl, 20 mM Tris HCl [pH 8.3], 1.5 mM $MgCl_2$, 0.005% Tween-20, and 1 mM EGTA), 50 μM dNTPs, 0.1 μg TS (or 1 μL end-labeling reaction), 0.1 μg ACX return primer (5'-GCG CGG [CTTACC]$_3$ CTA ACC-3'), 0.1 μg NT internal control primer (5'-ATC GCT TCT CGG CCT TTT-3'), 0.01 amol TSNT internal control (5'-AAT CCG TCG AGC AGA GTT AAA AGG CCG AGA AGC GAT-3'), 2 U Taq DNA polymerase, and 2 μL CHAPS cell extract. After the TRAPeze reaction mixture was added, the reaction mixture was incubated at 30°C for 30 min to allow telomerase-mediated extension of a substrate oligonucleotide. The extended products were then amplified by 30 cycles of PCR (94°C for 30 s and 60°C for 30 s). The PCR products were electrophoresed on 15% non-denaturing polyacrylamide gels, which were exposed in a Bio-Imaging Analyzer (LAS 2000, Fujifilm, Tokyo, Japan) or stained with SYBR Green I (Molecular Probes, Eugene, OR) following exposure. Positive enzyme activity was

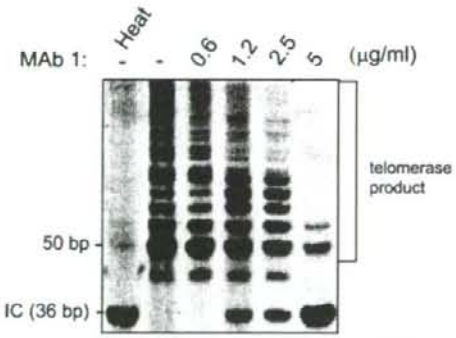

FIG. 1. Suppression of telomerase activity in cellular extracts from HeLa cells by anti-topoisomerase I monoclonal antibody, MAb 1. The mixtures (20 μL) containing MAb 1 (0[-], 0.6, 1.2, 2.5, or 5 μg/mL) and 10^3 fold-diluted cellular extracts from HeLa cells with CHAPS were incubated at 4°C for 20 min. After addition of dNTP substrates, the samples were further incubated at 37°C for 30 min. The samples containing heat-inactivated cellular extracts (heat) instead of the nitrated cellular extracts were also prepared. The products were then amplified by PCR and electrophoresed on 15% non-denaturing polyacrylamide gels. Products of telomerase activity starting from 50 bp are indicated as telomerase products. Some shorter products, which are resulted from weak primer-binding to telomere DNA, are visible. The position of internal control is indicated as IC (36 bp).

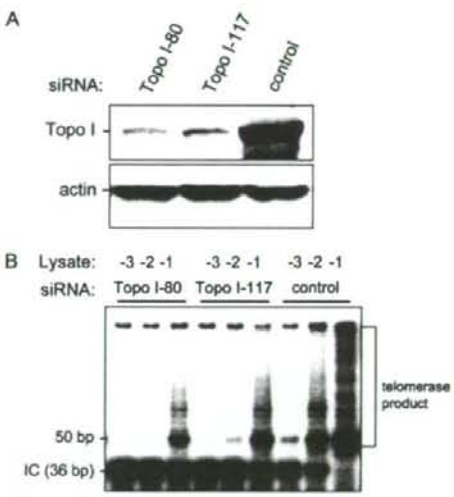

FIG. 2. Suppression of telomerase activity in 293T cells transfected with siRNA against topoisomerase I. (A) Immunoblotting analysis of cellular extracts from 293T cells transfected with siRNA against topoisomerase I (Topo I-80 or Topo I-117) or control siRNA, miR 16. The signal was detected by MAb 1 (Topo I) or anti-actin antibodies. (B) The TRAP assay using cellular lysates from 293T cells transfected with siRNA against topoisomerase I (Topo I-80 or Topo I-117) or control siRNA, miR 16. The cellular lysates were diluted with CHAPS buffer in 10, 10^2, or 10^3 fold (described as −1, −2, or −3) and were incubated at 37°C for 30 min after addition of dNTP substrates. The samples were amplified by PCR, electrophoresed on 15% non-denaturing polyacrylamide gels, and stained with Sybergreen. Products of telomerase activity, starting from 50 bp, are indicated as telomerase products. IC, position of internal control.

confirmed when a ladder of products with six-base increments starting at 50 nucleotides and a band representing the 36 bp internal control were observed. As a negative control, heat-inactivated cell extracts treated at 75°C for 10 min were used.

Results and Discussion

We initially examined the effect of a monoclonal antibody for topoisomerase I (MAb1)[8] on telomerase activity in cellular lysates prepared from HeLa cells. We could not observe any telomerase activity in the sample containing heat-inactivated cellular lysates (Fig. 1). In the absence of MAb 1, telomerase activity was clearly recognized and the internal control (IC), which is a semi-competitive internal control in that it shares only one of the primers (TS) used to amplify the telomerase products, was not detected. The telomerase activities were reduced in the presence of MAb 1 in a concentration-dependent manner, and reversibly products of internal control (IC) were increased (Fig. 1). Products of internal control (IC) were recognized in the presence of 1.2, 2.5, and 5 μg/mL of MAb 1, suggesting that MAb 1 did not interfere with PCR reaction (Fig. 1). The results suggested that MAb 1 reduced the telomerase activity in cellular lysates.

To examine the effects of topoisomerase I on telomerase activity, the teromerase acitivities in 293T cells that were transfected with siRNA-80, siRNA-117 against topoisomerase I, or control siRNA were examined. Both siRNAs for topoinosmerase I markedly decreased the expression level of topoisomerase I (Fig. 2A). Expression of actin was not altered in siRNA-treated cells (Fig. 2A). Cellular lysates from the siRNA-transfected 293T cells were diluted in 10, 10^2, or 10^3 fold, and the telomerase activity of the samples was examined by a TRAP assay (Fig. 2B). The telomerase activities of the samples containing 10^2 and 10^3 fold-diluted cell lysates transfected with siRNA-80 or siRNA-117 were clearly (>10-fold) suppressed compared to those containing cellular lysates transfected with control siRNA (Fig. 2B). We also observed that the internal control products were decreased in a dose-dependent manner by addition of cellular lysates (Fig. 2B). Because the internal control utilized in the assay, TSNT is amplified by primer TS and its own dedicated return primer, this TRAP internal control is a semi-competitive internal control.[9] These results suggested that knockdown of topoisomerase I by siRNA results in decrease of telomerase activity in 293T cells.

In the present study, we have demonstrated that anti-topoisomerase I monoclonal antibody, MAb 1, suppresses telomerase activity in cellular lysates from HeLa cells. We have also shown that siRNAs for topoisomerase I reduces telomerase activity in cultured 293T cells.

We previously found that topoisomerase I regulates binding of HIV-1 RT to a stem-loop RNA[5] and can regulate HIV-1 reverse transcription.[6,7] Although the mechanism by which topoisomerase I regulates telomerase activity is unclear, it seems likely that topoisomerase I might regulate dissociation of TERT from TR. The processive cycle of telomerase includes binding of TERT to TR, elongation of telomere, and translocation of TERT.[10] Further analysis will be necessary to clarify how the telomerase activity is regulated.

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