A single CTL clone can recognize a naturally processed HIV-1 epitope presented by two different HLA class I molecules

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Although it is known that a single peptide can be recognized by CTL restricted to two MHC class I alleles, there is no direct evidence for presentation of a single peptide by two MHC class I molecules. Furthermore, it is unclear whether such peptides are presented to the same T cell or to different T cells. Our previous study suggested that CTL recognition of the human immunodeficiency virus-1 (HIV-1) Pol HIV-B35-SF2-24 epitope (IPLTEEAEL) occurs via both HLA-B35 and HLA-B51 restriction. Here we provide the first direct evidence that a single CTL clone can recognize this peptide presented by both HLA-B35 and HLA-B51. Furthermore, we directly purified this peptide eluted from both HLA-B*3501 and HLA-B*5101 molecules isolated from target cells infected with HIV-1 recombinant vaccinia virus. These results demonstrate that HIV-B35-SF2-24 is a naturally processed peptide which is presented by both HLA-B*3501 and HLA-B*5101. TCR analysis of one CTL clone suggested that it is a single clone. B*3501-SF2-24-tetrameric complexes inhibited both HLA-B*3501- and HLA-B*5101-restricted recognition of this clone, suggesting that the TCR of this clone cross-recognize the structure of both HLA class I-peptide complexes.

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1 Introduction

Members of the HLA class I group bind peptides carrying similar anchor residues. For example, members of the HLA-A3 super type (HLA-A3, -A11, -A31, -A33 and -A68) bind peptides with Ala, Val, Ile, Leu, Met, Ser or Thr at position 2 (P2) and Arg or Lys at the C terminus [1]. This suggests that one peptide could bind to different HLA class I molecules and may be recognized by CTL. Indeed, it is well known that a single peptide is recognized by CTL restricted to different HLA class I molecules. CTL recognition of a hepatitis B virus core peptide (STLPETTVVRR) was restricted by both HLA-A31 and HLA-A68 [2]. The peptide KHPDATYSR, derived from hepatitis C virus, was recognized by Patr-A04- and Papa-A06-restricted chimpanzee CTL [3]. IVLPEKDSW, derived from HIV-1 RT protein, is a candidate CTL epitope restricted by both HLA-B57 and HLA-B58 [4]. LLGPGRPYR, derived from tyrosinase-related protein, was recognized by CTL with both HLA-A31 and HLA-A33 restriction [5]. In addition to CTL recognition of the same epitope restricted by different HLA class I molecules, mouse CTL can recognize the same peptide presented by different MHC molecules [6]. However, these studies did not show direct evidence that a single peptide bound to different MHC class I molecules is presented to CTL as a naturally processed peptide. Furthermore, it is not known whether a single peptide presented by different MHC class I molecules is recognized by a single CTL clone.

We previously showed that a bulk PBMC culture from an HIV-1-infected individual with both HLA-B35 and HLA-B51 stimulated with the HIV-1 Pol HIV-B35-SF2-24 epitope (IPLTEEAEL) killed both C1R-B*3501 and C1R-B*5101 cells infected with HIV-1 recombinant vaccinia virus [7]. This observation suggests that this epitope is presented by both HLA-B*3501 and HLA-B*5101. In the present study, we examined CTL recognition of this epi-

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Abbreviations: nano-LC/ESI IT MS: Nano-liquid chromatography/electrospray ionization ion trap mass spectrometry **CID MS:** Collisional-induced dissociation mass spectroscopy

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tope presented by both HLA class I molecules using specific CTL clones and attempted to directly identify this epitope in HLA class I peptide complexes isolated from cells infected with HIV-1 recombinant vaccinia virus. One CTL clone was further analyzed to clarify whether this epitope is presented to a single or different CTL clones.

2 Results

2.1 HLA-B*3501 and HLA-B*5101 cross-restricted CTL recognition of the HIV-B35-SF2-24 epitope

The CTL clones HIV-B35-SF2-24-562 and HIV-B35-SF2-24-589 were previously established as HLA-B*3501-restricted, HIV-B35-SF2-24-specific CTL clones [8]. We tested cross-recognition of these CTL clones for HIV-B35-SF2-24 presented by HLA-B*5101. These CTL clones killed both C1R-B*3501 cells and C1R-B*5101 cells pulsed with HIV-B35-SF2-24 peptide (Fig. 1A). The cytolytic activity of the CTL clones for peptide-pulsed C1R-B*3501 cells was much higher than that for peptide-pulsed C1R-B*5101 cells. The difference in cytotoxicity may reflect differing affinity of the peptide for the two HLA class I molecules: indeed, this peptide effectively binds to HLA-B*3501 [7] while its binding to HLA-B*5101 is very weak (data not shown). The CTL clones weakly killed peptide-pulsed C1R cells. This can be explained by the fact that C1R cells express HLA-B*3503 at very low level [9].

Since specific lysis of peptide-pulsed C1R-B*5101 cells by the CTL clones was not high, we attempted to confirm HIV-B35-SF2-24-specific, HLA-B*5101-restricted recognition of these CTL clones using the cell lines LKT2 and T1 which express HLA-B*5101 but not HLA-B*3501, as target cells. The CTL clones effectively killed both target cell lines pulsed with HIV-B35-SF2-24 peptide (Fig. 1B). Moreover, the cytolytic activity of the CTL clones was reduced following incubation with HLA-Bw4-specific mAb TÜ48 but not following the incubation with the HLA-Bw6-specific mAb SFR8-B6 (Fig. 1B). These results confirmed that the CTL clones recognize HIV-B35-SF2-24 peptide presented by HLA-B*5101 molecules.

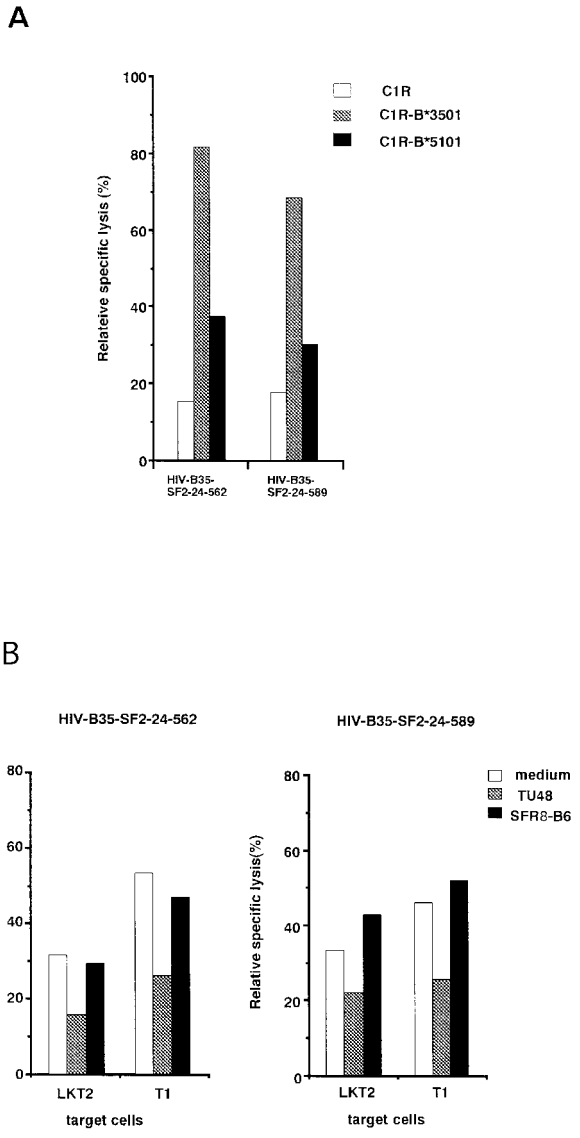

Fig. 1. HIV-B*3501 and HLA-B*5101 cross-restricted CTL recognition of the HIV-B35-SF2-24 epitope. (A) Recognition of HIV-B35-SF2-24-specific CTL clones for C1R-B*3501 and C1R-B*5101 cells pulsed with HIV-B35-SF2-24. Target cells prepulsed with HIV-B35-SF-24 peptide (1 μM) were mixed with the HIV-B35-SF2-24-562 and HIV-B35-SF2-24-589 CTL clones at an E:T ratio of 2:1. (B) Inhibition of HLA-B51-restricted CTL recognition by anti-Bw4 mAb. HLA-B51-positive T1 or LKT2 cells pulsed with HIV-B35-SF2-24 peptide (1 μM) were incubated with TÜ48 anti-Bw4 mAb or SFR8-B6 anti-Bw6 mAb (fivefold concentrated hybridoma culture soup) and then the cytotoxicity of the HIV-B35-SF2-24-562 and HIV-B35-SF2-24-589 CTL clones for these target cells was tested at an E:T ratio of 2:1.

2.2 Recognition of naturally processed HIV-B35-SF2-24 epitope by HIV-B35-SF2-24-specific CTL clones

Our previous study demonstrated that the HIV-B35-SF2-24-562 and HIV-B35-SF2-24-589 CTL clones killed C1R-B*3501 cells infected with HIV-1 recombinant vaccinia virus, suggesting that HIV-B35-SF2-24 is a naturally processed epitope presented by HLA-B*3501 [8]. Since the cytolytic activity of these CTL clones for

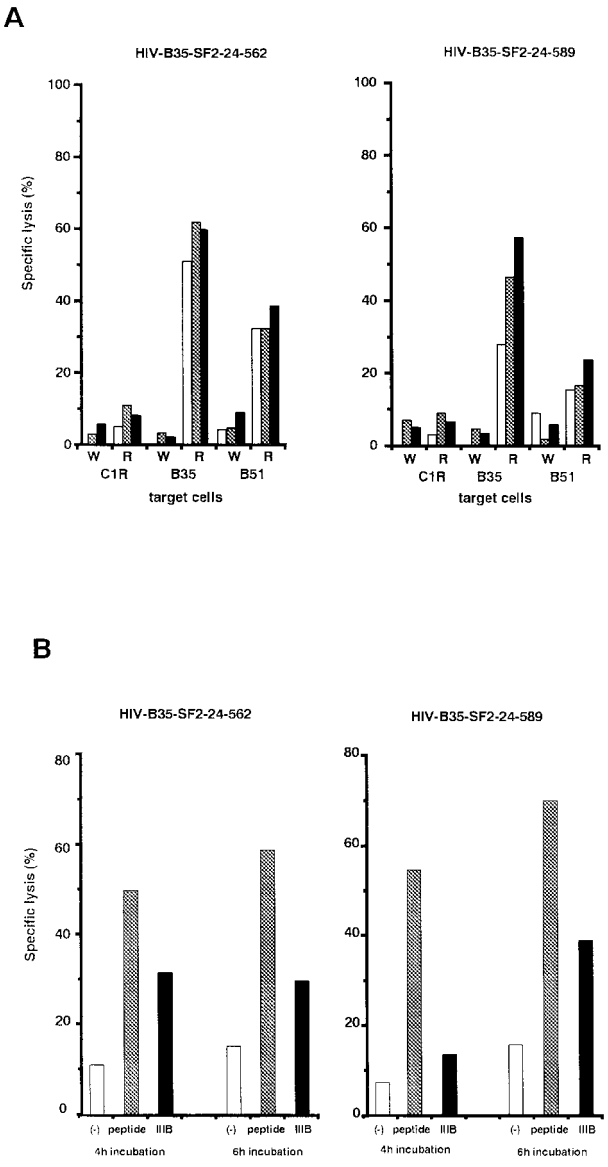

Fig. 2. HLA-B*3501 and HLA-B*5101 cross-restricted CTL recognition of naturally processed HIV-B35-SF2-24 epitope. (A) HIV-B35-SF2-24-specific CTL clones recognize the epitope presented on C1R-B*3501 and C1R-B*5101 cells infected with HIV-1 recombinant vaccinia virus. Cytotoxicity of the HIV-B35-SF2-24-562 CTL clone for C1R-B*3501 (B35), C1R-B*5101 (B51) or C1R cells (C1R) infected with HIV-1 recombinant vaccinia virus (R) and wild-type vaccinia virus (W) was tested at E:T ratios of 8: 1 (■), 4:1 (⊠) and 2:1 (□). (B) Killing of HIV-1-infected HLA-B51-positive cells by HIV-B35-SF2-24-specific CTL clones. Killing of T1 cells infected with HIV-1 IIIB and those pulsed with the HIV-B35-SF2-24 peptide by HIV-B35-SF2-24-562 and HIV-B35-SF2-24-589 CTL clones was tested at E:T ratios of 4:1 (HIV-B35-SF2-24-562) and 8:1 (HIV-B35-SF2-24-589). ^{51}Cr release was measured 4 and 6 h after effector cells were added to wells containing labeled target cells.

peptide-pulsed C1R-B*5101 cells was much weaker than that for peptide-pulsed C1R-B*3501 cells, it could be expected that the CTL clones would fail to lyse C1R-B*5101 cells infected with HIV-1 recombinant vaccinia virus. The CTL clones killed both C1R-B*5101 cells and C1R-B*3501 cells infected with HIV-1 recombinant vaccinia virus (Fig. 2A). These results suggest that HIV-B35-SF2-24 is a naturally processed epitope presented by both HLA-B*3501 and HLA-B*5101.

It is not known whether CTL recognition of the HIV-B35-SF2-24 epitope presented by HLA-B*5101 contributes to effective killing of HIV-1-infected cells. We examined the specific killing of T1 cells infected with HIV-1 IIIB by the HIV-B35-SF2-24-562 and HIV-B35-SF2-24-589 CTL clones. T1 cells were infected with HIV-1 and then cultured for 5 days before use as target cells for the ^{51}Cr-release assay. The CTL clones effectively killed T1 cells infected with HIV-1 IIIB (Fig. 2B). These results strongly suggest that these CTL clones can kill target cells infected with HIV-1 in HLA-B*5101-restricted manner.

2.3 Isolation of naturally processed HIV-B35-SF2-24 epitope presented by HLA-B*3501 and HLA-B*5101

To clarify whether HIV-B35-SF2-24 is a naturally processed epitope presented by both HLA-B*3501 and HLA-B*5101 molecules, we attempted to isolate this epitope from C1R-B*3501 and C1R-B*5101 cells infected with HIV-1 recombinant vaccinia virus. HLA class I molecules were purified from HIV-1 recombinant vaccinia virus-infected C1R-B*3501, C1R-B*5101 and C1R cells using an anti-HLA class I mAb W6/32 binding affinity column. Binding peptides were eluted with trifluoroacetic acid (TFA) and the eluted material was separated by reverse-phase HPLC. Synthesized peptide was detected in fraction 34. C1R-B*3501 target cells were, therefore, pulsed with an 80-fold dilution of HPLC fractions 31–37 from HIV-1 recombinant vaccinia virus-infected C1R-B*3501, C1R-B*5101 and C1R cells and then the cytolytic activity of the HIV-B35-SF2-24-562 CTL clone for the target cells was measured. Specific cytolytic activity was detected when target cells were pulsed with fraction 34 derived from C1R-B*3501 and C1R-B*5101 cells infected with HIV-1 recombinant vaccinia virus but was not observed in any fraction derived from C1R cells infected with HIV-1 recombinant vaccinia virus (Fig. 3). These results strongly suggest that HIV-B35-SF2-24 is a naturally processed peptide and is presented by both HLA-B*3501 and HLA-B*5101.

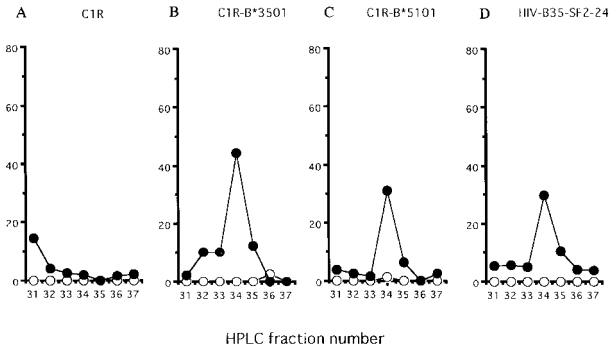

Fig. 3. Isolation of HIV-B35-SF2-24 from C1R, C1R-B*3501, and C1R-B*5101 cells infected with HIV-1-recombinant vaccinia virus. Cytotoxicity of the HIV-B35-SF2-24-562 CTL clone for C1R B*3501 cells pulsed with each HPLC fraction (●) or C1R-B*3501 cells alone (○) was tested. C1R-B*3501 cells were pulsed with 80-fold dilutions of HPLC fractions derived from C1R (A), C1R-B*3501 (B) and C1R-B*5101 (C) cells infected with HIV-1 recombinant vaccinia virus and then cytotoxicity of the HIV-B35-SF2-24-562 CTL clone for these cells was tested at an E:T ratio of 1:1. Cytotoxicity of this CTL clone for cells pulsed with a 2,500-fold dilution of an HPLC fraction containing synthesized peptide (D) was used as control.

2.4 Direct identification of the HIV-B35-SF2-24 epitope peptide in an HPLC fraction derived from C1R-B*3501 and C1R-B*5101 cells infected with HIV-1 recombinant vaccinia virus

To exclude the possibility that HPLC fraction 34 from C1R-B*3501 and C1R-B*5101 cells contained an HIV-1 epitope peptide of different length, we attempted to directly identify the epitope peptide in this fraction. Each HLA-B*3501 and HLA-B*5101 HPLC fraction was analyzed using nano-liquid chromatography/electrospray ionization ion trap mass spectrometry (nano-LC/ESI IT MS) (Fig. 4). Protonated molecular ions (m/z 1014) of the peptides in fraction 34 from C1R-B*3501 and C1R-B*5101 cells were the same as those of HIV-B35-SF2-24. The protonated molecular ions of the purified peptides were further analyzed by collisional-induced dissociation (CID) mass spectrometry to determine the amino acid sequence of the peptides. Five C-terminal amino acid residues (EEAEL) of peptides eluted from both C1R-B*3501 and C1R-B*5101 were identical to those of HIV-B35-SF2-24. These results together confirm that the naturally processed HIV-B35-SF2-24 epitope is presented by both HLA-B*5101 and HLA-B*3501.

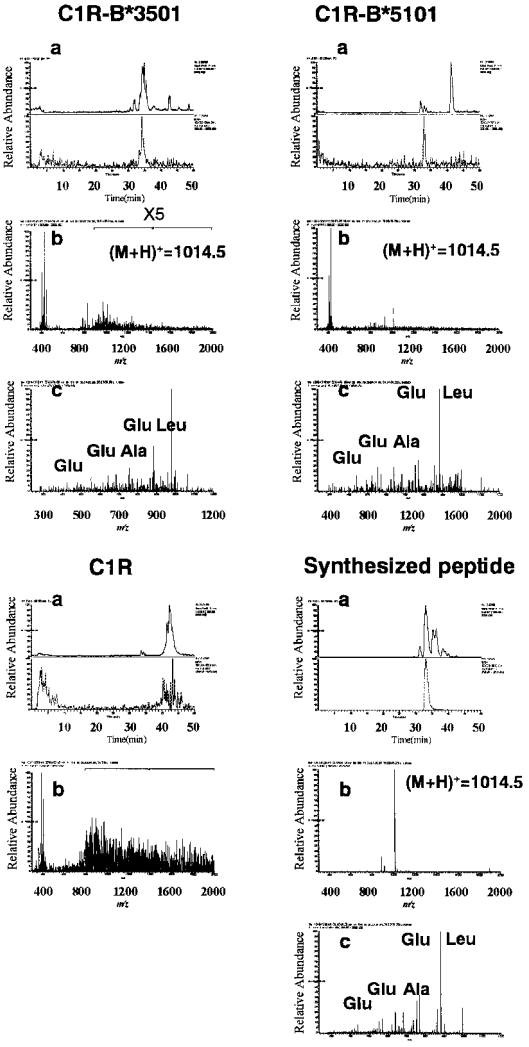

Fig. 4. Nano-LC/MS/MS analysis of peptides in HPLC fraction 34. (a) Base peak mass chromatograms (upper) and mass chromatograms of m/z 1014.5 for the HIV-SF2-24 peptide (lower) in HPLC fraction 34. In the case of C1R-B*3501 and C1R-B*5101, the peak of m/z 1014.5 was observed at 33 min which was the same as the mass chromatogram for synthesized peptide. (b) Mass spectrum of the peak at 33 min. In all nano-LC/MS analyses, a $(M+H)^+$ ion (m/z 1014.5) for the HIV-B35-SF2-24 peptide was observed at 33 min except for the sample from C1R cells. (c) Product ion spectrum of the $(M+H)^+$ ion (m/z 1014.5) of the peak at 33 min. The product ion spectra of peptides isolated from C1R-B*3501 and C1R-B*5101 was the same as the spectra of the synthesized peptide.

2.5 TCR analysis of HIV-B35-SF2-24-589 CTL clone

A single epitope presented by different HLA class I molecules may be presented to different CTL populations. That is, the CTL clones used in the present study may contain two subpopulations: one specific for the epitope presented by HLA-B*3501 and the other for that presented by HLA-B*5101. To exclude this possibility, we analyzed the TCR of the HIV-B35-SF2-24-589 CTL clone. The HIV-B35-SF2-24-562 CTL clone could not be analyzed because we could not obtain enough cells. The TCR gene of the HIV-B35-SF2-24-589 CTL clone was initially investigated. cDNA for both the TCR α and β chains was amplified by PCR using synthetic anchor primers and Cα-specific or Cβ-specific primers [10], then cloned and sequenced. Of nine Vα clones, six were identical to Vα10.1a-Jα7 while three were identical to Vα12.1a-JαK (Fig. 5). All 14 Vβ clones sequenced were identical to Vβ2.1a-Jβ2.1 (Fig. 5). There are two possible explanations for these results. One is that the HIV-B35-SF2-24-589 clone has two α chains, and the second is that this clone contains two subpopulations, each with different α chain. To clarify this, we examined surface expression of the Vα chain using the anti-Vα12 mAb. Flow cytometry showed that almost 100% of HIV-B35-SF2-24-589 cells stained with the anti-Vα12 mAb (Fig. 6A). An mAb against Vα10 is not available. However, these results demonstrate that HIV-B35-SF2-24-589 is a single clone regardless of whether or not Vα10 is expressed on the cell surface.

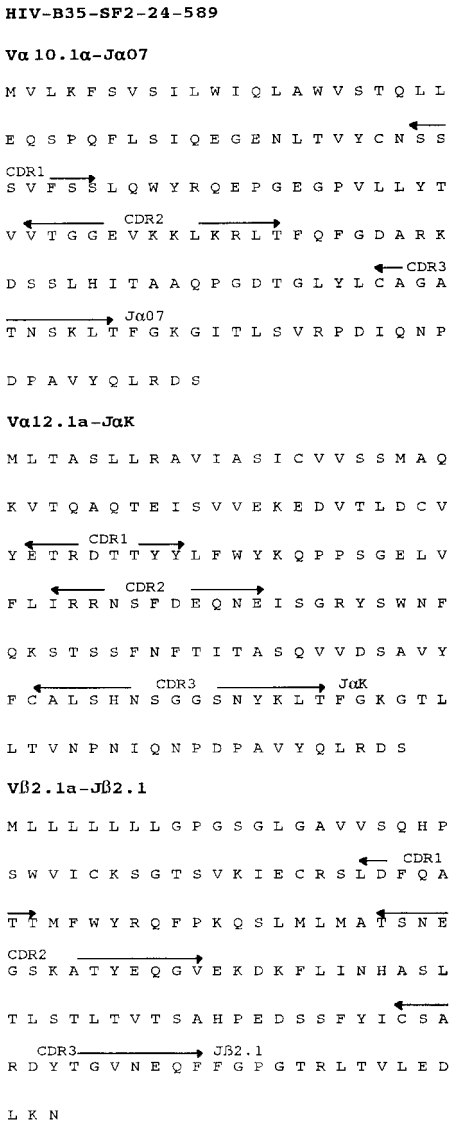

Fig. 5. TCR αβ chains derived from the HIV-B35-SF2-24-589 CTL clone.

The above results suggest that the TCR of the HIV-B35-SF2-24-589 CTL clone cross-recognizes the structures of HIV-B35-SF2-24 presented by both HLA-B*3501 and HLA-B*5101. To directly demonstrate this, we attempted to inhibit the cytolytic activity of this CTL clone for target

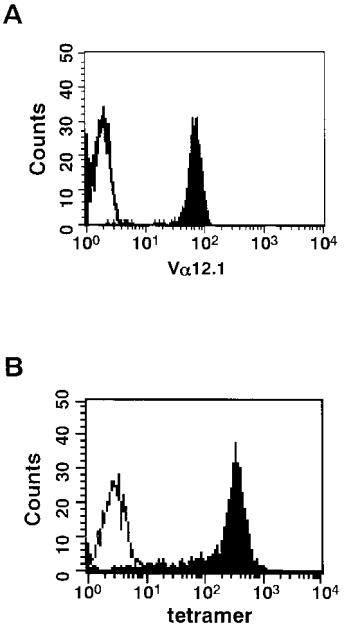

Fig. 6. Surface TCR expression on the HIV-B35-SF2-24-589 CTL clone. (A) Flow cytometry using an anti-TCR Vα12 mAb. Surface expression of TCR Vα12.1 on the HIV-B35-SF2-24-589 CTL clone was examined with an anti-TCR Vα12.1 mAb. (B) Flow cytometry using HLA-B*3501-SF2-24 tetramers. Surface expression of TCR specific for HIV-SF2-24 was examined using PE-labeled HLA-B*3501-HIV-B35-SF2-24 tetramers.

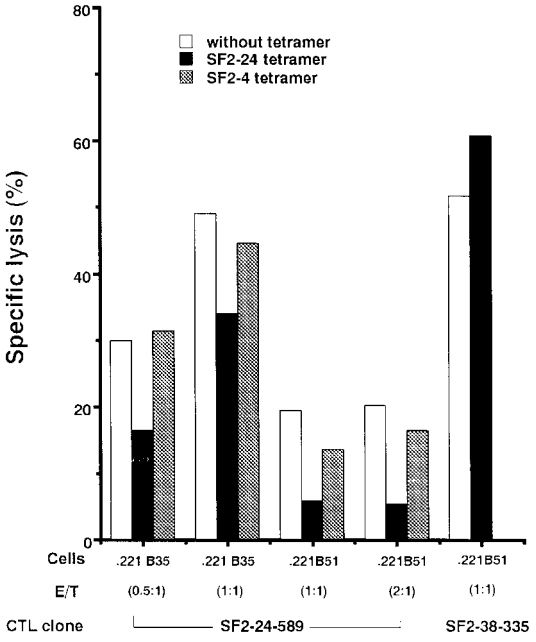

Fig. 7. HLA-B*3501-HIV-B35-SF2-24 tetramers block the cytolytic activity of the HIV-B35-SF2-24-589 CTL clone. Cytolytic activity of the HIV-B35-SF2-24-589 CTL clone was specifically blocked following incubation with HLA-B*3501-HIV-B35-SF2-24 (SF2-24) tetramers. .221-B*3501 (.221 B35) and .221-B*5101 (.221 B51) cells pulsed with HIV-B35-SF2-24 peptide were used as target cells. These tetramers failed to block the cytolytic activity of the SF2-38-specific CTL clone SF2-38-335 for .221 B51 cells pulsed with SF2-38 peptide.

cells expressing HLA-B*3501 and HLA-B*5101 using HLA-B*3501-HIV-B35-SF2-24 tetramers. These tetramers bound to all cells of this clone (Fig. 6B) and inhibited their cytolytic activity not only for target cells expressing HLA-B*3501 but also for those expressing HLA-B*5101 (Fig. 7). In contrast, HLA-B*3501-SF2-4 tetramer, which binds to TCR specific for SF2-4 (VPLDKDFRKY), failed to inhibit the cytolytic activity of this CTL clone. Thus, the TCR of the HIV-B35-SF2-24-589 CTL clone cross-recognizes the structure of HIV-B35-SF2-24 bound to both HLA-B*3501 and HLA-B*5101.

3 Discussion

Previous pool sequencing studies of HLA-B*3501 and HLA-B*5101 binding self-peptides [11, 12] and peptide binding studies of HLA-B*3501 and HLA-B*5101 [13, 14] showed that HLA-B*3501 and HLA-B*5101 binding peptides have similar motifs. These binding peptides have Pro or Ala at P2 and hydrophobic residues at the C terminus, although HLA-B*3501 favors peptides with Tyr at the C terminus. These findings imply that the same peptides may bind to both HLA-B*3501 and HLA-B*5101 and, furthermore, that they may be presented to T cells by both HLA-B*3501 and HLA-B*5101.

In the present study we demonstrated that HIV-B35-SF2-24 is a naturally processed peptide which is presented by both HLA-B*3501 and HLA-B*5101. Although CTL clones specific for this epitope killed C1R-B*5101 cells infected with HIV-1 recombinant vaccinia, they killed similarly infected C1R-B*3501 cells more effectively. This was most apparent in the case of C1R transfectant cells pulsed with HIV-B35-SF2-24 peptide. Since the affinity of HLA-B*5101 binding peptides is much weaker than that of HLA-B*3501 binding peptides [13–15], different binding affinity to HLA-B*3501 and HLA-B*5101 most likely accounts for this difference in CTL recognition. Indeed, the affinity of HIV-B35-SF2-24 for HLA-B*5101 is very low, while that for HLA-B*3501 is high. The amount of peptide eluted from HLA-B*5101 molecules isolated from C1R-B*5101 cells infected with the HIV-1 recombinant vaccinia virus was approximately one fourth of that eluted from HLA-B*3501 molecules isolated from similarly infected C1R-B*3501 cells. This suggests that, even though the binding affinity of the peptide to HLA-B*5101 is very weak, HLA-B*5101 can effectively assemble with this epitope peptide in the endoplasmic reticulum.

A recent study [5] of HLA-A31- and HLA-A33-restricted CTL recognition of a tyrosinase-related protein derived epitope suggested that a single TCR can recognize the same peptide presented by either HLA-A31 or HLA-A33 molecules. However, as the clonality of the CTL clones used in this study was confirmed by reverse transcription-PCR analysis of the TCR β chain only, the possibility remains that these clones contain two different CTL populations carrying the same TCR Vβ chain but different TCR Vα chains. In the present study, we analyzed both TCR α and TCR β from one CTL clone by PCR and showed that this CTL clone carries a single Vβ and two Vα chains, Vα10.1a and Vα12.1a. Flow cytometry using a Vα12.1-specific mAb demonstrated that almost 100 % of the cells express Vα12.1 on their surface. Thus, it is likely that this clone is a single CTL clone regardless of whether Vα10 is expressed on the cell surface or not. As a Vα10-specific mAb is not available, we can not clarify whether Vα10 is expressed on the cell surface. Therefore, it is possible that this clone has two types of TCR, Vα10.1 Vβ2.1 and Vα12.1 Vβ2.1 on the cell surface and that these TCR recognize different HLA class I-peptide complexes. Expression of two TCR α chains on a single T cell has been previously reported [16]. However, the observation that HLA-B*3501-HIV-B35-SF2-24 tetramer

complexes blocked both HLA-B*3501-restricted and HLA-B*5101-restricted CTL recognition excluded the possibility that two TCR recognize different HLA class I-SF2-24 complexes in this clone. This result shows that the TCR of this CTL clone cross-recognizes both HLA class-I peptide complexes regardless of whether this clone expresses a single or two TCR on the cell surface.

There is growing evidence that CTL play an important role in controlling HIV-1 replication [17–21]. Therefore, identification and characterization of HIV-1 CTL epitopes contributes to studies of AIDS immunopathogenesis and HIV-1 vaccine development. The HIV-B35-SF2-24-specific CTL clones examined in the present study not only killed HLA-B*3501-positive target cells infected with HIV-1 (data not shown) but also HLA-B*5101-positive target cells infected with HIV-1. Therefore, it is likely that both HLA-B35- and HLA-B51-restricted CTL for this epitope play a role in elimination of HIV-1-infected cells in this patient. Since this epitope is presented by more than one HLA class I molecule, it may be particularly useful for an HIV-1 vaccine.

Mass spectrometry analysis of peptides eluted from HLA-B*5101 and HLA-B*3501 directly demonstrated that the HIV-B35-SF2-24 peptide presented to T cells by HLA-B*5101 is identical to that presented by HLA-B*3501. Moreover, we showed that this peptide can be presented to a single T cell clone by both HLA-B*3501 and HLA-B*5101 and that the TCR of this clone cross-recognizes the structure of both HLA class I-peptide complexes. As CTL specific for this epitope can recognize HIV-1-infected cells in both an HLA-B*3501- and HLA-B*5101-restricted manner, it is likely that dual recognition of specific CTL contributes to effective elimination of HIV-1 in HIV-1-infected patients.

4 Materials and methods

4.1 Cells

C1R cells transfected with HLA-B*3501 or HLA-B*5101 (C1R-B*3501 and C1R-B*5101, respectively), were previously established [22, 23]. .221 cells transfected with HLA-B*3501 or HLA-B*5101 (.221-B*3501 and .221-B*5101, respectively) were generated. RMA-S-B*3501 and RMA-S-B*5101 have also been generated previously [13, 14]. The EBV-transformed cell line LKT-2, which expresses HLA-A2/A2, HLA-B51/B51 and Cw-/-, was cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS (R10 medium). C1R, C1R-B*3501 and T1 cells were cultured in R10 medium. C1R-B*5101, RMA-S-B*3501 and RMA-S-B*5101 cells were cultured in R10 medium supplemented with 0.15 mg/ml hygromycin B.

4.2 Antibodies

Hybridomas secreting W6/32 anti-HLA class I and SFR8·B6 anti-HLA-Bw6 mAb were purchased from the American Type Culture Collection. A hybridoma secreting TÜ48 anti-HLA-Bw4 mAb was a kind gift from Dr. Claudia Müller. FITC-labeled 6D6-6 anti-human TCR-Vα12.1 was purchased from Serotec Ltd. (Oxford, GB).

4.3 Peptide synthesis

The HIV-B35-SF2-24 peptide (IPLTEEAEL) derived from HIV-1 SF2 Pol protein (position 448–456) was synthesized with a peptide synthesizer (Shimadzu Model PSSM-8) using the Fmoc strategy followed by cleavage. Peptide purity was confirmed by mass spectrometry.

4.4 CTL clones

The HIV-B35-SF2-24-specific CTL clones, HIV-B35-SF2-24-562 and HIV-B35-SF2-24-589 were previously established from patient SG (HLA-A24/A26, B35/B51 and Cw3/-) [8]. These CTL clones were cultured in R10 medium supplemented with 100 U/ml human recombinant IL-2 and stimulated weekly with irradiated T2-B*3501 cells prepulsed with HIV-B35-SF2-24 peptide.

4.5 Cytotoxic assay

Cytotoxicity of CTL clones against target cells pulsed with peptides was determined by a standard ^{51}Cr-release assay. Target cells were incubated for 60 min at 37°C with 100 μCi $Na_2{}^{51}CrO_4$ in saline and then washed three times with R10 medium. Labeled target cells (5×10^3) were plated into 96-well round-bottom microtiter plates (Nunc) with the indicated amount of HIV-B35-SF2-24 peptide. After incubation for 1 h at 37°C, effector cells were added to each well and the mixture was incubated at 37°C for 4–5 h. The supernatants were collected and analyzed with a γ-counter. Spontaneous ^{51}Cr release (cpm spn) was determined by measuring the cpm of the supernatant from the wells containing only target cells. Maximum release (cpm max) was determined by measuring the cpm of the supernatant from wells containing 2.5% Triton X-100. Percent specific lysis was calculated as follows: % specific lysis = 100 x (cpm exp–cpm spn)/(cpm of max–cpm of spn), where cpm exp is the cpm in the supernatant from wells containing both target and effector cells.

4.6 CTL assay for target cells infected with HIV-1 recombinant vaccinia virus

Recombinant vaccinia virus encoding the HIV-1 SF2 *gag/pol* gene has been generated previously [24]. C1R-B*3501 or

C1R-B*5101 cells (5×10^5) were cultured with HIV-1 recombinant vaccinia virus or wild-type vaccinia virus (10 PFU/cell) at 37°C overnight. Infected target cells were labeled with $Na_2{}^{51}CrO_4$ as described above. CTL activity of the CTL clones against target cells infected with the recombinant or wild-type vaccinia virus was measured by a standard ^{51}Cr-release assay as described above.

4.7 Infection of target cells with HIV-1

T1 cells were incubated in R10 medium and 0.1% polybrene for 30 min at 37°C in a CO_2 incubator. The cells were washed once and incubated with HIV-1 IIIB at 37°C. These cells were used as target cells for the ^{51}Cr-release CTL assay after 5 days culture.

4.8 Peptide binding assay

Binding of HIV-1 peptides to HLA-B*5101 and HLA-B*3501 molecules was examined using the HLA-B*5101 stabilization assay as described previously [13, 14]. Briefly, RMA-S-B*5101 and RMA-S-B*3501 cells were cultured for 16 h at 26°C and then pulsed with peptides at concentrations of 10^{-3}–10^{-9} M for 3 h at 26 °C. After incubation for 3 h at 37°C, peptide-pulsed cells were stained with the TP25.99 anti-HLA class I mAb and a fluorescent-conjugated IgG fraction of sheep anti-mouse Ig (Silenius Laboratories, Hawthorn, Victoria, Australia). The mean fluorescence intensity (MFI) was measured with a FACScan (Becton Dickinson, Mountain View, CA).

4.9 Isolation of HIV-B35-SF2-24 peptide from cells infected with HIV-1 recombinant vaccinia virus

C1R, C1R-B*3501 and C1R-B*5101 cells (5×10^8 cells) infected with HIV-1 recombinant vaccinia virus were resuspended in lyzing buffer (pH 7.5, 10 mM TBS containing 0.1% NP40, 1 mM PMSF, 10 μg/ml trypsin inhibitor, 10^{-5}M pepstatin A, 10^{-5} M leupeptin, 10^{-4} M iodoacetamaide) and then agitated gently at 4 °C for 30 min. The resulting cell suspension was centrifuged (1,600 xg) for 30 min at 4°C. The supernatant was incubated with CNBr-activated Sepharose CL4B beads coupled to W6/32 mAb for 1 h. The beads were washed for 20 min with PBS, then 5 ml 0.1% TFA was added to the column. This procedure was repeated six times and then the eluted solution was lyophilized. Lyophilized sample was dissolved in 2 ml 0.1% TFA in double distilled water, and centrifuged (12,000 xg) for 10 min at 4°C. The supernatants were filtrated and separated by reverse-phase HPLC using a C18 column (YMC) as described previously [25]. Each fraction eluted between 30 and 40 min after loading was collected and lyophilized.

4.10 Analysis of antigenic peptides by nano-LC/ESI IT MS

To determine the molecular mass and partial amino acid sequence of antigenic peptides purified from HLA class I molecules isolated from C1R-B*3501 and C1R-B*5101 cells, the HPLC fractions were analyzed by nano-LC/ESI IT MS. Each fraction was loaded onto a nano-column chromatography column (0.075 mm i.d.×150 mm) and separated peptides were subjected to an electrospray ion source in an LCQ ion trap mass spectrometer (Finnigan, CA). The peptides were eluted at 300 nl/min with a gradient of 98% solvent A–85% solvent A (0–10 min), 85% solvent A-40% solvent A (10–50 min), 40% solvent A–2% solvent A (50–60 min), and 20 min isoclatic with 2% solvent A (A, 0.1% aqueous TFA; B, acetonitrile: water 9:1 with 0.095% TFA). ESI IT MS was performed on an LCQ equipped with a stainless electrospray ion source. The electrospray ion source was operated at a potential of 4.0–4.5 kV. To achieve stable electrospray over the course of the gradient elution, 50% methanol containing 1% acetic acid was used as a sheath liquid at a flow rate 300 nl/min. Mass spectra were obtained by scanning the range of mass to charge values between 300 and 2,000. The most abundant peptides were further analyzed by CID MS to identify the amino acid sequence of the peptides. LC/ESI MS and LC/ESI MS/MS were run in an automated LC/MS-LC/MS/MS mode that monitored for signal threshold and performed MS/MS on the base peak when the threshold criterion was exceeded. In CID mode, the trap was filled for up to 50 ms, depending on the number of ions entering the trap per unit time. In this mode, the threshold to trigger ion selection was 3.0×10^4, and the default collision energy was set to 35%.

4.11 Identification of TCR α and β in CTL clones specific for HIV-B35-SF2-24

mRNA was directly isolated from HIV-SF2-24-589 CTL clones using oligo(dT)-conjugated magnetic beads. Double-stranded TCR β cDNA was synthesized from mRNA and amplified by anchor-ligation PCR as previously described [10]. Similarly, double-stranded TCR α cDNA was synthesized and amplified using a forward primer complementary to the anchor and a reverse primer specific for the Cα. Following cloning into a plasmid vector randomly selected cDNA clones were subjected to sequence analysis of Vα and Vβ to the beginning of Cα and Cβ.

4.12 Production of HLA-peptide tetrameric complexes

HLA-B*3501-peptide tetrameric complexes were synthesized as previously described [26]. Briefly, recombinant HLA-B*3501 and human β2-microglobulin (β_2m) were purified from *E. coli* cells transformed with the relevant expression plasmids. The heavy chain was modified by deletion of the transmembrane cytosolic tail and C-terminal addition of

a sequence containing the BirA enzymatic biotinylation site. HLA-B*3501 complexes were folded *in vitro* using the HIV-1-B35-SF2-24 epitope peptide. The resulting 45-kDa complexes were purified by gel filtration using a Superdex G75 column (Amersham Pharmacia Biotech, Uppsala, Sweden). Purified complexes were biotinylated enzymatically with BirA enzyme (Avidity, Denver Co), and then the biotinylated complexes were purified by gel filtration using a Superdex G75 column followed by further purification using a Mono Q column (Amersham Pharmacia Biotech). HLA-B*3501-HIV-B35-SF2-24 peptide tetrameric complexes were generated by mixing phycoerythrin-labeled ExtrAvidin (Sigma) at a molar ratio of 4:1.

4.13 Blocking of HIV-B35-SF2-24-589 CTL clone cytotoxic activity by HLA-B*3501-HIV-B35-SF2-24 tetramers

^{51}Cr-labeled .221-B*3501 and .221-B*5101 cells pulsed with or without peptide (1 nM for .221-B*3501, 1 μM for .221-B*5101) were used as target cells. Effector cells were incubated with 50 μl of R10 medium containing HLA-B*3501-HIV-B35-SF2-24 tetramer or HLA-B*3501-SF2-4 tetramer (0.039 μg/ml) for 30 min at 37°C. Serially diluted effector cells were incubated in a 96-well round-bottom microtiter plate with 2×10^3 target cells for 1 h at 37°C, after which the supernatant was collected and measured using a γ-counter.

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