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Original article

Identification and characterization of HLA-B*5401-restricted HIV-1-Nef and Pol-specific CTL epitopes

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Abstract

The identification of HIV-1 cytotoxic T lymphocyte (CTL) epitopes presented by each HLA allele and the characterization of their CTL responses are important for the study of pathogenesis of AIDS and the development of a vaccine against it. In the present study, we focused on identification and characterization of HIV-1 epitopes presented by HLA-B*5401, which is frequently found in the Asian population, because these epitopes have not yet been reported. We identified these epitopes by using 17-mer overlapping peptides derived from HIV-1 Gag, Pol, and Nef. Seven of these 17-mer peptides induced HLA-B*5401-restricted $CD8^+$ T cell responses. Only five HLA-B*5401-restricted Pol- or Nef-specific $CD8^+$ T cell responses were detected in the analysis using 11-mer overlapping peptides. Three Pol and two Nef optimal peptides were identified by further analysis using truncated peptides. These epitope-specific CTLs effectively killed HLA-B*5401-expressing target cells infected with HIV-1 recombinant vaccinia virus, indicating that these peptides were naturally processed by HLA-B*5401 in HIV-1-infected cells. These epitope-specific $CD8^+$ T cells were elicited in more than 25% of chronically HIV-1-infected individuals carrying HLA-B*5401. Therefore, these epitopes should prove useful for studying the pathogenesis of AIDS in Asia and developing a vaccine against HIV-1.
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Keywords: Cytotoxic T lymphocytes; Epitopes; HIV-1

1. Introduction

Human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocytes (CTLs) play an important role in HIV-1 infections [1–4]. Previous studies demonstrated that HIV-1-specific CTL can inhibit viral replication in vitro [5,6] and that depletion of $CD8^+$ T cells by an anti-CD8 mAb results in failure of the clearance of the virus in rhesus macaques infected with chimeric simian/human immunodeficiency virus [7]. These studies suggest that the $CD8^+$ CTLs contribute to viral clearance and disease progression in HIV-1-infected individuals. Although high HIV-1-specific CTL activity is detected in the early phase of infection in HIV-1-infected individuals, CTL escape mutants are selected by these CTLs [8,9]. The patients in which these HIV-1 escape mutants appear may progress to AIDS. The CTL escape mutants are selected by strong immunological pressure via HIV-1-specific CTLs [10], and the disease progression to AIDS is associated with HLA class I alleles [11,12]. Therefore, the characterization of HIV-1 epitope-specific CTLs is important for understanding the pathogenesis of HIV and developing an AIDS vaccine. However, the number of identified HIV-1 CTL epitopes is limited and CTLs specific for a restricted number of epitopes have been investigated in detail.

Abbreviations: CTL, cytotoxic T lymphocytes; HLA, human leukocyte antigens.

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To identify HIV-1 epitopes, we previously used the strategy of reverse immunogenetics: (i) identification of the motif of HLA class I-binding peptides, (ii) selection of sequences matched to the motif of HLA class I-binding peptides from HIV proteins and synthesis of peptides, (iii) identification of HLA class I-binding HIV-1 peptides by a peptide-binding assay such as the HLA stabilization assay, (iv) induction of CTL by HLA class I-binding peptides in PBLs from HIV-1-infected individuals. We identified many HIV-1 CTL epitopes by using reverse immunogenetics and showed that it is a useful method to identify HLA-class-I-restricted HIV-1 epitopes [13–19]. However, some CTL epitopes may not be identified by this method, since some reported epitopes do not match the motif [20,21]. Identification of CTL epitopes by using overlapping peptides is another useful method [22–26]. This method is advantageous to identify epitopes that are inconsistent with HLA class I-binding motifs.

HLA-B54 is one of the serotypes in HLA-B22, which is a common allele in Asia. HLA-B*5401 is the only genotype of HLA-B54 in the Japanese population and is found in approximately 13% of the Japanese [27]. Therefore, the identification of HLA-B*5401-restricted HIV-1 epitopes is important in studies of immunopathogenesis and for vaccine development in Asia. So far, no HLA-B*5401 HIV-1 epitopes have been reported.

In the present study, we utilized 17-mer overlapping peptides to identify HLA-B*5401-restricted HIV-1 epitopes because those that are inconsistent with HLA-B*5401 motif can be identified by the method using overlapping peptide. Only Pol, Gag, and Nef were focused upon in the present study because these major proteins provide many CTL epitopes, and they are considered as vaccine targets. $CD8^+$ T cells specific for HLA-B*5401-restricted HIV-1 epitopes were further investigated in chronically HIV-1-infected individuals to clarify the immunodominancy of these epitopes.

2. Materials and methods

2.1. Patients

Blood samples were obtained from HIV-1-seropositive individuals carrying HLA-B*5401. The study was approved by the ethics committees of Kumamoto University and the International Medical Center of Japan. Informed consent was obtained from all subjects according to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood.

2.2. Cells

The EBV-transformed B-lymphoblastoid cell lines (B-LCL) were established by transforming B cells from PBMC of laboratory volunteers and an HIV-1-seropositive individual. The PBMC were plated at $3–4 \times 10^6$ cells per well in flat-bottomed 24-well plates in RPMI-1640 medium supplemented with 2 μg/ml cyclosporin A and the supernatant derived from B95-8 cultures. C1R cells expressing HLA-B*5401 (C1R-B*5401) were generated by transfecting C1R cells with the HLA-B*5401 gene. The C1R-B*5401 cells were maintained in the RPMI-1640 medium containing 10% FCS and 0.2 mg/ml of neomycin.

2.3. Synthetic peptides

We designed a panel of 281 overlapping peptides consisting of 17 amino acids in length and spanning Gag, Pol, and Nef of HIV-1 clade B sequences. Each 17-mer peptide was overlapped by at least 11 amino acids. The 281 peptides were synthesized by utilizing an automated multiple peptide synthesizer. Several peptides having difficult sequences were manually synthesized by monitoring of peptide-chain elongation. All peptides were purified by high-performance liquid chromatography (HPLC). The purity was examined by HPLC and mass spectrometry. Peptides with more than 90% purity were used in the present study.

2.4. Induction of peptide-specific T cells

The peptide-specific T cells were induced from PBMCs of HIV-1-seropositive individuals carrying HLA-B*5401. PBMCs were cultured with each peptide cocktail including eight kinds of 1 μM 17-mer peptides (totally 8 μM) or each 17-mer single peptide (1 μM) in culture medium (RPMI-1640 containing 10% FCS and 200 U/ml IL-2). Two weeks later, they were used in intracellular IFN-γ staining assays or CTL assays.

2.5. Intracellular IFN-γ staining assay

After B-LCL, C1R-B*5401 or C1R cells had been incubated for 60 min with each peptide cocktail containing eight kinds of 1 μM 17-mer peptide (totally 8 μM) or each 17-mer single peptide (1 μM), they were washed twice with RPMI-1640 containing 10% FCS. These peptide-pulsed autologous B-LCL (2×10^5 cells per well) and peptide-stimulated PBMCs cells (1×10^5 cells per well) were added to a 96-well round-bottomed plate, which was incubated for 2 h. Subsequently, Brefeldin A (10 μg/ml) was added, and incubation was continued for an additional 4 h. After the cells had been stained with anti-CD8 mAb (DAKO Corporation, Flostrup, Denmark), they were fixed with 4% paraformaldehyde at 4 °C for 20 min and then permeabilized with PBS containing 20% newborn calf serum (Summit Biotechnology, Greely, Co.) and 0.1% saponin (permeabilizing buffer) at 4 °C for 10 min. Thereafter, the cells were resuspended in the permeabilizing buffer and then stained with anti-IFN-γ mAb (BD Bioscience, CA, USA). The cells were finally resuspended in PBS containing 2% paraformaldehyde and then the percentage of $CD8^+$ cells positive for intracellular IFN-γ was analyzed by FACSCalibur (BD Bioscience).

2.6. CTL assay

The cytotoxicity of HIV-1-specific CTL was measured by the standard ^{51}Cr release assay. The HLA-B*5401$^+$ B-LCL

infected with recombinant vaccinia virus encoding *gag*/*pol*, or *nef* gene of HIV-1 SF2 or WT vaccinia virus were used as target cells. Target cells were incubated for 60 min with $Na_2^{51}CrO_4$ (150 μCi) in saline, and washed three times with RPMI-1640 medium containing 10% NCS. The labeled target cells were added to each well of a 96-well round-bottomed plate with peptides and they were incubated for 1 h at 37 °C. Then, HIV-1-specific bulk CTL or clones as effector cells were added to the target cells and the mixtures were incubated for 4 h at 37 °C. The supernatants were collected and analyzed with a gamma counter. The spontaneous ^{51}Cr release (cpm spn) was determined by measuring the cpm in the supernatant in the wells containing only target cells. The maximum release (cpm max) was determined by measuring the release of ^{51}Cr from the target cells in the presence of 2.5% TritonX-100. Percent specific lysis was calculated as follows: percentage specific lysis = 100 × (cpm exp − cpm spn)/(cpm max − cpm spn), where cpm exp is the cpm in the supernatant from wells containing both target and effector cells. In another experiment, labeled C1R-B*5401 cells were pulsed with various concentrations (0.1–10^3 nM) of the corresponding peptide.

3. Results

*3.1. Induction of HIV-1 peptide-specific CD8$^+$ T cells by using 17-mer overlapping peptide cocktails from PBMCs of chronically HIV-1-infected HLA-B*5401$^+$ individuals*

PBMCs from KI-119, a chronically HIV-1-infected HLA-B*5401$^+$ individual, were stimulated in vitro for 12–14 days with Gag, Pol, and Nef peptide cocktails including eight 17-mer overlapping peptides. IFN-γ production by each bulk culture in response to autologous B-LCL pre-pulsed with the corresponding peptide cocktail was assessed by staining for intracellular IFN-γ. Bulk cultures from KI-119 responded to six Gag, seven Pol, and three Nef cocktails (data not shown). To determine which peptides in the each cocktail induced the specific CD8$^+$ T cell, we stimulated the bulk cultures with autologous B-LCL pre-pulsed with single 17-mer peptides found in the cocktails. Twelve Gag, nine Pol, and four Nef peptides induced CD8$^+$ T cells to produce IFN-γ (data not shown).

*3.2. Candidates of HLA-B*5401-restricted 17-mer peptides*

HLA restriction of the T cell response specific for these 17-mer peptides was subsequently determined by using the bulk cultured cells having a specific ability to recognize 17-mer peptide as well as a panel of B-LCLs sharing one HLA class allele with KI-119 carrying HLA-A*0206/A*0206 and HLA-B*5401/B*4801. Bulk cultured cells were incubated with either autologous B-LCL, HLA-A,-B-mismatched B-LCL or B-LCL sharing only one HLA class I allele with the donor. A representative result of flow cytometric analysis is shown in Fig. 1A. Pol300–316 peptide-pulsed autologous B-LCL or B-LCL expressing HLA-B*5401 induced IFN-γ production from CD8$^+$ T cells in the bulk culture cells having a specific ability for the Pol300–316 peptide. No significant response was found by stimulation with Pol300–316 peptide-pulsed HLA-B*5401-negative B-LCL. These results suggest that these peptide-specific CD8$^+$ T cells were restricted by HLA-B*5401. Similar results were obtained with bulk culture cells having a specific ability to recognize Pol151–167, Pol786–802, Pol792–808, Nef119–135, Nef125–141 or Nef149–165 peptide, suggesting that CD8$^+$ T cells specific for these peptides were also restricted by HLA-B*5401 (Fig. 1B). For some peptides, we could not test the entire panel at the same time due to sample limitation, while other 17-mer peptides were restricted by HLA-B*4801 or HLA-A*0206 (data not shown). Thus, these seven 17-mer peptides may include candidates of HLA-B*5401-restricted HIV-1 epitopes.

3.3. Identification of optimal epitope peptides

To identify the optimal epitope recognized by CD8$^+$ T cells specific for these peptides, we designed 11-mer peptides which were overlapping nine amino acids each in the sequence of the 17-mer peptide. IFN-γ production of each bulk culture in response to autologous B-LCL pre-pulsed with a 1 μM concentration of the corresponding 11-mer or 17-mer peptides was assessed by intracellular IFN-γ staining. The Pol151–167 (CTLNFPISPIETVPVKL)-induced CD8$^+$ T cells recognized LNFPISPIETV and FPISPIETVPV but not ISPIETVPVKL (Fig. 2). Since Pro is an anchor for HLA-B*5401 [28], 6P in this 17-mer is the anchor for HLA-B*5401 rather than 9P or 14P. Thus, we expected that the epitope would be included in FPISPIETVPV (Pol155–165). To identify the optimal peptide, we generated three truncated peptides (FP10: FPISPIETVP, FV9: FPISPIETV, and FT8: FPISPIET). Pol151–167 (CTLNFPISPIETVPVKL)-induced CD8$^+$ T cells recognized all of them (Fig. 3A), but at lower concentrations of the peptide they recognized FV9 and FP10 more than FT8 (Fig. 3B). The difference in T cell recognition between FV9 and FP10 is not significant though they seem to recognize FV9 more than FP10 at a lower concentration. These results suggest that a shorter peptide, FV9 (Pol 155–163), might be the optimal epitope rather than FP10, but it still remains possible that both peptides are presented and recognized by T cells.

Pol300–316 (YNVLPQGWKGSPAIFQS)-induced CD8$^+$ T cells recognized VLPQGWKGSPA but not the other three 11-mer peptides (Fig. 2), indicating that 5P in this 17-mer peptide is the anchor for HLA-B*5401 rather than 12P. We therefore generated three truncated peptides (LA10: LPQGWKGSPA, LP9: LPQGWKGSP, and LS8: LPQGWKGS) from Pol300–316. Pol300–316 (YNVLPQGWKGSPAIFQS)-induced CD8$^+$ T cells recognized both LA10 and LS8 (Fig. 3A), but they failed to recognize LS8 at lower concentrations of the peptide (Fig. 3C). These findings indicate that LA10 (Pol303–312) is the optimal epitope.

Bulk cultured cells stimulated with Pol786–802 or Pol792–808 responded to the same 11-mer peptide, HVASGYIEAEV (Fig. 2), suggesting that both bulk cultured

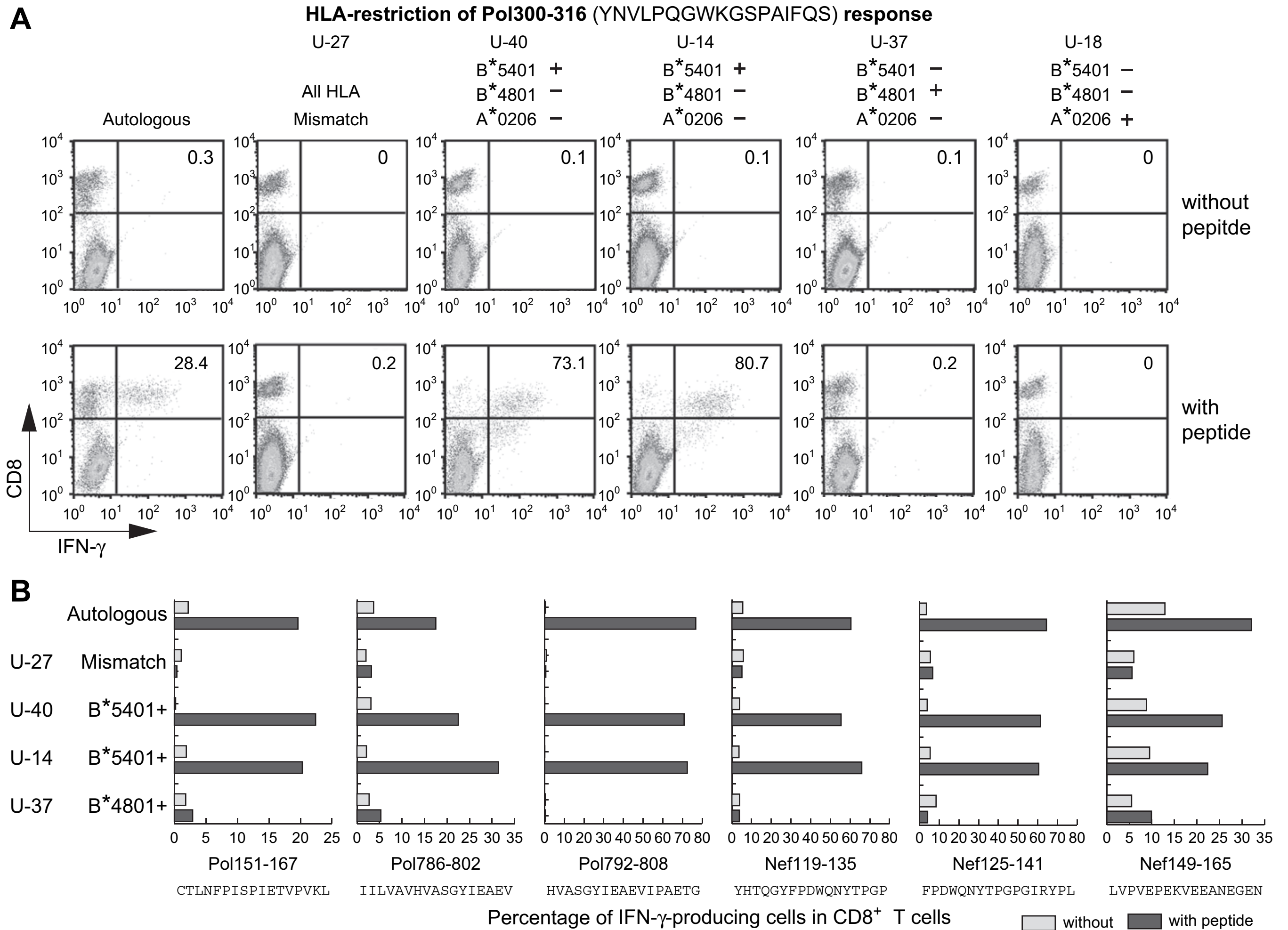

Fig. 1. Identification of HLA-B*5401-restricted HIV-1 CTL epitope candidates by using a panel of B-LCL pulsed with 17-mer peptides. A. PBMC from an HIV-1-seropositive individual KI-119 (A*0206/-, B*5401/B*4801) were stimulated with Pol300–316 peptide and then cultured for 2 weeks. The cultured cells were stimulated with Pol300–316 peptide-pulsed autologous B-LCL or allo B-LCL sharing only one HLA class I allele with the donor. Pol 300–316-specific $CD8^+$ T cells were detected by using the intracellular IFN-γ staining assay. The percentage of IFN-γ-producing cells among $CD8^+$ T cells are shown in each plot. B. The same assays shown in "A" were performed by using other 17-mer HIV-1 Pol and Nef peptides (Pol151–167, Pol786–802, Pol792–808, Nef119–135, Nef125–141 and Nef149–165). The percentage of IFN-γ-producing cells among $CD8^+$ T cells are shown in each figure.

$CD8^+$ T cells recognize this peptide (Pol790–800). Pol790–800 did not include the B*5401 anchor residue Pro. Since Ala is an amino acid with characteristics similar to those of Pro, we synthesized three peptides carrying A at position 2 (VV10: VASGYIEAEV, VE9: VASGYIEAE, and VA8: VASGYIEA). Pol790–800-specific bulk $CD8^+$T cells failed to recognize these three peptides (Fig. 3A). We therefore synthesized three truncated peptides (HA9: HVASGYIEA, AE10: AVHVASGYIE, and VA10: VHVASGYIEA) and tested whether Pol790–800-specific bulk $CD8^+$ T cells could recognize them. The result showed that they recognized VA10 and HA9 but not AE10 (Fig. 3A). However, they failed to recognize lower concentrations of VA10 peptide (Fig. 3D). These results indicate that HA9 (Pol 792–800) is the optimal epitope.

Similarly FPDWQNYTPGP was recognized by bulk cultured $CD8^+$ T cells stimulated with either Nef119–135 or Nef125–141. Bulk cultured $CD8^+$ T cells stimulated with Nef119–135 recognized both FPDWQNYTPGP and GYFPDWQNYTP, whereas Nef125–141-induced $CD8^+$ T cells recognized FPDWQNYTPGP but not the other 11-mers (Fig. 2). According to peptide-binding motif of HLA-B*5401, which has Pro at position 2, we speculated that FPDWQNYTP (overlapped between GYFPDWQNYTP and FPDWQNYTPGP) would be the optimal epitope peptide, and so we synthesized three truncated peptides (GT10: GYFPDWQNYT, FP9: FPDWQNYTP, and PP8: PDWQNYTP) from Nef123–133. The result showed that Nef125–141-induced $CD8^+$ T cells recognized FP9 but not GT10 and PP8 (Fig. 3A), thus indicating FP9 (Nef125–133) to be the optimal peptide.

In the case of Nef149–165, we found that the bulk culture cells stimulated with the Nef149–165 peptide failed to produce IFN-γ by stimulation with B-LCL pre-pulsed with four 11-mer peptides in Nef149–165 (Fig. 2). Nef149–165 has two Pro residues, but the bulk cells failed to respond to EPEKVEEANEG, suggesting that Pro at position 2 of

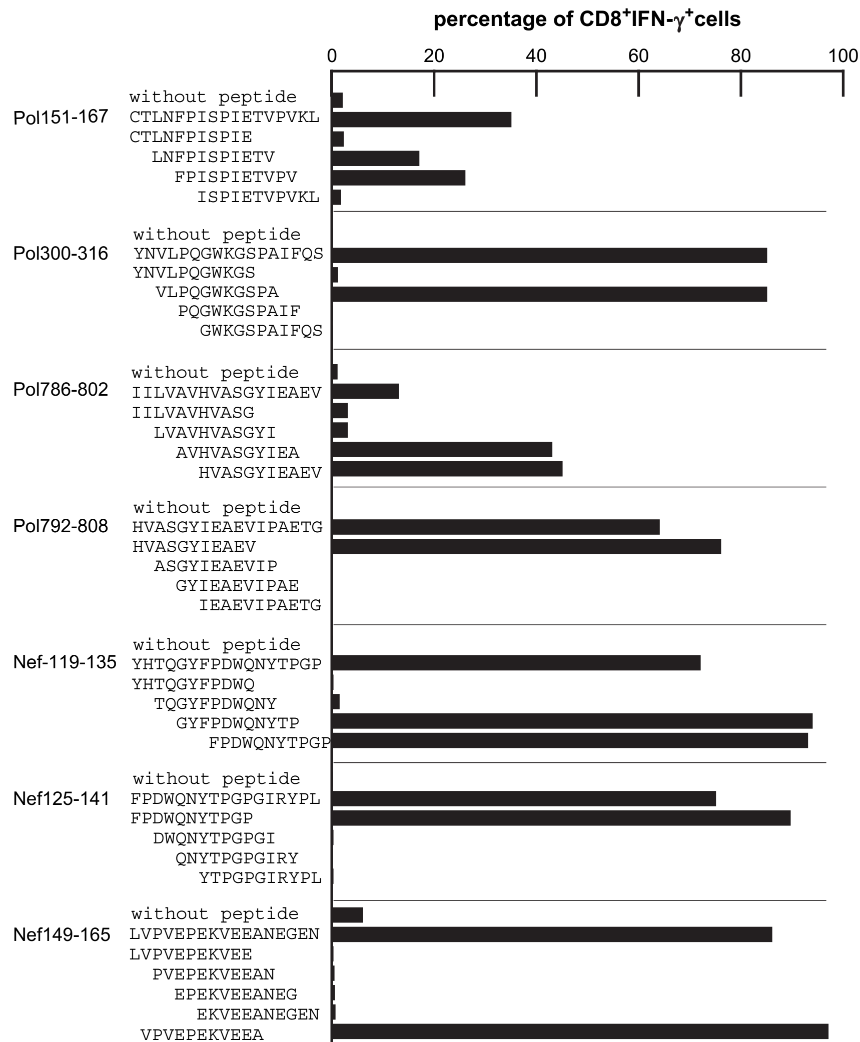

Fig. 2. Selection of 11-mer HIV-1 Pol and Nef peptides including HLA-B*5401-restricted epitopes. The 17-mer peptide-specific bulk CD8$^+$ T cells were stimulated with autologous B-LCL pre-pulsed with each overlapping 11-mer peptide included in the 17-mer peptides. The responsibility of the bulk CD8$^+$ T cells toward each 11-mer peptide was measured by using the intracellular IFN-γ staining assay. The percentages of IFN-γ-producing cells among CD8$^+$ T cells are shown in the figure.

VPVEPEKVEEA (Nef150–160) is the anchor residue of the epitope. Therefore, we generated the Nef150–160 peptide and investigated whether the bulk cultured cells would respond to the stimulator cells pre-pulsed with this 11-mer peptide. The results showed that they produced IFN-γ production in response to Nef150–160 (Fig. 2). The finding that the bulk cells did not respond to LVPVEPEKVEE (Nef149–159) excluded the possibility that one of the three shorter peptides (VPVEPEKV, VPVEPEKVE or VPVEPEKVEE) was the epitope. These results strongly suggest that Nef150–160 is the optimal epitope peptide.

3.4. Killing of HIV-1-recombinant vaccinia-infected cells by specific CTLs

To clarify whether Pol155–163, Pol303–312, Pol792–800, Nef125–133, and Nef150–160 epitopes are naturally occurring peptides, we investigated the ability of these peptide-specific CD8$^+$ T cells to kill HLA-B*5401 expressing B-LCL infected with recombinant HIV-1 (r-HIV-1) vaccinia virus. They effectively killed HLA-B*5401 expressing B-LCL infected with r-HIV-1 vaccinia virus but not the cells infected with the wild-type vaccinia virus (Fig. 4). There was a difference in killing activity toward r-HIV vaccinia-infected cells between Pol- and Nef-specific bulk CTLs. A previous study showed that HLA class I is downregulated in cells infected with HIV-1 nef recombinant vaccinia [28]. The difference might be explained by Nef-mediated HLA-A and -B down-regulation. These results confirm these peptides to be naturally occurring ones presented by HLA-B*5401.

3.5. Confirmation of HLA-B*5401-restriction in five HIV-1-epitope-specific CTLs

To confirm the restriction molecule of these five HIV-1 epitopes, we generated CTL clones specific for these

A

percentage of $CD8^+IFN\text{-}\gamma^+$ cells

0 20 40 60 80 100

Pol155-165
without peptide
FPISPIETVPV (FV11)
FPISPIETVP (FP10)
FPISPIETV (FV9)
FPISPIET (FT8)

Pol302-312
without peptide
VLPQGWKGSPA (VA11)
LPQGWKGSPA (LA10)
LPQGWKGSP (LP9)
LPQGWKGS (LS8)

Pol790-800
without peptide
AVHVASGYIEA (AA11)
AVHVASGYIE (AE10)
VASGYIEAEV (VV10)
VASGYIEAE (VE9)
VASGYIEA (VA8)
VHVASGYIEA (VA10)
HVASGYIEA (HA9)

Nef123-133
without peptide
GYFPDWQNYTP (GP11)
GYFPDWQNYT (GT10)
FPDWQNYTP (FP9)
PDWQNYTP (PP8)

B
Pol155-165
IFN-γ-producing cells in CD8+ T cells (%)
50 40 30 20 10 0
FPISPIETVP (FP10)
FPISPIETV (FV9)
FPISPIET (FT8)
0.1 1 10 100 1000
Peptide concentration (nM)

C
Pol302-312
IFN-γ-producing cells in CD8+ T cells (%)
80 70 60 50 40 30 20 10 0
LPQGWKGSPA (LA10)
LPQGWKGSP (LP9)
LPQGWKGS (LS8)
0.1 1 10 100 1000
Peptide concentration (nM)

D
Pol790-800
IFN-γ-producing cells in CD8+ T cells (%)
25 20 15 10 5 0
AVHVASGYIEA (AA11)
VHVASGYIEA (VA10)
HVASGYIEA (HA9)
0.1 1 10 100 1000
Peptide concentration (nM)

Fig. 3. Recognition of the 8- to 10-mer truncated peptides by HIV-1 Pol- or Nef-specific CD8+ T cells. A. The 17-mer peptide-specific bulk $CD8^+$ T cells were stimulated with autologous B-LCL pre-pulsed with each 8- to 10-mer truncated peptide. For determination of the optimal epitopes in Pol155–165 (B), Pol302–312 (C), and Pol790–800 (D), bulk CTL were co-cultured with autologous B-LCL pre-pulsed with each truncated peptide at concentrations from 0.1 to 1000 nM. The responsiveness of the bulk $CD8^+$ T cells toward each truncated peptide was measured by conducting the intracellular IFN-γ staining assay. The percentages of IFN-γ-producing cells among $CD8^+$ T cells are shown in the figure.

epitopes as well as HLA-B*5401-transfected C1R cells (C1R-B*5401 cells). We used both C1R-B*5401 cells and C1R cells as target cells for the CTL clones specific for these epitopes. These CTL clones killed C1R-B*5401 cells pre-pulsed with the corresponding peptide but failed to kill the C1R cells that were similarly treated (Fig. 5). These results confirm that these CTLs recognized HLA-B*5401-restricted epitopes.

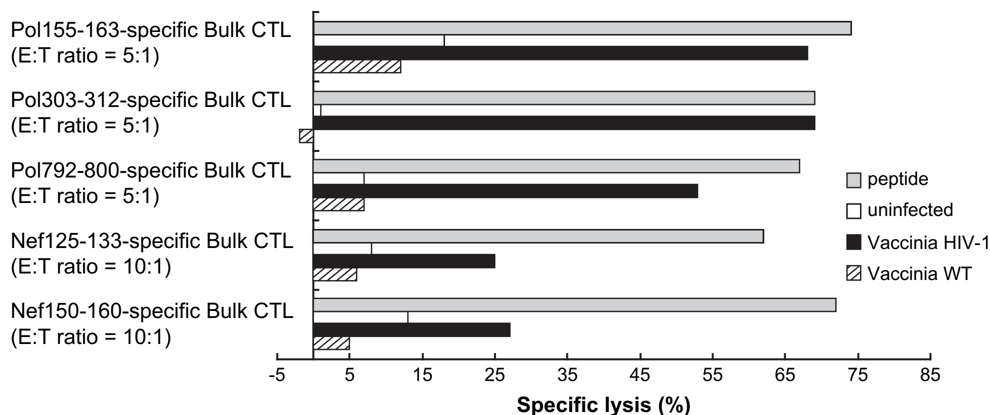

Fig. 4. Killing of r-HIV-1 vaccinia-infected or peptide-pulsed cells by the Pol- or Nef-specific CTLs. The activities of the five HIV-1-specific T cells toward $B*5401^+$B-LCL pre-pulsed with the corresponding peptide (1 μM), or those infected with recombinant vaccinia virus expressing the corresponding proteins Pol and Nef (vaccinia-HIV-1) or wild-type vaccinia virus (vaccinia-WT) were measured at an effector-to-target (E:T) ratio of 5:1 or 10:1.

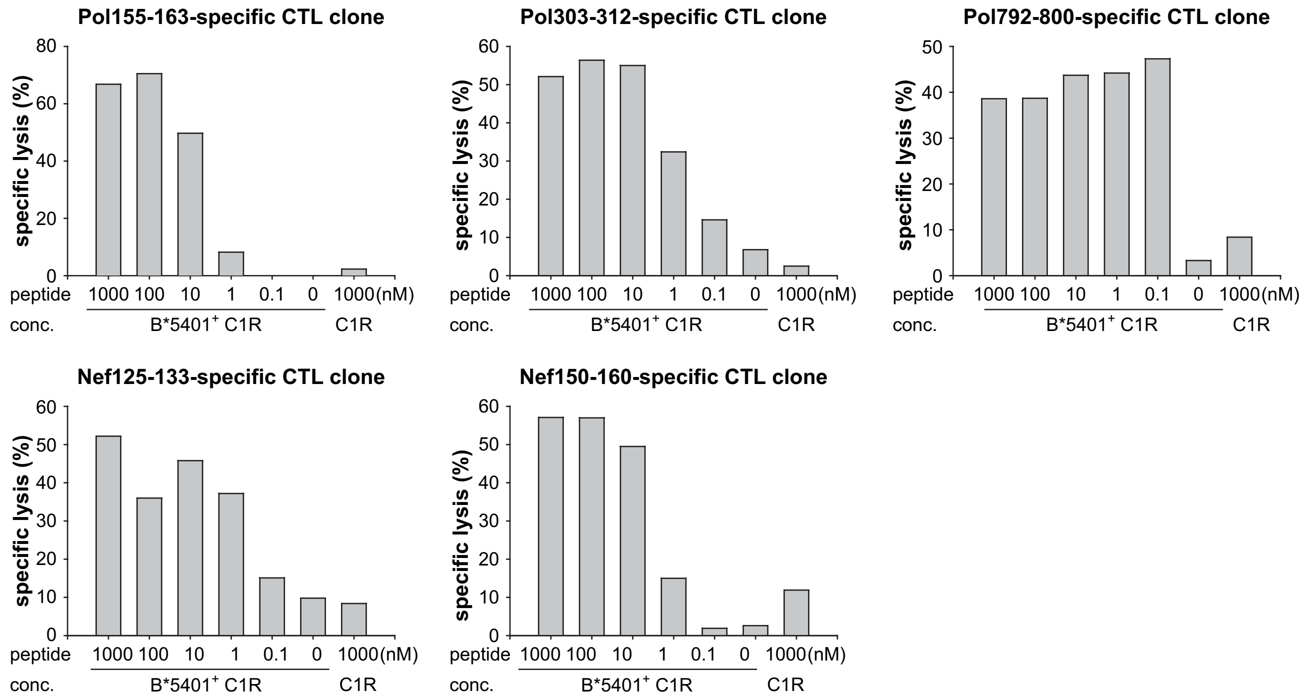

Fig. 5. Confirmation of HLA-B*5401-restricted recognition. Specific lysis of C1R-B*5401 cell lines by Pol155–163-specific, Pol303–312-specific, Pol792–800-specific, Nef125–133-specific, or Nef150–160-specific CTL clone was determined by performing the ^{51}Cr-release assay. The target cells were pulsed with each peptide at concentrations from 0.1 to 1000 nM, and the assays were performed at a 2:1 ratio of effector cells to target cells. The percentage of specific lysis is shown in each graph.

3.6. Frequency of HLA-B*5401-restricted HIV-1-specific CD8$^+$ T cells in HIV-1-infected individuals with HLA-B*5401

To clarify whether CD8$^+$ T cells specific for these epitopes were predominantly induced in chronically HIV-1-infected individuals bearing HLA-B*5401, we investigated the induction of the specific CD8$^+$ T cells in PBMCs from eight chronically HIV-1-infected HLA-B*5401-positive individuals by stimulating them with these epitope peptides. Pol155–163-specific CD8$^+$ T cells were found in four of the eight HIV-1-infected individuals. Pol792–800-, Nef125–133-, and Nef150–160-specific CD8$^+$ T cells were found in three individuals; and Pol303–312-specific CD8$^+$ T cells, in two of them (Table 1).

4. Discussion

In the present study, we could identify 5 HLA-B*5401-restricted epitopes in HIV-1 Pol and Nef by using 17-mer overlapping peptides. A previous study had shown that Pro at position 2 is the primary anchor residue and that Phe, Met, Arg, Tyr or Asp at position 3, and Ala at position 9 is the secondary anchor residue for HLA-B*5401 [29]. However, CTL epitopes are not always consistent with the peptide-binding motif of HLA [20,21]. In fact, out of the five HLA-B*5401-restricted HIV-1 epitopes, one does not have Pro at position 2. Pol792–800 epitope (HVASGYIEA) has Ala residue at position 3. This Ala is a candidate of the anchor at position 2 because Ala has similar characteristics as Pro, but the specific

Table 1
Induction of epitope-specific CD8$^+$ T cells among PBMCs from HLA-B*5401$^+$ HIV-1-infected individuals

Patients[a]	Viral load[b]	CD4[c]	CD8[c]	Percentage of IFN-γ-producing cells in CD8$^+$ T cells				
				Pol155–163	Pol303–312	Pol792–800	Nef125–133	Nef150–160
KI-119	3.0×10^3	536	1268	16.1	58.0	39.8	26.3	21.1
KI-160	3.5×10^4	360	831	3.9	0.0	0.5	0.0	0.0
KI-172	1.8×10^4	512	558	0.0	0.0	0.0	8.1	0.0
KI-115	5.3×10^3	264	721	19.9	7.0	0.0	0.1	2.1
KI-150	2.4×10^4	307	1411	0.0	0.0	3.5	0.0	0.0
KI-167	4.2×10^4	281	1055	0.0	0.0	0.0	48.5	0.0
KI-141	1.7×10^5	578	1414	0.8	0.0	0.0	0.4	0.0
KI-201	<50	518	374	7.2	0.0	12.8	0.0	3.5

[a] HIV-1-infected individuals with HLA-B*5401.
[b] Copies/ml.
[c] Cells/μl.

CD8+ T cells failed to recognize three truncated peptides carrying Ala at position 2. Thus, this epitope is not consistent with the HLA-B*5401 peptide motif. We note that the Pol792—800 epitope cannot be identified by reverse immunogenetics. Interestingly, the Pol792—800-specific CTL clone showed high cytotoxicity toward B*5401-transfected C1R cells pulsed with peptides at low concentrations (Fig. 5), thus suggesting that the Pol792—800 peptide may be a high-affinity HLA-B*5401-binding peptide.

In the present study, we used 17-mer overlapping peptides to identify HIV-1-specific CTLs, because the cost of making shorter peptides is much cheaper than that for the longer ones. The optimal length of epitope peptides presented by HLA class I molecules is thought to be 8—11 amino acid residues [30]. Therefore, the affinity of 17-mer peptides toward HLA class I molecules is thought to be low. This suggests that some epitopes are not identified by this approach using 17-mer overlapping peptides.

Interestingly, Gag-specific epitopes were not identified in the present study, although Pol- and Nef-specific CTL ones were. We used PBMCs from only HIV-1-infected individual KI-119. Therefore, we speculate that this individual does not have any ability to elicit CTL specific for Gag. However, KI-119 showed strong HLA-A*0206-restricted or HLA-B*4801-restricted CD8+ T cells responses to Gag (data not shown), suggesting that HLA-B*5401-restricted Gag-specific T cell responses are hardly induced. This suggests the possibility that Gag does not include a high-affinity peptide carrying HLA-B*5401 motif. Recent studies reported that Gag-specific CTLs play a critical role in the control of viral replication, because their frequency was correlated with viral loads in HIV-1-infected individuals [31]. If this is also the case in Japanese and other Asian populations, HLA-B*5401 may be associated with rapid progression to AIDS. The role of these HLA-B*5401-restricted CTLs still remains unknown. Further analysis of these CTLs will be required to clarify the role of HLA-B*5401-restricted CTLs in Asian populations.

When we examined the frequency of these five epitope-specific CTL in eight chronically HIV-1-infected individuals, these CTLs were detected in two to four of eight chronically HIV-1-infected individuals with HLA-B*5401 (Table 1), indicating that these five epitopes were relatively recognized ones in chronically HIV-1-infected individuals. These epitopes except Nef150—160 are relatively conserved in clade B (approximately more than 80% of clade B has consensus sequences: Los Alamos National Laboratory HIV Molecular Immunology Database, http://www.hiv.lanl.gov/content/immunology/maps/ctl/ctl.pdf). In contrast, many substitutions are found in Nef150—160. They include D at position 4, D at position 6, Q/E/R at position 8, I at position 8, and K at position 10. These results imply that these CTLs play an important role in the control of HIV-1. Further analysis of these epitopes such as escape mutants is now under investigation.

In summary, we identified five novel HLA-B*5401-restricted HIV-1 epitopes in HIV-1-infected individuals by using 17-mer overlapping peptides derived from HIV-1 Gag, Pol, and Nef. In addition, one of them, Pol792—800, did not have an amino acid sequence matching the HLA-B*5401 peptide motif. These epitopes identified by using 17-mer overlapping peptides will be useful to clarify immune response toward HIV-1 and to develop a population-based AIDS vaccine.

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