Ⅲ. 研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表

研究分担者　滝口 雅文

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研究分担者　潟永 博之

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研究分担者　天野 将之

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研究分担者　前仲 勝実

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IV. 研究成果の刊行物・別刷
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Original article

Effective recognition of HIV-1-infected cells by HIV-1 integrase-specific HLA-B*4002-restricted T cells

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Abstract

HLA-B*4002 is one of the common HLA-B alleles in the world. All 7 reported HLA-B*4002-restricted HIV epitopes are derived from Gag, Nef, and Vpr. In the present study we sought to identify novel HLA-B*4002-restricted HIV epitopes by using overlapping 11-mer peptides of HIV-1 Nef, Gag, and Pol, and found that 6 of these 11-mer Pol peptides included HLA-B*4002-restricted epitopes. Analysis using truncated peptides of these 6 peptides defined 4 optimal Pol (integrase) epitopes. All epitopes previously reported had Glu at position 2 (P2), suggesting that Glu at P2 is the anchor residue for HLA-B*4002; whereas only 2 of the integrase epitopes that we here identified had Glu at P2. CTL clones specific for the 2 epitopes effectively recognized HIV-1-infected cells whereas those for other 2 epitopes only weakly recognized them. The antigen sensitivity of the former clones for the epitope peptide was much higher than that of the latter clones, suggesting 2 possibilities: 1) the former T cells have high-affinity TCRs and/or 2) the epitope peptides recognized by the former T cells are highly presented by HLA-B*4002 in HIV-1-infected cells. These integrase-specific T cells with high antigen sensitivity may contribute to the suppression of HIV-1 replication in HIV-1-infected HLA-B*4002$^+$ individuals.

Keywords: HIV-1; Cytotoxic T lymphocytes; HLA-B*4002; Integrase

1. Introduction

Human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocytes (CTL) play an important role in HIV-1 infections [1–4]. Previous studies demonstrated that HIV-1-specific CTL can inhibit viral replication in vitro [5–7] and that depletion of CD8$^+$ T cells by treatment with an anti-CD8 mAb results in failure of the clearance of the virus in rhesus macaques infected with chimeric simian/human immunodeficiency virus [8]. These studies suggest that the CD8$^+$ CTLs contribute to viral clearance and disease progression in HIV-1-infected individuals. The study of CTL responses in an African cohort demonstrated that HLA-B-restricted T cell responses are associated with lower viral load than HLA-A-restricted or HLA-C-restricted ones [9], suggesting that HLA-B-restricted responses are important for the control of HIV-1. Therefore, the characterization of HIV-1 epitope-specific HLA-B-restricted CTLs is important for understanding the pathogenesis of HIV and developing an AIDS vaccine.

HLA-B*4001 and HLA-B*4002 are common HLA-B alleles in the world. These alleles are found in 10.8% and 16.6% of Japanese population, respectively, and the frequency of HLA-B*4002 is the third highest among HLA-B alleles [10]. Only residue 97 differs between these 2 alleles. So far 10 HLA-B*4001-restricted and 7 HLA-B*4002-restricted HIV epitopes have been reported in Caucasian cohorts [11–16]. These HLA-B*4002-restricted epitopes were derived from

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Gag, Nef, and Vpr; whereas the HLA-B*4001-restricted ones came from Gag, Nef, Pol, and Env.

In the present study, we sought to identify HLA-B*4001-restricted and HLA-B*4002-restricted HIV-1 epitopes in chronically HIV-1-infected Japanese cohorts by using 11-mer overlapping peptides derived from Pol, Gag, and Nef. We focused on these 3 proteins in the present study because these major proteins, which provide many CTL epitopes, are considered as vaccine targets. In addition, $CD8^+$ T cell clones specific for these newly identified epitopes were generated and used to clarify their ability to recognize HIV-1-infected cells. In the present study, we found 4 novel integrase epitopes presented by HLA-B*4002 and further characterized the $CD8^+$ T cells specific for these epitopes. Two of these epitopes were considered as immunodominant epitopes, because the specific T cells effectively recognized HIV-1-infected cells.

2. Materials and methods

2.1. Samples of HIV-1-infected individuals

This study was approved by the National Center for Global Health and Medicine and the Kumamoto University Ethical Committee. Informed consent was obtained from all subjects according to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood. The HLA type of the patients was determined by standard sequence-based genotyping.

2.2. Synthetic peptides

We previously designed and generated overlapping peptides consisting of 11-mer amino acids and spanning Gag, Pol, and Nef of HIV-1 clade B consensus sequences [17]. Each 11-mer peptide was overlapped by 9 amino acids. Truncated peptides of some 11-mer peptides were synthesized by utilizing an automated multiple peptide synthesizer and 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.

2.3. Cells

The EBV-transformed B-lymphoblastoid cell lines (B-LCL) were established by transforming B cells from PBMC of KI-400. C1R cells expressing HLA-A*0207 (C1R-A*0207) and those expressing HLA-B*4002 (C1R-B*4002) were generated by transfecting C1R cells with the HLA-A*0207 and HLA-B*4002 genes, respectively. C1R-A*3101 cells were previously generated [18]. 721.221-CD4 cells expressing HLA-B*4002 (.221-CD4-B*4002), HLA-Cw*0102 (.221-CD4-Cw*0102), and HLA-Cw*0304(.221-CD4-Cw*0304) were generated by transfecting 721.221-CD4 cells with the HLA-B*4002, HLA-Cw*0102, and HLA-Cw*0304 genes, respectively, and maintained in RPMI 1640 medium supplemented with 10% FCS and 2.0 mg/ml hygromycin B.

2.4. Intracellular cytokine production (ICC) assay

PBMCs from chronically HIV-1-infected patient KI-400 were stimulated with HIV-1-derived peptide (1 μM) in culture medium (RPMI 1640 medium supplemented with 10% FCS and 200 U/ml recombinant human IL-2). After 14 days in culture, the cells were assessed for IFN-γ production activity by using a FACSCalibur. Briefly, bulk cultures were stimulated with stimulator cells pulsed with HIV-1-derived peptide (1 μM) for 2 h at 37 °C. Brefeldin A (10 μg/ml) was then added, and the cultures were continued for an additional 4 h. Cells were collected and stained with phycoerythrin (PE)-labelled anti-CD8 monoclonal antibody (mAb; Dako Corporation, Glostrup, Denmark). After having been treated with 4% paraformaldehyde solution, the cells were made permeable by incubation in permeabilization buffer (0.1% saponin and 20% NCS in phosphate-buffered saline) at 4 °C for 10 min and then stained with fluorescein isothiocyanate (FITC)-labeled anti-IFN-γ mAb (PharMingen, San Diego, CA). After a thorough washing with the permeabilization buffer, the cells were analyzed by using the FACSCalibur. Similarly IFN-γ production of established CTL clones was analyzed by use of this assay.

2.5. Generation of CTL clones

Peptide-specific CTL clones were generated from established peptide-specific bulk CTLs by seeding 0.8 cells/well into U-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μl of cloning mixture (RPMI 1640 medium containing 10% FCS, 200 U/ml human recombinant interleukin-2, 5×10^5 irradiated allogeneic PBMCs from a healthy donor, and 1×10^5 irradiated C1R-B*4002 cells pulsed with a 1 μM concentration of the appropriate HIV-1-derived peptides. Wells positive for growth after about 2 weeks were examined for CTL activity by performing the ICC assay. All CTL clones were cultured in RPMI 1640 containing 10% FCS and 200 U/ml recombinant human interleukin-2. CTL clones were stimulated biweekly with irradiated target cells pulsed with the corresponding peptides.

2.6. HIV-1 clones

NL-432, which is an infectious proviral clone of HIV-1, was previously reported [7,19].

2.7. HIV-1 infection of .221-CD4-B*4002 and .221-CD4 cells

.221-CD4-B*4002 and 721.221-CD4 cells were exposed to NL-432 for several days. These infected cells were used as stimulator cells for ICC assays when approximately 60% of cells had been infected, which was confirmed by intracellular staining for HIV-1 p24 antigen.

3. Results

3.1. Identification of 11-mer peptides recognized by HLA-B*4001-restricted and HLA-B*4002-restricted HIV-1-specific CD8$^+$ T cells

To identify novel HLA-B*4001-restricted CTL epitopes, we analyzed 5 HIV-seropositive HLA-B*4001$^+$ Japanese individuals by Elispot assays with cocktails of overlapping 11-mer peptides spanning Gag (p17^{Gag}, p24^{Gag}, p2p7p1p6^{Gag}), Pol (Protease, RT, integrase), and Nef. The overlapping 11-mer peptide cocktails that gave more than 200 spots per 10^6 cells were used to stimulate PBMC of each patient in order to identify the epitopes. After the PBMC had been cultured for 2 weeks, their IFN-γ production was analyzed by using the ICC assay. We found that 3 peptide cocktails induced IFN-γ production. Further analysis using 10 peptides in the peptide cocktails showed that three 11-mer peptides included HLA-B*4001-restricted epitopes but all of these peptides contained reported HLA-B*4001-restricted epitope sequences. Thus, we could not find any novel HLA-B*4001-restricted epitopes.

In order to identify CTL epitopes restricted by HLA-B*4002, we analyzed fresh CD8$^+$ T cells from patient KI-400 (A*0207/A*3101, B*4002/B*4601, Cw*0102/Cw*0304) by performing Elispot assays with the cocktails of the overlapping 11-mer peptides. More than 200 spots per 10^6 cells were observed with 7 out of 25 Gag cocktails, 11 out of 50 Pol cocktails, and 1 out of 10 Nef cocktails (data not shown). To find novel HLA-B*4002-restricted CTL epitopes, we focused on analyzing 5 peptide cocktails (Gag21-49, Pol781-809, Pol801-829, Pol901-929, and Pol921-949) that did not contain reported epitopes restricted by the 6 HLA-class I alleles this patient expressed. To determine which peptide in each cocktail induced the specific CD8$^+$ T cells, we stimulated PBMCs from KI-400 with these peptide cocktails and then cultured the cells for 2 weeks. The responsiveness of the cultured CD8$^+$ T cells toward ten 11-mer peptides in each peptide cocktail was measured by using the ICC assay. IFN-γ production was found in the bulk CD8$^+$ T cells stimulated with autologous B-LCLs pre-pulsed with 2 Gag (Gag31-41 and Gag33-43) and 6 Pol peptides (Pol799-809, Pol807-817, Pol909-919, Pol911-921, Pol919-929, and Pol921-931).

For determination of HLA restriction molecules of CD8$^+$ T cells specific for these 11-mer peptides, the responsiveness of the bulk CD8$^+$ T cells towards peptide-pulsed C1R cells expressing one of the HLA-A or -B alleles or .221 cells expressing one of the HLA-C alleles was measured by performing the ICC assay. HLA-B*4002-restricted responses were found in the bulk culture cells stimulated with the cells pre-pulsed with Pol799-809, Pol807-817, Pol909-919, Pol911-921, Pol919-929 or Pol921-931 (data not shown). These results indicate that these six 11-mer peptides included HLA-B*4002-restricted epitopes.

3.2. Identification of HLA-B*4002-restricted optimal epitope peptides

To determine the optimal epitopes for these 11-mer peptides, we stimulated bulk T cells with C1R-B*4002 cells pre-pulsed with truncated peptide of Pol799-809, Pol807-817, Pol909-919, Pol911-921, Pol919-929 or Pol921-931 at concentrations of 1000 nM and then measured the IFN-γ production of each bulk T cells was measured by conducting the ICC assay. Previous studies on HLA-B*4002-restricted epitopes suggested that Glu at position 2 is an anchor for HLA-B*4002 (11–16). Judging from the finding that Pol801-811 did not include HLA-B*4002-restricted epitopes, we speculated that 2E in Pol799-809 (IG11: IEAEVIPAETG) would be the anchor for HLA-B*4002 rather than 4E. We therefore generated 5 truncated peptides (IT10: IEAEVIPAET, IA8: IEAEVIPA, ET9: EAEVIPAET, AT8: AEVIPAET, and AG9: AEVIPAETG) of Pol799-809 and investigated whether CD8$^+$ T cells induced by Pol799-809 would recognize these peptides. The T cells recognized only IG11 and IT10 at 1000 nM (Fig. 1A), whereas they showed higher sensitivity to IT10 than to IG11 (Fig. 1B). These findings indicate that Pol799-808 (IT10) was the optimal epitope.

For Pol807-817 (EL11: ETGQETAYFLL), we generated 4 truncated peptides (TL10: TGQETAYFLL, GL9: GQETAYFLL, GL8: GQETAYFL, and QL8: QETAYFLL). CD8$^+$ T cells induced by the Pol807-817 peptide recognized EL11, TL10, GL9 and QL8, but not GL8 (Fig. 1A), indicating that L at position 11 was critical for the epitope. On the other hand, the T cells showed higher sensitivity to EL11 than to the other 3 peptides (Fig. 1C). These findings indicate that Pol807-817 (EL11) was the optimal epitope.

For Pol909-919 (YI11: YSAGERIVDII) and Pol911-921 (AT11: AGERIVDIIAT), we assumed 2 possibilities: 1) the two 11-mer peptides shared the same epitope, or 2) the two peptides included different epitopes. To clarify these possibilities, we analyzed Pol909-919 and Pol911-921 independently. For Pol909-919, we generated 5 truncated peptides (SI10: SAGERIVDII, SI9: SAGERIVDI, AI8: AGERIVDI, AI9: AGERIVDII, and GI8: GERIVDII). CD8$^+$ T cells induced by Pol909-919 peptide recognized YI11, SI10, AI9, and GI8, but not SI9 and AI8 (Fig. 1A), indicating that I at position 11 was critical for this epitope. On the other hand, they showed higher sensitivity to GI8 than to the other 3 peptides (Fig. 1D). These findings indicate that Pol909-919 (GI8) was the optimal epitope. Regarding Pol911-921 (AT11), we generated 4 truncated peptides (AI9: AGERIVDII, AI8: AGERIVDI, GI8: GERIVDII, and GA9: GERIVDIIA). CD8$^+$ T cells induced by Pol911-921 peptide recognized AT11, AI9, GI8 and GA9, but not AI8 (Fig. 1A), indicating I at position 11 to be critical for this epitope. They also showed higher sensitivity to GI8 than to the other 3 peptides (Fig. 1E), indicating that GI8 (Pol912-919) was the optimal epitope. Thus, these results confirmed that Pol909-919 and Pol911-921 included the same epitope.

For Pol919-929 (IQ11: IATDIQTKELQ) and Pol921-931 (TQ11: TDIQTKELQKQ), we assumed that these two 11-mer peptides shared the same epitope. Therefore, we analyzed Pol919-929 and Pol921-931 independently. Regarding Pol919-929 (IQ11: IATDIQTKELQ) we speculated that 10L would be the C-terminus of the epitope because no hydrophilic residue is found in the C-terminus of HLA class I-binding peptides.

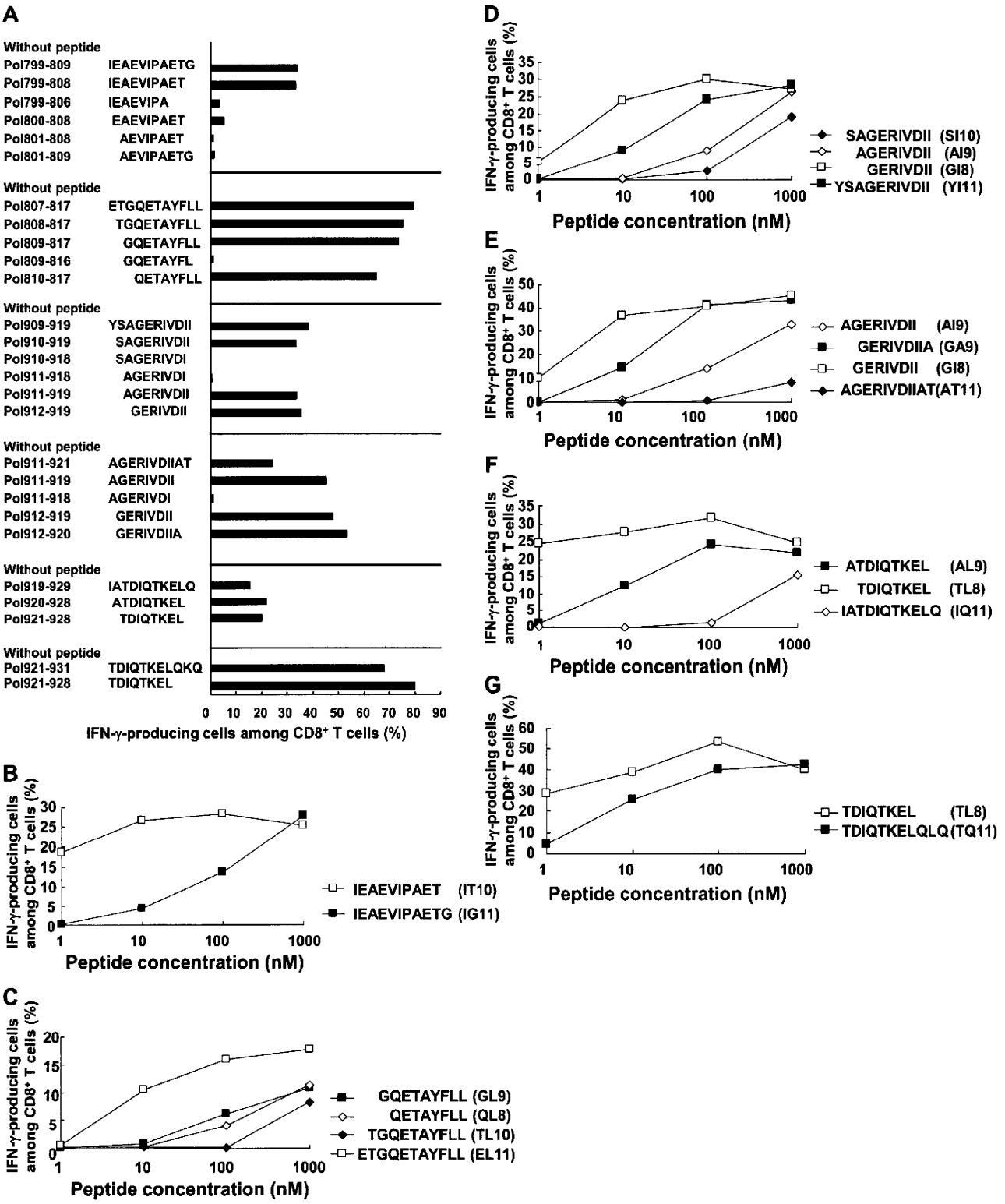

Fig. 1. Identification of HLA-B*4002-restricted HIV-1 CTL epitopes. A. For determination of the optimal epitopes of Pol799-809, Pol807-817, Pol909-919, Pol911-921, Pol919-929 and Pol921-931, the recognition of the bulk T cells for the truncated peptides was examined by using C1R-B*4002 cells pre-pulsed with each truncated peptide at a concentration of 1000 nM. The responsiveness of the bulk $CD8^+$ T cells toward each truncated peptide was measured by using the ICC assay. The percentages of IFN-γ-producing cells among the $CD8^+$ T cells are shown in the figure. B–G. Optimal epitopes were not determined at concentrations of 1000 nM for Pol799-809 (B), Pol807-817 (C), Pol909-919 (D), Pol911-921 (E), Pol919-929 (F) or Pol921-931 (G). The responsiveness of the bulk $CD8^+$ T cells was examined for C1R-B*4002 cells pre-pulsed with each truncated peptide at concentrations from 1 to 1000 nM. The responsiveness of the bulk $CD8^+$ T cells toward each truncated peptide was measured by performing the ICC assay. The percentages of IFN-γ-producing cells among $CD8^+$ T cells are shown in the figure.

Therefore, we generated 2 truncated peptides (AL9: ATDIQTKEL and TL8: TDIQTKEL). Bulk $CD8^+$ T cells induced by Pol919-929 peptide recognized all 3 peptides (Fig. 1A) and showed higher sensitivity to TL8 than to the other 2 peptides (Fig. 1F), indicating that Pol921-928 (TL8) was the optimal epitope. Similarly we speculated TL8 to be optimal epitope for Pol921-931 (TQ11: TDIQTKELQKQ), because no hydrophilic residue is found in the C-terminus of HLA-class I-restricted epitopes. Although bulk $CD8^+$ T cells induced by Pol921-931 peptide recognized both TQ11 and TL8 peptides (Fig. 1A), they showed higher sensitivity to TL8 than to TQ11 (Fig. 1F). These findings indicate that Pol919-929 and Pol921-931 11-mer peptides included the same epitope, Pol921-928(TL8).

Thus, we identified 4 HLA-B*4002-restricted optimal peptides. Interestingly, these 4 Pol epitopes were all derived from integrase.

*3.3. Generation and antigen sensitivity of HLA-B*4002-restricted Pol-specific CTL clones*

To analyze the $CD8^+$ T cells specific for these 4 integrase epitopes, IT10 (Pol799-808), EL11 (Pol807-817), GI8 (Pol912-919), and TL8 (Pol921-928), we established the specific $CD8^+$ T cell clones and analyzed them for their antigen sensitivity by using the ICC assays. The result was shown in Fig. 2. The T cell clones and their EC_{50} values were as follows: Pol799-808-specific T cells (27.7), Pol807-817-specific T cells (191.7), Pol912-919-specific T cells (443.1), and Pol921-928-specific T cells (7.6). These results indicate that Pol799-808-specific and Pol921-928-specific $CD8^+$ T cell clones had higher antigen sensitivity than Pol807-817-specific and Pol912-919-specific ones.

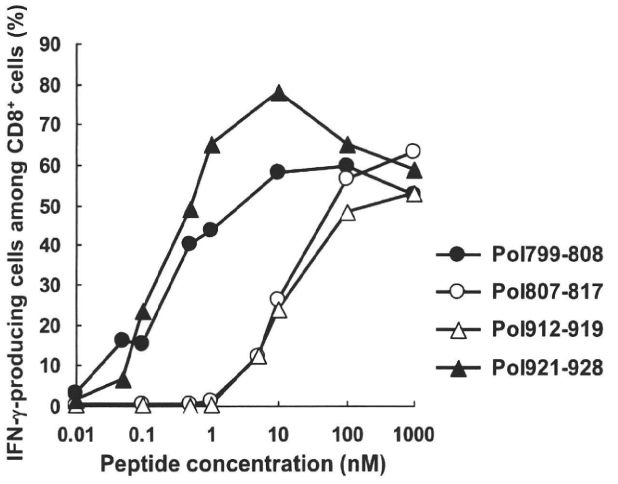

Fig. 2. Antigen Sensitivity of 4 HIV-1 integrase-specific $CD8^+$ T cells. Antigen sensitivity of 4 HIV-1 integrase-specific $CD8^+$ T cells was examined by using the ICC assay. The responsiveness of these CTL clones was examined for C1R-B*4002 cells pre-pulsed with each truncated peptide at concentrations from 0.01 to 1000 nM.

3.4. Recognition of HIV-1-infected cells by specific T cells

To clarify whether Pol799-808, Pol807-817, Pol912-919, and Pol921-928 were naturally occurring peptides and whether CTLs specific for these epitopes had the ability to recognize HIV-1-infected cells, we investigated the response of these peptide-specific $CD8^+$ T cell clones toward HIV-1 (NL-432)-infected .221-CD4 cell lines expressing HLA-B*4002. NL-432 includes wild-type sequences of these 4 epitopes. .221-CD4 cell lines and those expressing HLA-B*4002 were infected with NL-432, and then cultured for 4 days. The responses of the T cell clones toward these infected cells were measured by using the ICC assay. The percentage of the HIV-1-infected cells was determined by staining intracellular HIV-1 p24 (Fig. 3A). The Pol799-808-specific, Pol807-817-specific, Pol912-919-specific, and Pol921-928-specific CTL clones responded to .221-CD4-B*4002 cells infected with HIV-1 but not to uninfected .221-CD4-B*4002 cells or to HLA-B*4002-negative .221-CD4 cells infected with HIV-1. These results indicate that Pol799-808, Pol807-817, and Pol921-928 peptides were naturally processed and presented by HLA-B*4002 and that the T cells specific for these epitopes could recognize HIV-1-infected cells (Fig. 3B). On the other hand, the responses of Pol807-817-specific and Pol912-919-specific CTL clones was much weaker than those of the other CTL clones (Fig. 3B), indicating that the former CTLs only weakly recognized HIV-1-infected cells.

4. Discussion

There is only 1 amino acid substitution, at residue 97, on the peptide binding floor between HLA-B*4001 and HLA-B*4002. A previous study on the peptide motif of HLA-B*4001 showed that HLA-B*4001-binding peptide anchors are Glu at P2 (2E) and Leu at the C-terminus [20]. Indeed, 7 of 8 reported HLA-B*4001-restricted HIV-1-specific T cell epitopes have 2E and Leu at their C-terminus [11–13]. Although no HLA-B*4002-binding peptide motif had not yet been identified, we speculated that this motif would be similar to the HLA-B*4001-binding one. Indeed, all 7 HLA-B*4002-restricted epitopes previously reported have 2E (Table 1). However, 2 of the 4 epitopes identified in the present study did not have the 2E anchor. In addition, only 5 of 11 HLA-B*4002-restricted epitopes had Leu at their C-terminus. These findings suggest that the substitution from Ser to Arg at residue 97 may partially affect the structure of the F and B pockets. Pol807-817 (ETGQETAYFLL) does not have the 2E anchor. QL8 (QETAYFLL) is speculated to be an HLA-B*4002-restricted epitope because this peptide has 2E. However, the antigen sensitivity of the T cells specific for QL8 is much weaker than that for EL11. This result excludes the possibility that QL8 is the epitope peptide. Thr at position 2 of Pol807-817 may bind to the residues facing the B-pocket by hydrogen-bonding. Nine of the 11 HLA-B*4002-restricted epitopes have 2E, suggesting that the 2E is still anchor residue for HLA-B*4002.

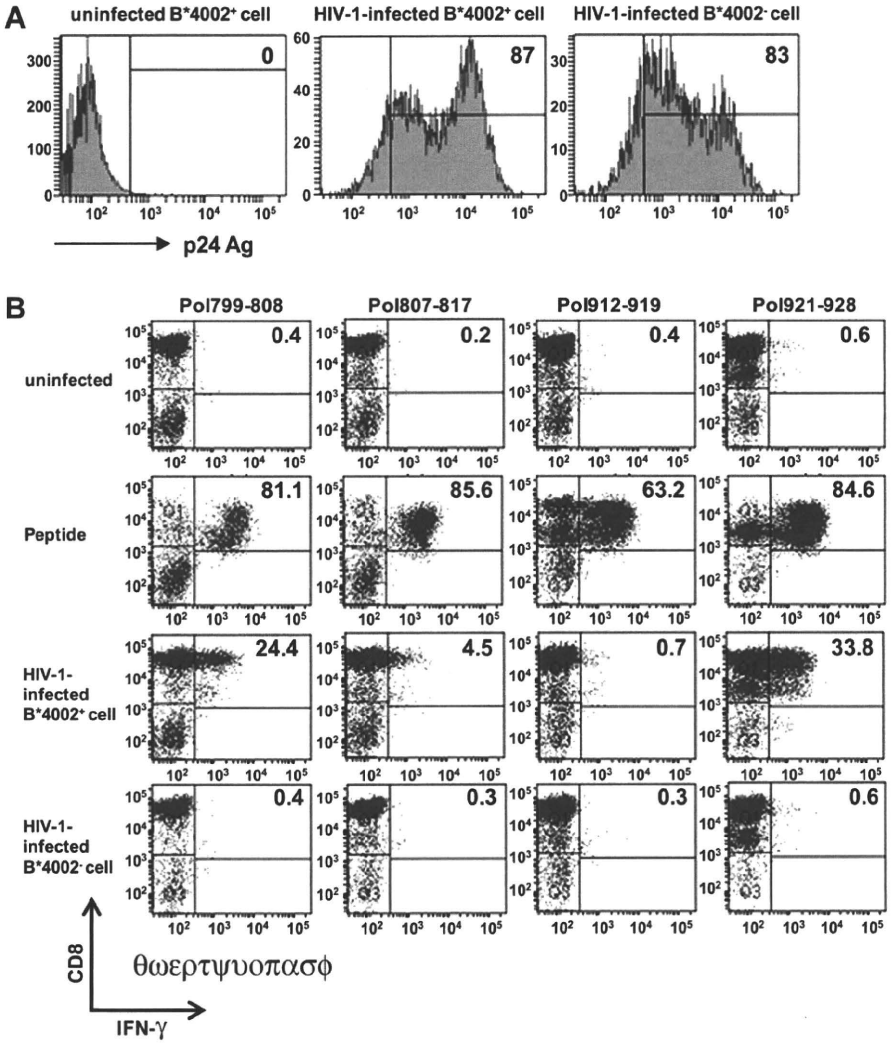

Fig. 3. Ability of 4 HIV-1 integrase-specific $CD8^+$ T cells to recognize HIV-1-infected cells. A. The .221-CD4 and $B*4002^+$.221-CD4 cell lines were infected with HIV-1 (NL-432) and cultured for 4 days. The frequency of HIV-1-infected cells was detected by using staining of intracellular p24 with anti-p24 mAb. The percentage of HIV-1-infected cells is shown in each figure. B. Recognition of HIV-1-infected cells by the Pol799-808-, Pol807-817-, Pol912-919- or Pol921-928-specific $CD8^+$ T cell clones. The activities of these peptide-specific $CD8^+$ T cell clones to recognize $B*4002^+$.221-CD4 cell lines infected with HIV-1 or those pre-pulsed with the corresponding peptide (1000 nM) were measured by use of the ICC assay. The percentages of IFN-γ-producing cells among $CD8^+$ T cells are shown in each figure.

Although the 7 HLA-B*4002-restricted epitopes previously reported do not include Pol-derived ones, we identified novel 4 HLA-B*4002-retricted Pol-specific T cell epitopes in the present study. Interestingly, all of these Pol epitopes were derived from integrase. Though 29 integrase epitopes were reported as 20 different HLA class I-restricted epitopes (Los Alamos HIV Molecular Immunology Data), integrase epitopes were not found among HLA-B*4001-restricted Pol epitopes. Regarding the integrase epitopes, HLA-B*4201 and HLA-B*1503 present 3 different epitopes, whereas the other 18 alleles present 1 or 2 epitopes. Thus, HLA-B*4002 is so far the only HLA-class I allele that can present more than 3 integrase epitopes.

Pol799-808-specific and Pol921-928-specific T cells strongly recognized HIV-1-infected cells, whereas Pol807-817-specific and Pol912-919-specific ones weakly recognized these cells. Antigen sensitivity of the former T cells was much higher than that of the latter ones. Thus, the ability to recognize HIV-1-infected cells was associated with the antigen sensitivity. However, it is difficult to clarify why the 2 T cells weakly recognize HIV-1-infected cells because we did not measure the bindings of these epitope peptides to HLA-B*4002 molecules and of the specific tetramers to the specific T cells. We can suggest 2 possibilities from the data shown in Fig. 2 and Fig. 3: 1) The former T cells may have higher affinity TCR and/or 2) these former epitope peptides are more highly presented than the latter by HLA-B*4002 in HIV-1-infected cells. Since Pol799-808-specific and Pol921-928-specific T cells strongly recognized HIV-1-infected cells, we proposed that they would effectively recognize and kill HIV-1-infected cells *in vivo*.

HLA-B*4001 and HLA-B*4002 are found in 10.8% and 16.6% of the Japanese population, respectively. Since both

Table 1
A list of HLA-B*4002-restricted epitopes identified previously and in this study.

Sequence	Protein	Reference
GELDRWEKI	Gag (p17)	*15
KETINEEAA	Gag (p24)	*15
AEWDRVHPV	Gag (p24)	*15
AEAMSQVTNS	Gag (p2p7p1p6)	*16
TERQANFL	Gag (p2p7p1p6)	*15
REPHNEWTL	Vpr	*14
KEKGGLEGL	Nef	*15
IEAEVIPAET	Pol (Integrase)	This study (Pol799-808)
ETGQETAYFLL	Pol (Integrase)	This study (Pol807-817)
GERIVDII	Pol (Integrase)	This study (Pol912-919)
TDIQTKEL	Pol (Integrase)	This study (Pol921-928)

HLA-class I alleles are detected in approximately 25% of Japanese individuals, T cell epitopes presented by these alleles are useful for studies on HIV-1 immunopathogenesis and the development of AIDS vaccines.

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Different *In Vivo* Effects of HIV-1 Immunodominant Epitope-Specific Cytotoxic T Lymphocytes on Selection of Escape Mutant Viruses[▽‡]

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HIV-1 escape mutants are well known to be selected by immune pressure via HIV-1-specific cytotoxic T lymphocytes (CTLs) and neutralizing antibodies. The ability of the CTLs to suppress HIV-1 replication is assumed to be associated with the selection of escape mutants from the CTLs. Therefore, we first investigated the correlation between the ability of HLA-A*1101-restricted CTLs recognizing immunodominant epitopes *in vitro* and the selection of escape mutants. The result showed that there was no correlation between the ability of these CTLs to suppress HIV-1 replication *in vitro* and the appearance of escape mutants. The CTLs that had a strong ability to suppress HIV-1 replication *in vitro* but failed to select escape mutants expressed a higher level of PD-1 *in vivo*, whereas those that had a strong ability to suppress HIV-1 replication *in vitro* and selected escape mutants expressed a low level of PD-1. *Ex vivo* analysis of these CTLs revealed that the latter CTLs had a significantly stronger ability to recognize the epitope than the former ones. These results suggest that escape mutations are selected by HIV-1-specific CTLs that have a stronger ability to recognize HIV-1 *in vivo* but not *in vitro*.

HIV-1-specific cytotoxic T lymphocytes (CTLs) have an important role in the control of HIV-1 replication during acute and chronic phases of an HIV-1 infection (5, 28, 33). On the other hand, HIV-1 can escape from the host immune system by various mechanisms. These may include the appearance of HIV-1 carrying escape mutations in its immunodominant CTL epitopes as well as Nef-mediated downregulation of HLA class I molecules. There is a growing body of evidence for the former mechanism, i.e., that CTLs targeting immunodominant HIV-1 epitopes select escape mutants in chronically HIV-1-infected individuals (18, 20, 36), whereas the latter mechanism was proved by demonstrating that HIV-1-specific CTLs fail to kill Nef-positive-HIV-1-infected $CD4^+$ T cells but effectively kill Nef-defective-HIV-1-infected ones or that they suppress the replication of Nef-defective HIV-1 much more than that of Nef-positive HIV-1 (12, 13, 42, 45).

It is speculated that HIV-1 immunodominant epitope-specific CTLs have the ability to suppress HIV-1 replication and effectively select escape mutants. However, the correlation between this ability of the CTLs and the appearance of escape mutants is still unclear, because it is not easy to evaluate the ability of HIV-1-specific CTLs to exert a strong immune pressure *in vivo*. To examine this ability, most previous studies measured the number of HIV-1-specific CTLs or $CD8^+$ T cells and the CTL activity against target cells prepulsed with the epitope peptide or those infected with HIV-1 recombinant vaccinia virus (6, 7, 23, 46). However, the results obtained from such experiments do not reflect the ability of the CTLs to exert immune pressure *in vivo*. We and other groups previously utilized an assay to directly evaluate the ability of the CTLs to suppress HIV-1 replication *in vitro* (1, 17, 18, 42, 43). This assay may be better for evaluation of immune pressure by HIV-1-specific CTLs than other assays, because the ability of the CTLs to suppress HIV-1 replication is directly measured in cultures of HIV-1-infected $CD4^+$ T cells incubated with HIV-1-specific CTL clones. But it still remains unknown whether this assay reflects immune pressure *in vivo*.

In the present study, we investigated whether HIV-1-specific CTLs having a strong ability to suppress HIV-1 replication could positively select escape mutants. Since HLA-A*1101 is known to be an HLA allele relatively associated with a slow progression to AIDS (32), it is speculated that some HLA-A*1101-restricted CTLs would have a strong ability to suppress HIV-1 replication *in vitro*. Therefore, we first focused on 4 well-known HLA-A*1101-restricted CTL epitopes in the present study. We investigated the frequency of CTLs specific for these epitopes in chronically HIV-1-infected individuals, the ability of these CTLs to suppress HIV-1 replication *in vitro*, and whether the escape mutants were selected by the CTLs. Furthermore, we analyzed the expression of Programmed Death-1 (PD-1) on these CTLs *ex vivo* and antigen recognition of them.

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MATERIALS AND METHODS

Patient samples. Informed consent was obtained from all subjects according to the Declaration of Helsinki. For sequence analysis, blood specimens were collected in EDTA. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood. Patient HLA type was determined by standard sequence-based genotyping.

Sequence of autologous virus. Viral RNA was extracted from samples of plasma from HIV-1-infected patients by the use of a QIAamp MinElute virus spin kit (Qiagen), and cDNA was synthesized from the RNA with SuperScript RNase H-reverse transcriptase and random primers (Invitrogen). The Nef region and the Gag region were amplified by nested PCR using *Taq* DNA polymerase (Promega). The PCR products were then agarose gel purified and sequenced directly or cloned by use of a TOPO TA cloning kit (Invitrogen). All DNA sequencing was performed by using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) and an ABI Prism 310 genetic analyzer. The regions of Gag349, Nef73, and Nef84 epitopes were sequenced directly in 124, 121, and 122 individuals, respectively, while those of Nef73 and Nef84 epitopes were sequenced for cloned samples from 10 and 11 individuals, respectively.

Cells. C1R cells expressing HLA-A*1101 (C1R-A*1101) and transporter associated with antigen processing (TAP)-defective RMA-S cells expressing HLA-A*1101 (RMA-S-A*1101) were previously generated and were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.15 mg/ml hygromycin B.

Generation of CTL clones. Peptide-specific CTL clones were generated from an established peptide-specific bulk CTL culture by seeding 0.8 cell/well into U-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μl of cloning mixture (RPMI 1640 medium supplemented with 10% FCS and 200 U/ml human recombinant interleukin-2, 5×10^5 irradiated allogeneic PBMC from a healthy individual, and 1×10^5 irradiated C1R-A*1101 cells prepulsed with a 1 μM concentration of the corresponding peptide, Gag349 [ACQGVG GPGHK], Nef73 [QVPLRPMTYK], or Nef84 [AVDLSHFLK]). Wells positive for growth after about 2 weeks were transferred to 48-well plates together with 1 ml of the cloning mixture. The clones were examined for CTL activity by the standard ^{51}Cr release assay. All CTL clones were cultured in RPMI 1640-10% FCS supplemented with 200 U/ml recombinant human interleukin-2 and were stimulated weekly with irradiated target cells prepulsed with the appropriate HIV-1-derived peptide.

HIV-1 clones. Infectious proviral clones of HIV-1, pNL-432, and its Nef mutant, pNL-M20A (containing a substitution of Ala for Met at residue 20 of Nef), reported previously, were used (2). For pNL-432-Nef84-2L9R, the mutation was introduced by site-directed mutagenesis (Invitrogen).

CTL assay for target cells pulsed with HIV-1 peptide. Cytotoxicity activity was measured by the standard ^{51}Cr release assay, as previously described (34). Target cells (2×10^5) were incubated for 60 min with 100 μCi $Na_2{}^{51}CrO_4$ in saline and then washed three times with RPMI 1640 medium containing 10% newborn calf serum (NCS). Labeled target cells (2×10^3/well) were added to 96-well round-bottom microtiter plates (Nunc) along with the appropriate amount of the corresponding peptide. After a 1-h incubation, effector cells were added, and the mixtures were then incubated for 4 h at 37°C. The supernatants were collected and analyzed with a gamma counter.

Intracellular cytokine (ICC) production assay. PBMCs from HLA-A*1101-positive HIV-1-infected patients were stimulated with a given peptide (1 μM) in culture medium (RPMI 1640 medium supplemented with 10% FCS and 200 U/ml recombinant human interleukin-2). After 14 days in culture, the cells were assessed for gamma interferon (IFN-γ) production activity by using a FACSCalibur instrument. Briefly, bulk cultures were stimulated by C1R-A*1101 cells pulsed with or without the corresponding peptide (1 μM) for 2 h at 37°C. Brefeldin A (10 μg/ml) was then added, and the cultures were continued for an additional 4 h. Cells were collected and stained with 7-amino-actinomycin D (7-AAD) at room temperature for 10 min. After 2 washes with RPMI 1640 medium supplemented with 10% FCS, cells were stained with phycoerythrin (PE)-labeled anti-CD8 monoclonal antibody (MAb) (Dako Corporation, Glostrup, Denmark). After having been treated with 4% paraformaldehyde solution, the cells were permeabilized in permeabilization buffer (0.1% saponin and 20% NCS in phosphate-buffered saline) at 4°C for 10 min and stained with fluorescein isothiocyanate (FITC)-labeled anti-IFN-γ MAb (PharMingen, San Diego, CA). After a thorough washing with the permeablization buffer, the cells were analyzed by using the FACSCalibur instrument. Nonspecific binding of anti-IFN-γ MAb and nonspecific production of IFN-γ were excluded by subtracting the data of the negative control, which was the same sample stimulated with C1R-A*1101 cells without the specific peptide and stained with the same MAbs.

For *ex vivo* analysis, PBMCs from HLA-A*1101-positive HIV-1-infected patients were stimulated with the corresponding peptide (1 μM), and IFN-γ production was measured 6 h later, as described above.

HLA class I stabilization assay. The binding of peptides to HLA-A*1101 molecules was tested as previously described (11). RMA-S-A*1101 cells transfected with HLA-A*1101 and human β_2-microglobulin were used. These cells express a very low level of HLA class I molecules on their cell surface when they are cultured at 37°C, whereas empty HLA class I molecules are stably expressed if they are cultured at 26°C. The stabilization of HLA class I molecules is dependent on peptide binding affinity (22, 30, 40). Briefly, RMA-S-A*1101 cells were cultured at 26°C for 14 to 18 h. The cells were incubated at 26°C for 1 h with Nef84 (AVDLSHFLK), Nef84-2L (ALDLSHFLK), or Nef84-2L9R (ALDLSHFLR) peptide at various concentrations and then at 37°C for 3 h. After 2 washes with phosphate-buffered saline (PBS) supplemented with 20% FCS (PBS-20% FCS), they were subsequently incubated for 30 min on ice with an appropriate dilution of MAb TP25.99 (41). After 2 washes with PBS-20% FCS, the cells were incubated for 30 min on ice with an appropriate dilution of FITC-conjugated sheep IgG with anti-mouse Ig specificity (Silenus Laboratories, Hawthorn, Australia). Finally, they were washed three times with PBS-20% FCS, after which the fluorescence intensity was measured by using a flow cytometer (Becton Dickinson, Mountain View, CA).

Surface expression of HLA class I molecules on HIV-1-infected cells. To assess HLA class I expression on HIV-1-infected $CD4^+$ T cells, we stained the cells with anti-HLA-A11 MAb followed by PE-labeled anti-mouse Ig (Pharmingen International, San Diego) and thereafter fixed and permeabilized them for intracellular HIV-1 p24 staining with FITC-labeled anti-p24 MAb KC-57. The expression of HLA class I molecules on HIV-1infected $CD4^+$ T cells was examined by using the FACSCalibur instrument with Cell Quest software (Becton Dickinson, San Jose, CA).

Suppression of HIV-1 replication by HIV-1-specific CTL clones. The ability of HIV-1-specific CTL clones to suppress HIV-1 replication was examined as previously described (42). $CD4^+$ T cells purified by means of anti-human CD4 MAb-coated magnetic beads (MACS beads; Miltenyi Biotec) from PBMCs of an HIV-1-seronegative individual with HLA-A*1101 were cultured and infected with HIV-1 clones. Cultured $CD4^+$ T cells were incubated with an HIV-1 clone for 4 h at 37°C with intermittent agitation and then washed three times with RPMI 1640 medium supplemented with 10% FCS. HIV-1-infected $CD4^+$ T cells were cocultured with an HIV-1-specific CTL clone in culture medium. From day 2 to day 7 postinfection, 10 μl of culture supernatant was collected, and the concentration of p24 antigen (Ag) in the supernatant was measured by conducting an enzyme immunoassay (HIV-1 p24 Ag enzyme-linked immunosorbent assay [ELISA] kit; ZeptoMetrix). Percent suppression was calculated as follows: (concentration of p24 Ag in the supernatant of HIV-1-infected $CD4^+$ T cells cultured with HIV-1-specific CTLs/concentration of p24 Ag in the supernatant of HIV-1-infected $CD4^+$ T cells cultured without the CTLs) × 100.

HLA-peptide tetrameric complexes. The tetrameric complexes of HLA-A*1101, HLA-A*2402, and HLA-A*2601 were synthesized as previously described (3). The purified complexes were enzymatically biotinylated at a BirA recognition sequence located at the C terminus of the heavy chain and were mixed with PE- or allophycocyanin (APC)-conjugated avidin (Molecular Probes) at a molar ratio of 4:1.

Analysis of PD-1 or CD27 CD28 CD45RA expression on HIV-1-specific $CD8^+$ T cells. For the analysis of PD-1 expression, cryopreserved PBMCs of HIV-positive individuals were first stained with Pacific Blue-conjugated CD8 MAb (BD Bioscience) and FITC-conjugated CD3 MAb (Dako Corporation, Glostrup, Denmark) at 4°C for 30 min followed by PE-conjugated PD-1 MAb (BD Bioscience) at the room temperature for 30 min. After 2 washes with RPMI 1640 medium supplemented with 10% FCS, the cells were stained with allophycocyanin (APC)-conjugated tetramer at 37°C for 30 min. After 2 additional washes, the cells were stained with 7-AAD (BD Bioscience) at room temperature for 10 min and analyzed by using flow cytometry (FACS Canto II; BD Bioscience). For the phenotypic analysis of HIV-1-specific $CD8^+$ T cells, the PBMCs were first stained with PE-Cy7-conjugated anti-CD3 (BioLegend), Pacific Blue-conjugated CD8 (BD Bioscience), FITC-conjugated anti-CD27 (BD Bioscience), PE-conjugated anti-CD28 (BioLegend), and phycoerythrin-Texas red (ECD)-conjugated anti-CD45RA (Beckman Coulter) MAbs at 4°C for 30 min. After 2 washes with RPMI 1640 medium supplemented with 10% FCS, the cells were stained with APC-conjugated tetramer at 37°C for 30 min. After 2 additional washes, the cells were stained with 7-AAD at room temperature for 10 min and analyzed by using flow cytometry.

Enzyme-linked immunospot (ELISPOT) assay. Cryopreserved PBMCs of 2 HLA-A*1101$^+$ HIV-1-infected individuals (KI-015 and KI-036) were plated out in 96-well polyvinylidene plates (Millipore, Bedford, MA) which had been pre-