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Identification of HLA-A*3101-restricted cytotoxic T-lymphocyte response to human immunodeficiency virus type 1 (HIV-1) in patients with chronic HIV-1 infection

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Key words
CTL; epitope; HIV-1; HLA-A*3101; peptide

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Abstract

Virus-specific cytotoxic T-lymphocyte (CTL) responses are critical in the control of human immunodeficiency virus type 1 (HIV-1) infections. As several HIV-1 CTL epitopes restricted to many HLA types are already known, we aimed at identifying the CTL epitopes restricted by HLA-A*3101 in an effort to expand the epitope repertoire available for the development of potential T cell-mediated therapeutic measures and protective vaccines. Scanning of HIV-1 clade B SF2 strain proteins for the presence of peptides containing HLA-A*3101-binding motifs revealed 88 nine- to 11-mer peptides that had been synthesized and assayed for binding to HLA-A*3101 molecules. Peptides with medium to high HLA-binding affinity were tested for their ability to stimulate a CTL response in the peripheral blood mononuclear cells (PBMCs) from selected HIV-1-infected patients. Two of these binding peptides, Env769–779 (RLRDLLLIAAR) and Nef192–200 (KLAFHHMAR), induced peptide-specific CTLs in PBMCs from at least two of five HIV-1-seropositive individuals. CTL clones specific for the two peptides killed HLA-A*3101-expressing target cells infected with HIV-1 recombinant vaccinia virus, indicating that these peptides were naturally processed HLA-A*3101-restricted CTL epitopes. Identification of T-cell epitopes on HIV-1 proteins will increase our understanding of the role of $CD8^+$ T cells in HIV-1 infections and assist in the design of new protective strategies.

Introduction

Human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocytes (CTLs) are considered to play a central role in the immune response against HIV-1 (1–3). High levels of HIV-1-specific CTLs are detectable in subjects with asymptomatic chronic infection (4–8), but their level generally declines with disease progression (9). Furthermore, *in vitro* studies have demonstrated potent inhibition of viral replication by HIV-1-specific CTL, mediated by both lytic and non-lytic mechanisms (10); and *in vivo*, there is strong evidence that AIDS viruses evolve to escape CTL recognition by undergoing epitope-specific mutations (11–14). The critical role of virus-specific CTL responses for the control of viraemia has been directly demonstrated by $CD8^+$ T-cell depletion studies in simian immunodeficiency virus infection in macaques, which show that $CD8^+$ T cells effectively suppress viral replication (15, 16). Recent data derived from HIV-1-infected individuals who were treated during an acute HIV-1 infection showed enhancement of both CTL and T-helper cell responses against HIV-1 associated with subsequent viral control following supervised treatment interruptions (17). These data suggest that the induction of HIV-1-specific CTL responses *in vivo* may help prevent infection of normal individuals or attenuate HIV-1 disease in infected individuals.

The precise characterization of epitope-specific CTL responses is important for assessing HIV pathogenesis and vaccine candidates. However, viral escape from CTL recognition may limit the durability of the potential

protection afforded by such vaccines (18, 19). Moreover, association of escape mutations with particular host HLA class I alleles (20) may further confine the use of universal vaccines and support the development of population-based vaccines. To achieve this goal, we need to identify and characterize CTL epitopes restricted to particular HLA class I alleles in a given area.

HLA-A31 is one of the common alleles in Asia (21). Of the 12 reported HLA-A31 subtypes (A*3101–A*3112) (IMGT/HLA database: http://www.ebi.ac.uk/imgt/hla) (22), HLA-A*3101 is the most common (21). Therefore, the identification of HLA-A*3101-restricted HIV-1 epitopes is important in studies of immunopathogenesis and for vaccine development in Asia. We previously showed reverse immunogenetics to be a useful method to identify HIV-1-specific CTL epitopes presented by HLA-A*2402, HLA-A*2601, HLA-A*3303 and HLA-B*5101 (23–26). In this study, potential epitopes were identified by scanning HIV-1 proteins for peptides containing the HLA-A*3101 motif. Peptide-specific $CD8^+$ T cells were measured by counting interferon-γ (IFN-γ)-producing $CD8^+$ T cells after stimulating peripheral blood mononuclear lymphocytes (PBMCs) from HIV-1-infected HLA-A*3101$^+$ individual with HLA-A*3101-binding HIV-1 peptides. $CD8^+$ T-cell epitopes were finally identified by testing whether peptide-specific $CD8^+$ T-cell clones could kill target C1R-A*3101 cells infected with HIV-1 recombinant (r-HIV-1) vaccinia virus.

Materials and methods

Patients

Blood samples were collected with informed consent from five HLA-A*3101$^+$ individuals with HIV-1 infection (KI-033, KI-039, KI-126, KI-147 and KI-178).

Cells

RMA-S cells expressing HLA-A*3101 (RMA-S-A*3101) were previously generated (22). C1R cells expressing HLA-A*3101 (C1R-A*3101) were generated by transfecting C1R cells, which express HLA-Cw4 and HLA-B*3503, with the HLA-A*3101 gene. C1R-A*3101 and RMA-S-A*3101 cells were maintained in RPMI-1640 medium containing 10% foetal calf serum (FCS) and 0.15 mg/ml of hygromycin B. C1R and RMA-S cells were cultured in RPMI-1640 medium supplemented with 10% FCS.

Synthetic peptides

Sequences derived from proteins of HIV-1 SF2 strain were screened for HLA-A*3101-binding motifs. Peptides were prepared by an automated multiple peptide synthesizer and examined by high-performance liquid chromatography and mass spectrometry (HiPep Laboratories, Kyoto, Japan). Peptides with more than 90% purity were used in this study (Table 1).

HLA stabilization assay

Binding of HIV-1-derived peptides to HLA-A*3101 was examined by the HLA stabilization assay as previously described (22). Briefly, RMA-S-A*3101 cells were cultured for 16 h at 26°C and then pulsed with peptides (10^{-3}–10^{-7} M) for 1 h at 26°C. After further incubation for 3 h at 37°C, the peptide-pulsed cells were stained with anti-HLA class I α3 domain-specific monoclonal antibody TP25.99 (a generous gift from Dr S. Ferrone, Roswell Park Cancer Institute Buffallow, NY) followed by fluorescein isothiocyanate-conjugated IgG fraction of sheep anti-mouse immunoglobulin (Silenius Laboratories, Hawthorn, Victoria, Australia). The mean fluorescence intensity (MFI) was measured by flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA). The MFI of RMA-S-A*3101 cells was obtained by subtracting the MFI value of RMA-S-A*3101 cells incubated for 3 h at 37°C from that of control cells cultured at 26°C. HLA-A*3101-binding peptides were defined as those that at a concentration of 10^{-3} M caused $>25\%$ increase in MFI compared with control RMA-S-A*3101 cells cultured at 26°C. The peptide concentration that yielded the half-maximal MFI level (BL_{50} value) was calculated. Binding peptides were classified into three categories according to their BL_{50}: high binding ($BL_{50} < 10^{-5}$ M), medium binding (BL_{50} 10^{-5}–10^{-4} M) and low binding ($BL_{50} \geq 10^{-4}$ M) (Table 1).

Detection of peptide-specific interferon-γ-producing $CD8^+$ T cells in PBMCs from HIV-1-infected HLA-A*3101$^+$ individuals

PBMCs (5×10^5) from HIV-1-seropositive or HIV-1-seronegative individuals expressing HLA-A*3101 were stimulated with HLA-A*3101-binding peptides. After 10–14 days of culture, IFN-γ production activity was determined by flow cytometry. Briefly, bulk cultures were stimulated by C1R-A*3101 cells pulsed with the corresponding peptide cocktail or individual peptide (1 μM) for 2 h at 37°C (Table 2). Brefeldin A (10 μg/ml) was then added, and the culture was continued for an additional 4 h. The cells were then collected and stained with anti-CD8 monoclonal antibody (Dako Corporation, Glostrup, Denmark). After having been treated with 4% paraformaldehyde solution, the cells were permeabilized in permeabilization buffer [0.1% saponin and 20% newborn calf serum (NCS) in phosphate-buffered saline] at 4°C for 10 min and stained with anti-IFN-γ monoclonal antibody (PharMingen, San Diego, CA). After thorough washing with the permeabilization buffer, the cells were analysed by flow cytometry (FACS Calibur; BD Biosciences).

Table 1 HLA-A *3101-binding HIV-1 SF2-derived peptides

Peptide	Protein	Sequence	BL_{50}
SF2-Env373–381	Env	IVMHSFNCR	8.1×10^{-6}
SF2-Env769–779	Env	RLRDLLLIAAR	1.2×10^{-5}
SF2-Env835–845	Env	AYRAILHIHRR	1.3×10^{-5}
SF2-Env835–844	Env	AYRAILHIHR	1.4×10^{-5}
SF2-Env827–837	Env	RVIEVAQRAYR	1.8×10^{-5}
SF2-Env193–201	Env	NYRLIHCNR	1.9×10^{-5}
SF2-Env568–578	Env	TVWGIKQLQAR	2.5×10^{-5}
SF2-Env761–769	Env	SLCLFSYRR	2.6×10^{-5}
SF2-Env578–587	Env	RVLAVERYLR	2.7×10^{-5}
SF2-Env696–706	Env	IVFAVLSIVNR	3.5×10^{-5}
SF2-Env574–584	Env	QLQARVLAVER	3.6×10^{-5}
SF2-Env579–587	Env	VLAVERYLR	3.7×10^{-5}
SF2-Env763–771	Env	CLFSYRRLR	4.1×10^{-5}
SF2-Env761–771	Env	SLCLFSYRRLR	4.2×10^{-5}
SF2-Env699–708	Env	AVLSIVNRVR	4.2×10^{-5}
SF2-Env839–847	Env	ILHIHRRIR	1.0×10^{-4}
SF2-Env464–474	Env	EVFRPGGGDMR	$>10^{-3}$
SF2-Env247–255	Env	TVQCTHGIR	$>10^{-3}$
SF2-Env758–768	Env	DLRSLCLFSYR	$>10^{-3}$
SF2-Env710–719	Env	GYSPLSFQTR	$>10^{-3}$
SF2-Env531–541	Env	AVSLTLTVQAR	$>10^{-3}$
SF2-Env700–708	Env	VLSIVNRVR	$>10^{-3}$
SF2-Env547–556	Env	IVQQQNNLLR	$>10^{-3}$
SF2-Env719–728	Env	RLPVPRGPDR	$>10^{-3}$
SF2-Pol60–70	Pol	TLWQRPLVTIR	1.6×10^{-5}
SF2-Pol430–439	Pol	KVKQLCKLLR	1.7×10^{-5}
SF2-Pol228–238	Pol	KLVDFRELNKR	3.0×10^{-5}
SF2-Pol813–822	Pol	AYFLLKLAGR	4.3×10^{-5}
SF2-Pol894–902	Pol	AVFIHNFKR	8.4×10^{-5}
SF2-Pol974–984	Pol	VVPRRKAKIIR	$>10^{-3}$
SF2-Gag30–39	Gag	KLKHIVWASR	6.4×10^{-6}
SF2-Gag34–43	Gag	IVWASRELER	9.2×10^{-6}
SF2-Gag144–152	Gag	MVHQAISPR	7.8×10^{-5}
SF2-Gag81–91	Gag	TVATLYCVHQR	$>10^{-3}$
SF2-Gag298–307	Gag	YVDRFYKTLR	$>10^{-3}$
SF2-Gag278–288	Gag	MYSPTSILDIR	$>10^{-3}$
SF2-Gag484–492	Gag	ELYPLTSLR	$>10^{-3}$
SF2-Gag297–307	Gag	DYVDRFYKTLR	$>10^{-3}$
SF2-Gag354–363	Gag	GVGGPGHKAR	$>10^{-3}$
SF2-Nef192–200	Nef	KLAFHHMAR	8.9×10^{-5}
SF2-Nef73–81	Nef	PVRPQVPLR	1.6×10^{-4}
SF2-Nef100–110	Nef	GLEGLIWSQRR	$>10^{-3}$
SF2-Vif9–19	Vif	IVWQVDRMRIR	5.3×10^{-6}
SF2-Vif165–173	Vif	SVKKLTEDR	8.9×10^{-6}
SF2-Vif6–15	Vif	QVMIVWQVDR	3.5×10^{-5}
SF2-Vif1–9	Vif	IVWQVDRMR	8.8×10^{-5}
SF2-Vif124–132	Vif	ILGYRVSPR	2.1×10^{-4}
SF2-Vif80–90	Vif	HLGQGVAIEWR	$>10^{-3}$
SF2-Vif68–77	Vif	TYWGLHTGER	$>10^{-3}$
SF2-Tat42–52	Tat	GLGISYGRKKR	$>10^{-3}$
SF2-Tat46–55	Tat	SYGRKKRRQR	$>10^{-3}$
SF2-Tat46–56	Tat	SYGRKKRRQRR	$>10^{-3}$
SF2-Tat68–78	Tat	SLSKQPASQSR	$>10^{-3}$
SF2-Vpu26–35	Vpu	VLIEYRKILR	3.5×10^{-5}
SF2-Vpu25–35	Vpu	IVLIEYRKILR	$>10^{-3}$
SF2-Vpr63–73	Vpr	ILQQLLFIHFR	5.2×10^{-5}

HIV-1, human immunodeficiency virus type 1.

Table 2 Induction of peptide cocktail-specific $CD8^+$ T cells in bulk cultures

	Percentage of $CD8^+$ IFN-γ^+ cells	
Peptide cocktail	KI-033	KI-126
Cocktail 1 (Env373–381, Gag30–39, Gag34–43)	0	0
Cocktail 2 (Gag144–152, Pol894–902, Nef192–200)	2.8	13.2
Cocktail 3 (Env699–708, Env761–771, Env763–771, Pol813–822)	0	0
Cocktail 4 (Env696–706, Env574–584, Env579–587)	0	0
Cocktail 5 (Env568–578, Env578–587, Env761–769, Pol228–238)	0	0
Cocktail 6 (Env193–201, Env827–837, Env835–844, Pol430–439)	0	0
Cocktail 7 (Env835–845, Env839–847, Env769–779, Nef73–81)	8.7	68.5

IFN-γ, interferon-γ.

Generation of CTL clones

Nef192–200-specific and Env769–779-specific CTL clones were generated from an established peptide-specific bulk CTL culture by limiting dilution in 96-well U-bottomed microtiter plates (Nunc, Roskilde, Denmark) by using the following cloning mixture: RPMI-1640 medium supplemented with 10% FCS and recombinant human interleukin-2 (200 U/ml; Ajinomoto, Tokyo, Japan), 1×10^6 irradiated allogeneic PBMCs from a healthy donor and 1×10^5 irradiated C1R-A*3101 cells pulsed with the corresponding peptide (1 μM). Wells positive for growth after about 14 days were tested for cytolytic activity by the ^{51}Cr-release assay described below.

CTL assay

The cytotoxic activity of CTL clone was determined by using a standard ^{51}Cr-release assay. C1R-A*3101 cells (2×10^5) were first incubated with 100 μCi $Na_2{}^{51}CrO_4$ (PerkinElmer, Boston, MA) in saline for 1 h at 37°C and then washed three times with RPMI-1640 medium containing 10% NCS. The ^{51}Cr-labelled cells (2×10^3) were pulsed with the corresponding peptide (1 μM) for 1 h at 37°C, after which a CTL clone (4×10^3) was added and incubation was continued further for 4 h at 37°C. A portion of the culture supernatant (100 μl) was then removed and analysed by a γ counter (Perkin Elmer, CT). The percentage of specific lysis was calculated as described previously (25). In another experiment, labelled C1R-A*3101 cells were pulsed with various concentrations (10^3–0.1 nM) of the corresponding peptide.

Confirmation of epitope authenticity by cytotoxic assay

C1R-A*3101 cells were infected for 12–18 h with 10 plaque-forming units of either recombinant vaccinia virus expressing a given protein (Env or Nef) or wild-type Vaccinia. The infected cells (2×10^5) were then labelled with 100 μCi $Na_2{}^{51}CrO_4$ in saline, washed three times and used as target cells in the cytotoxic assay described above. The activities of CTL clones against recombinant vaccinia virus-infected target cells were tested at an effector : target ratio of 4:1.

Results

Identification of HLA-A*3101-binding peptides derived from HIV-1 SF2 proteins

Nine- to 11-mer peptide sequences with two anchor residues of HLA-A*3101 peptides (Leu, Phe, Tyr or Val at position 2 and Arg at the C terminus) (27) were selected from the amino acid sequence of different HIV-1 SF2 strains, and then 88 peptides matched to these sequences were synthesized. The binding affinity of the synthesized peptides for HLA-A*3101 molecule was tested by conducting a stabilization assay using RMA-S-A*3101 cells (22). Fifty-six of the 88 peptides bound to HLA-A*3101 (Table 1). Among these binding peptides, five peptides were high binders ($BL_{50} < 10^{-5}$ M), 25 peptides were medium binders (BL_{50} 10^{-5} to $<10^{-4}$ M) and 26 peptides were low binders ($BL_{50} \geq 10^{-4}$ M). Peptides with high or medium binding affinity to HLA-A*3101 were then grouped into cocktails and used to induce CTLs in PBMCs from HIV-1-infected individuals expressing HLA-A*3101.

Induction of HIV-1 peptide-specific $CD8^+$ T cells from PBMCs of HLA-A*3101$^+$ HIV-1-infected individuals

PBMCs from two HLA-A*3101$^+$ HIV-1-seropositive individuals (KI-033 and KI-126) were stimulated *in vitro* with HLA-A*3101-binding peptide cocktails for 10–14 days. IFN-γ production by each bulk culture in response to C1R-A*3101 prepulsed with the corresponding peptide cocktail was assessed by intracellular staining. Bulk cultures from both KI-033 and KI-126 responded specifically to cocktails 2 and 7, as summarized in Table 2. Cocktail 2 induced a relatively low number of IFN-γ-producing $CD8^+$ T cells in bulk cultures from both patients KI-033 and KI-126 (2.8 and 13.2%, respectively). By contrast, cocktail 7 markedly induced a high number of IFN-γ-producing $CD8^+$ T cells, particularly in the bulk culture

from KI-126 (68.5%). To determine the peptide(s) responsible for the specific response to each cocktail, we stimulated the bulk cultures with C1R-A*3101 prepulsed with single peptides. Two peptides, Nef192–200 and Env769–779, induced $CD8^+$ T cells to produce IFN-γ in both patients KI-033 and KI-126 (Figure 1).

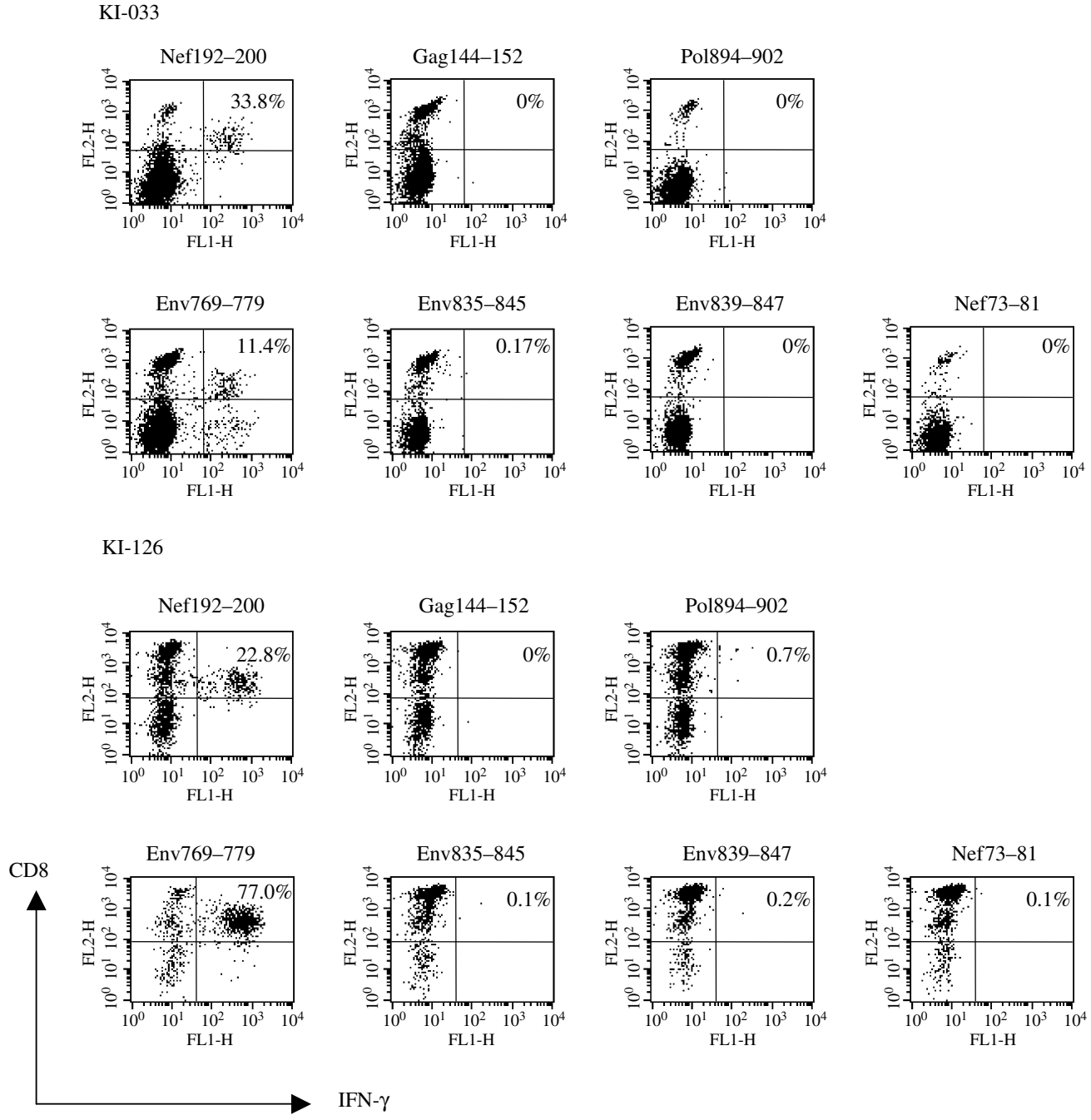

Figure 1 Induction of HIV-1-specific $CD8^+$ T cells from PBMCs of HIV-1-infected individuals bearing HLA-A*3101. PBMCs from patients KI-033 and KI-126 were stimulated *in vitro* with HLA-A*3101-binding peptide cocktails (Table 2) and were then cultured for 10–14 days. After the cultured PBMCs had been stimulated with the peptide cocktail-prepulsed C1R-A*3101 cells for 6 h, the number of IFN-γ-producing $CD8^+$ T cells was measured by flow cytometry. Cultured cells containing IFN-γ-producing $CD8^+$ T cells were again stimulated with C1R-A*3101 cells prepulsed with the individual peptides comprising cocktails 2 and 7, followed by intracellular IFN-γ staining. Percentages in upper right quadrants indicate IFN-γ-producing $CD8^+$ T cells. HIV-1, human immunodeficiency virus type 1; IFN, interferon; PBMCs, peripheral blood mononuclear cells.

Identification of HIV-1-specific CD8$^+$ T-cell epitopes endogenously processed and presented by HLA-A*3101

In order to further analyse these two peptides, we generated CTL clones specific for Nef192–200 and Env769–779 from patients KI-033 and KI-126, respectively. These CTL clones were capable of killing C1R-A*3101 cells pulsed with the corresponding peptide in a concentration-dependent manner (Figure 2).

To clarify whether these two peptides were endogenously processed and presented by HLA-A*3101, we investigated the ability of these CTL clones to kill C1R-A*3101 cells infected with recombinant vaccinia virus expressing the HIV-1 Nef or Env protein. The CTL clones effectively killed C1R-A*3101 cells infected with the recombinant vaccinia virus, but not those cells infected with wild-type vaccinia virus or uninfected cells (Figure 3). These results indicate that Nef192–200 and Env769–779 are naturally processed HLA-A*3101-restricted CTL epitopes.

Detection of Env769–779-specific and Nef192–200-specific CD8$^+$ T cells in chronically HIV-1-infected individuals

To investigate whether CD8$^+$ T cells specific for Nef192–200 and Env769–779 epitopes could be frequently found in HLA-A*3101$^+$ HIV-1-infected individuals, we expanded our investigation to include three more patients (KI-039, KI-147 and KI-178) by stimulating their PBMCs with epitope peptides. Env769–779-specific CD8$^+$ T cells were observed in the bulk culture from only one (KI-039) of these three patients (Table 3). No Nef192–200-specific CD8$^+$ T cells were induced in any of these individuals. Taken together, our data indicate that Env769–779-specific and Nef192–200-specific CD8$^+$ T cells were detected in the cultures from three and two of HLA-A*3101$^+$ HIV-1-infected individuals, respectively.

Discussion

This study focused on the identification and characterization of HIV-1-derived CTL epitopes restricted by the HLA-A*3101 allele. Fifty-six HLA-A*3101-binding peptides were identified based on their strong binding to HLA-A*3101 molecules. These peptides were tested for specific recognition by CTLs in five HIV-1-infected individuals and four HIV-1-seronegative controls expressing the HLA-A*3101 allele. Two of the 56 HLA-A*3101 binders induced peptide-specific T-cell responses in at least two HIV-1-infected subjects. Our results include the first description of HLA-A*3101-restricted CTL epitope Nef192–200. The other epitope, Env769–779, was a variant version of the previously described HLA-A3.1- and HLA-A31-restricted CTL epitope (28, 29).

While the association between the peptide-binding motif and the amino acid sequence of the actual CTL epitopes is not always absolute (30–32), the identification of two HLA-A*3101-restricted HIV-1 CTL epitopes in this study provides further evidence that the prediction of CTL epitopes from the binding motif of the corresponding HLA class I molecule is an efficient approach for epitope identification. Previous pool sequencing analysis showed that peptide binding to HLA-A*3101 molecule requires Arg as the C-terminal anchor and four possible hydrophobic residues, Leu, Phe, Tyr or Val, as the P2 anchor (33). Using the HLA stabilization assay, we further demonstrated that positively charged P1 residues have a positive effect on the binding of epitope peptides to HLA-A*3101 molecules (33). When the sequence of the Env769–779 epitope (RLRDLLLIAAR) is compared to that of the

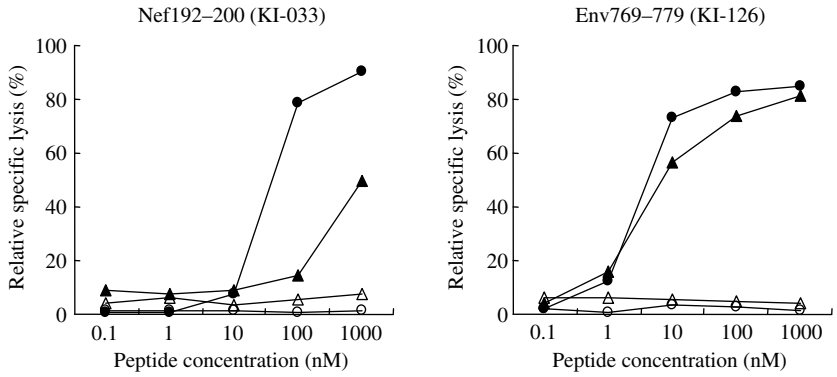

Figure 2 Recognition of HIV-1 Nef and Env peptides by established CTL clones. The CTL activity of Nef192–200-specific and Env769–779-specific clones against target cells pulsed with 10-fold serial dilution of the corresponding peptide was tested at an effector : target (E : T) ratio of 4:1. C1R-A*3101 cells (closed symbols) and C1R cells (open symbols) were used as target cells. Two CTL clones (circle and triangle) were tested for each epitope. Nef192–200-specific and Env769–779-specific CTL clones were established from KI-033 and KI-126, respectively. HIV-1, human immunodeficiency virus type 1; CTL, cytotoxic T lymphocyte.

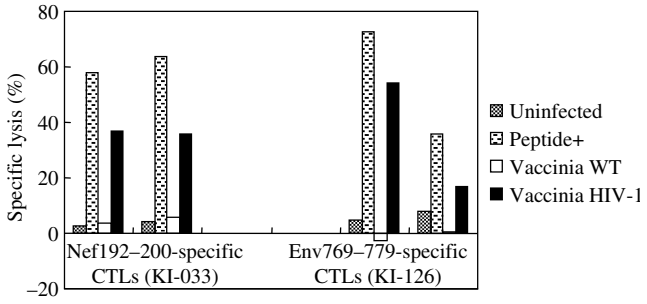

Figure 3 Recognition of HIV-1 epitopes presented on C1R-A*3101 cells infected with HIV-1 recombinant vaccinia virus. The CTL activity of Nef192–200-specific and Env769–779-specific clones against C1R-A*3101 cells prepulsed with 1 μM of epitope peptide (peptide$^+$) or infected with recombinant vaccinia virus (vaccinia HIV-1) expressing the corresponding HIV-1 protein, Nef or Env, or wild-type vaccinia virus (vaccinia WT) was tested at an effector : target (E : T) ratio of 4:1. HIV-1, human immunodeficiency virus type 1; CTL, cytotoxic T lymphocyte.

Nef192–200 epitope (KLAFHHMAR), both epitopes have a positively charged residue, Arg and Lys, respectively, at P1. These epitope peptides also bound to HLA-A*3101 molecules with high and medium affinity, respectively (Table 1). These results further support the importance of positively charged P1 residues in HLA-A*3101-binding peptides.

An increasing body of data demonstrates that virus-specific CTL responses play a crucial role in the immune response against HIV-1 (1, 3). Vaccines inducing strong CTL responses against HIV-1 may therefore represent a possible avenue to prevent infection or attenuate HIV-1 disease. However, the large degree of HLA polymorphism represents a significant challenge for this approach, if specific epitopes for a large number of different HLA class I specificities have to be defined. The identification of peptides capable of binding to common HLA class I molecules in a nonethically biased population may simplify the epitope selection with potential practical implications for development of epitope-based vaccines and immunotherapy. This study therefore focused on the identification of CTL epitopes that have high binding affinities for the HLA-A*3101 molecule, which is a common allele in Asia (9.3% in the Japanese population) (33, 34). CTL responses against the identified HLA-A*3101 binders were analysed in Japanese individuals expressing predominately HLA-A*3101. Our data obtained from individuals who were treated shortly after an acute HIV-1 infection demonstrate that both epitopes were recognized during the initial CTL response and suggest that they might subsequently participate in the early control of viraemia. However, it is important to assess whether these CTL clones are able to recognize the autologous virus.

As Nef is an early regulatory protein which is not expressed in infectious particles, the Nef-specific CTL response could play a critical role in host cytotoxic defences against infected cells at an early step of the HIV-1 replication. We report here a new HLA-A*3101-restricted Nef epitope (aa 192–200) located at the carboxyl-terminal region of the Nef protein, which is recognized by CTL in the context of many HLA molecules such as A1 and B35 (35). This region appears to be particularly interesting with respect to vaccine development, because it has been previously reported to be an immunodominant region for T helper cell and antibody responses (36).

The induction of the two types of epitope-specific CD8$^+$ T cells varied among five HIV-1-infected individuals expressing HLA-A*3101 (Table 3). Env769–779-specific and Nef192–200-specific CD8$^+$ T cells were found in three and two of them, respectively. These results indicate that CD8$^+$ T cells specific for these epitopes, particularly Env769–779, were frequently induced in HLA-A*3101$^+$ HIV-1-infected individuals. However, the induction of these HIV-1-specific CD8$^+$ T cells was correlated neither with viral load nor with CD4$^+$ or CD8$^+$ T cell number. For those subjects in whom these CTL responses were not

Table 3 Induction of epitope-specific CD8$^+$ T cells among PBMCs from HLA-A*3101$^+$ HIV-1-infected individuals

				Percentage of IFN-γ-producing cells in CD8$^+$ T cells	
Patients	Viral load[a]	CD4[b]	CD8[b]	Nef192–200	Env769–779
KI-033	4.3×10^5	225	578	33.8	11.4
KI-039	2.4×10^4	646	850	0	4.2
KI-126	2.1×10^4	524	883	22.8	77.0
KI-147	7.2×10^3	169	434	0	0
KI-178	9.9×10^4	407	1544	0	0
HIV-1-seronegative individuals ($n = 4$)				0.4 ± 0.3	0.5 ± 0.4

HIV-1, human immunodeficiency virus type 1; IFN-γ interferon-γ PBMCs, peripheral blood mononuclear cells.
[a]Copies/ml.
[b]Cells/l.

Table 4 Variation in HLA-A*3101-restricted epitopes in clades A–E

Epitope	Sequence											Clade A	Clade B	Clade C	Clade D	Clade E
Nef192–200	K	L	A	F	H	H	M	A	R			0/10	2/40	0/19	0/21	0/16
	R	–	–	–	–	–	–	–	–			0/10	26/40	0/19	0/21	0/16
	R	–	–	–	–	–	V	–	–			0/10	12/40	0/19	0/21	0/16
	R	–	–	–	E	–	K	–	–			0/10	0/40	0/19	21/21	0/16
	S	–	–	R	R	–	L	–	–			0/10	0/40	19/19	0/21	1/16
	A	–	–	R	K	–	I	–	–			0/10	0/40	0/19	0/21	15/16
Env769–779	R	L	R	D	L	L	L	I	A	A	R	0/29	9/52	3/22	6/26	0/19
	–	–	–	–	F	I	–	–	–	–	–	25/29	0/52	12/22	0/26	19/19
	–	–	–	–	–	–	–	–	V	T	–	0/29	28/52	1/22	1/26	0/19
	–	–	–	–	–	–	–	–	V	–	–	0/29	15/52	0/22	0/26	0/19
	–	–	–	–	–	I	–	–	–	–	–	4/29	0/52	5/22	7/26	0/19
	–	–	–	–	–	I	–	–	–	T	–	0/29	0/52	1/22	12/26	0/19

observed (KI-147 and KI-178), autologous virus sequencing should reveal whether escape mutations can account for the absence of these CTLs.

We searched reported HIV-1 sequences of clades A–E (HIV sequence database, Los Alamos, NM) for major variants of these epitopes. The sequences of Env769–779 (RLRDLLLIAAR) and Nef192–200 (KLAFHHMAR) were found in nine of 52 and two of 40 HIV-1 clade B isolates, respectively. The Env769–779 sequence was also found in clades D and C (Table 4). These sequences were rarely or not found in isolates of clades A, C, D and E. However, epitope variants with one or two amino acid changes were predominantly found among HIV-1 isolates. An Env769–779 mutant carrying Phe and Ile at positions 5 and 6, respectively, was a predominant sequence in clades A and E, whereas one carrying Val and Thr in positions 9 and 10, respectively, was frequently found in clade B. On the other hand, a Nef192–200 mutant carrying Arg at position 1 alone or with Val at position 7 was a common variant in clade B. At least three amino acid changes within this epitope were observed as major variants in clades C, D and E (Table 4). Given that amino acid changes in both epitope variants do not affect the anchor residues, it would be interesting to investigate whether the established CTL clones can recognize those epitope variants. Given that the two anchor residues (P2 and C-terminus) are conserved for these two epitopes among all HIV-1 isolates, it is tempting to assess the effect of predominant amino acid changes in these epitopes on their binding to HLA-A*3101 molecule and CTL recognition.

In conclusion 56 peptides were identified based on their binding to the HLA-A*3101 allele. Two of these peptides, including a variant of the previously described CTL epitope on HIV-1 Env, were recognized by CTL in acutely HIV-1-infected individuals. Further studies are needed to determine at what level the individual epitope-specific CTL response contributes to the entire CTL response in infected individuals so that we can assess whether these epitopes would be useful for the development of prophylactic or therapeutic vaccines.

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