

# Cytotoxic T-cell recognition of HIV-1 cross-clade and clade-specific epitopes in HIV-1-infected Thai and Japanese patients

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**Objective:** To identify and characterize cytotoxic T-cell (CTL) epitopes for HIV-1 clade E using eight known HLA-A\*1101-restricted HIV-1 clade B epitopes.

**Methods:** Induction of clade E-specific CTL was examined by stimulating peripheral blood mononuclear cells (PBMC) from clade E-infected Thai individuals with the clade E-specific peptide corresponding to the clade B epitopes. Cross-clade and clade-specific CTL recognition for these epitopes was analysed using CTL clones and bulk CTL specific for these epitopes. To clarify the presentation of these epitopes in HIV-1-infected T cells, CTL recognition for the clade E-specific and cross-clade epitopes was investigated using CD4CXCR4 cells infected with an HIV-1 clade E clone.

**Results:** Three epitopes, which are identical among clades A–E, were recognized as cross-clade CTL epitopes in both individuals. Clade B and E sequences corresponding to three epitopes were recognized as clade-specific epitopes in clade B-infected and clade E-infected individuals, respectively. In contrast, clade E-specific peptides corresponding to two other clade B epitopes failed to elicit clade E-specific CTL. CTL specific for the three cross-clade and three clade E-specific epitopes effectively lysed target cells infected with HIV-1 clade E virus.

**Conclusions:** These six epitopes are found to be processed naturally in HIV-1 clade E-infected cells. We show here that a strategy utilizing HIV-1 clade B epitopes is very useful for identifying clade E CTL epitopes.

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## Introduction

There is growing evidence that HIV-1-specific cytotoxic T lymphocytes (CTL) inhibit HIV-1 replication in HIV-1-infected individuals [1–5] and prevent HIV-1 infection [6,7]. Recent studies of SIV demonstrated an inverse relationship between SIV-specific CTL activity and disease onset [8–11]. These findings

strongly suggest that a vaccine which elicits HIV-1-specific CTL would prevent HIV-1 infection and delay progression to AIDS.

Geographically distinct epidemics of HIV-1 subtypes have been identified. In North America and Europe, most HIV-1-seropositive individuals are infected with clade B virus, while in Africa and Asia, two largest

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epicentres of global HIV/AIDS epidemic, most HIV-1-seropositive individuals are infected with non-clade B virus [12–14]. In Asian countries, HIV-1 clade E and clade C infection were observed in the majority of HIV-1-seropositive individuals [14]. The former is the most prevalent strain in South-East Asia while the latter is spreading rapidly in India. Both HIV-1 clade C and clade E infections are observed in south China [15,16]. In Japan, haemophilia patients are infected with HIV-1 clade B while clade E infection is increasing through heterosexual transmission.

Most HIV-1-specific CTL epitopes have been defined for clade B [17]. Several studies provided CTL epitopes for HIV-1 clades A [18,19] and C [18], but there are no reported CTL epitopes for HIV-1 clade E. Identification of CTL epitopes for HIV-1 clades E and C is critical for investigating the role of CTL in individuals infected with these HIV-1 clades and for designing HIV-1 vaccines for trial in Asia.

In the present study, we focused on HLA-A\*1101-restricted epitopes because HLA-A\*1101 is a common allele in East and South-East Asia, and is the most frequent allele in Thailand [20]. We previously identified four HLA-A\*1101-restricted CTL epitopes for HIV-1 clade B by reverse immunogenetics, and four others have been reported by other investigators [21,22]. We used these HIV-1 clade B epitopes to identify CTL epitopes for HIV-1 clade E. Cross-clade and clade-specific CTL recognition for these epitopes was analysed using CTL clones and bulk CTL cultures established from Thai individuals infected with HIV-1 clade E and Japanese individuals infected with HIV-1 clade B. Furthermore, to investigate the antigenicity of these epitopes in HIV-1-infected CD4 T cells, CTL recognition for the identified clade E-specific and cross-clade epitopes was investigated using CD4CXCR4 T cells infected with an HIV-1 clade E clone.

## Materials and methods

### Cells

C1R and RMA-S cells were maintained in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS). C1R cells expressing HLA-A\*1101 (C1R-A\*1101) and TAP-defective RMA-S cells expressing HLA-A\*1101 (RMA-S-A\*1101) were previously generated [23] and were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.15 mg/ml hygromycin B. The Epstein–Barr virus-transformed cell line Tm was previously generated from an individual Tm (HLA-A\*1101/A\*2402, B\*5201/B\*5201, Cw\*0701/Cw\*1202) [24] and was maintained in RPMI 1640 medium supplemented with 10% FCS. 721.221-CD4 cells were generated by transfection of

the CD4 gene (pBMG hyg CD4) into 721.221 cells that express CXCR4 (data not shown) but lose HLA class I. 721.221-CD4-A\*1101 cells were subsequently generated by transfection of the HLA-A\*1101 gene into 721.221-CD4 cells.

### CTL clones

Pol 424–432–34, Pol 424–432–64, Pol 675–683–133, Pol 894–903–14, Pol 894–903–18 and Env 202–210–56 CTL clones were generated previously [21,22].

### Synthetic peptides

Peptides were prepared by using an automated multiple peptide synthesizer (Shimadzu Model PSSM-8; Shimadzu Co., Kyoto, Japan) and then examined by mass spectrometry. Peptides with more than 90% purity were used in this study.

### Peptide binding assay

Binding of HIV-1-derived peptides to HLA-A\*1101 was examined by a peptide stabilization assay as described previously [23]. Briefly, RMA-S-A\*1101 cells were cultured for 16 h at 26°C and then pulsed with peptides ( $10^{-3}$  to  $10^{-7}$  M) for 1 h at 26°C. After further incubation for 3 h at 37°C, peptide-pulsed cells were stained by the anti-HLA class I  $\alpha_3$  domain monoclonal antibody (mAb) TP25.99 [25] and the fluoresceine isothiocyanate-conjugated IgG fraction of sheep anti-mouse Ig (Silenius Laboratories, Hawthorn Victoria, Australia). The mean fluorescence intensity (MFI) was measured using a FACS Calibur (BD Biosciences, San Jose, California, USA). The MFI of RMA-S-A\*1101 cells was obtained by subtracting the MFI value of RMA-S-A\*1101 cells incubated for 3 h at 37°C from that of control cells cultured at 26°C. HLA-A\*1101-binding peptides were defined as those which at a concentration of  $10^{-3}$  M caused > 25% increase in MFI compared to control RMA-S-A\*1101 cells cultured at 26°C. The peptide concentration that yielded the half-maximal MFI level (the BL<sub>50</sub> value) was calculated.

### Patients

Blood samples were collected with informed consent from seven HLA-A\*1101<sup>+</sup> patients (TT-005, TT-007, TT-008, TT-009, TU-002, TU-003 and TU-007) with chronic HIV-1 clade E infection at Siriraj Hospital and five HLA-A\*1101<sup>+</sup> patients (KI-005, KI-015, KI-030, KI-035 and KI-036) with chronic HIV-1 clade B infection at the International Medical Center of Japan. HLA typing was determined by HLA genotyping. All patients included in the present study had HLA-A\*1101. Identification of HIV-1-subtype was performed by sequencing the *env* gene.

### Induction of peptide-specific CTL in PBMC from HIV-1-infected individuals carrying HLA-A\*1101

Specific CTL were induced from peripheral blood

mononuclear cells (PBMC) of HIV-1-infected Thai and Japanese individuals carrying HLA-A\*1101. PBMC ( $1 \times 10^6$ ) were stimulated with HLA-A\*1101-binding peptide ( $10^{-6}$  M) in culture medium [RPMI 1640 medium supplemented with 10% FCS and 200 U/ml recombinant human interleukin (IL)-2]. The culture was maintained for 2 weeks before CTL activity was assayed as described below. As relative specific lysis of the bulk cultured T cells for non-specific peptide (HLA-B\*3501 binding peptide: DP NPQEVVL) was  $2.7 \pm 3.0\%$  in HIV-1 clade B-infected individuals and  $3.5 \pm 3.5\%$  in HIV-1 clade E-infected individuals, more than 10% of relative specific lysis was evaluated as positive CTL lysis.

### Statistical analysis

Statistical significance of the values for the relative specific lysis of the bulk cultured T cells for each HIV-1 clade B and clade E peptide was compared to those of the T cells for non-specific peptide using the non-parametric Mann-Whitney U test with StatView software (StatView 4.02; Abacus Concepts, Berkeley, California, USA). A difference with  $P < 0.05$  was considered to be significant.

### Generation of CTL clones specific for Pol 675-683-5E

Pol 675-683-5E-specific CTL clones were generated from an established Pol 675-683-5E-specific bulk CTL culture by seeding 0.8 cells/well into U-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200  $\mu$ l cloning mixture [RPMI 1640 medium supplemented with 10% FCS and 200 U/ml recombinant human IL-2,  $5 \times 10^5$  irradiated allogeneic PBMC from a healthy donor and  $1 \times 10^5$  irradiated C1R-A\*1101 cells pre-pulsed with the corresponding peptide ( $10^{-6}$  M)]. Forty out of 576 wells (6.9%) were positive for growth after about 2 weeks. They were transferred into 48-well plates together with 1 ml cloning mixture. The clones were examined for CTL activity by a standard  $^{51}\text{Cr}$  release assay.

### CTL assay for target cells pulsed with peptide

CTL activity was measured by a standard  $^{51}\text{Cr}$  release assay as follows. Target cells ( $5 \times 10^5$ ) were incubated for 60 min with 3.7 MBq  $\text{Na}_2^{51}\text{CrO}_4$  in saline, then washed three times with RPMI 1640 medium containing 10% newborn calf serum. Labelled target cells ( $5 \times 10^3$ /well) were added into U-bottom 96-well microtiter plates with the indicated amount of peptide. After incubation for 1 h, effector cells were added and the mixtures were incubated for 4 h at 37°C. The supernatants were collected and analysed with a gamma counter. Spontaneous  $^{51}\text{Cr}$  release was determined by measuring the counts per minute (c.p.m.) in supernatants from wells containing only target cells (c.p.m. spn). Maximum  $^{51}\text{Cr}$  release was determined by measuring the c.p.m. in supernatants from wells containing

target cells in the presence of 2.5% Triton X-100 (c.p.m. max). Specific lysis was calculated by  $[(\text{c.p.m. exp} - \text{c.p.m. spn}) / (\text{c.p.m. max} - \text{c.p.m. spn})] \times 100$ , where c.p.m. exp is the c.p.m. in supernatants of wells containing both target and effector cells. Relative specific lysis was calculated by (specific lysis of target cells pulsed with peptides) - (specific lysis of target cells pulsed with no peptides).

### CTL assay for target cells infected with recombinant vaccinia virus expressing HIV-1 Nef or Gag protein

Two recombinant vaccinia viruses, vT141 (expressing the Nef protein of HIV-1 clade A isolate 90CR402, which contains the Nef 84-92-2F6F sequence) and vT157 (expressing the Gag protein of HIV-1 clade D isolate 94UG114, which contains the Gag 349-359-9S sequence), were provided by the National Institutes of Health AIDS Research and Reference Reagent Program (National Institute of Health, Rockville, Maryland, USA). Target cells were cultured overnight with 10 plaque forming units of either recombinant or wild-type virus. The infected cells ( $1 \times 10^5$ ) were labelled with  $^{51}\text{Cr}$  as described above. Labelled target cells ( $5 \times 10^3$ /well) were added to effector cells into U-bottom 96-well microtiter plates. The mixtures were incubated for 4 h (targets infected with vT157) or 6 h (targets infected with vT141) at 37°C, as the HIV-1 Nef recombinant vaccinia virus-infected target cells are weakly recognized by CTL if the mixtures were incubated for 4 h (data not shown). The activities of bulk T-cell cultures for target cells infected with recombinant vaccinia virus were tested at an effector:target (E:T) ratio of 10:1.

### HIV-1 clade E clone p93JP-NH1

The replication-competent molecular clone of HIV-1 clade E (CRF01\_AE), p93JP-NH1, was isolated from a Japanese individual infected with HIV-1 clade E virus. The full-length nucleotide sequence was determined to confirm that this clone belongs to clade E.

### CTL assay for target cells infected with HIV-1 clade E

The virus stock was produced from HeLa cells transfected with p93JP-NH1. 721.221-CD4-A\*1101 cells were exposed to the virus for several days. The cells were used as target cells for a CTL assay when infection of > 80% cells was confirmed by intracellular staining of HIV-1 p24 antigen. Infected cells ( $1 \times 10^5$ ) were labelled with  $^{51}\text{Cr}$  as described above. Labelled target cells ( $5 \times 10^3$ /well) were added with effector cells into U-bottom 96-well microtiter plates, and the mixtures were incubated for 4 h at 37°C. The activities of CTL clones or bulk T-cell cultures for the target cells were tested at the indicated E:T ratios.

## Results

### Variants of HLA-A\*1101-restricted CTL epitopes in HIV-1 clades A-E

In addition to four previously reported HLA-A\*1101-restricted clade B epitopes (Pol 313–321, Nef 84–92, Gag 83–90 and Gag 349–359) [26–29], we recently identified further four epitopes (Pol 424–432, Pol 675–683, Pol 894–903 and Env 202–210) [21,22]. We searched reported HIV-1 sequences (HIV sequence database, Los Alamos National Laboratory, Los Alamos, New Mexico, USA) for major variants of these epitopes in HIV-1 clades A–E (Table 1). This search revealed that Pol 894–903 (AVFIHNFKRK) and Pol 313–321 (AIFQSSMTK) are highly conserved between clades A–E and that Pol 424–432 (QIYA GIKVK) is conserved between clade B and clade E. The majority of sequences corresponding to the other five epitopes were found to be different between HIV-1 clade B and clade E. These observations imply that Pol 894–903- and Pol 313–321-specific CTL are induced in all individuals infected with HIV-1, regardless of the clade, while clade- or clade group-specific CTL are induced by the other six epitopes. We focused on CTL recognition of these eight epitopes in HIV-1 clade E-infected individuals to identify clade E epitopes and to investigate cross-clade or clade-specific recognition of clade B and E epitopes by CTL between the clades B and E.

### Cross-clade CTL epitopes between HIV-1 clade B and clade E

In order to clarify whether the conserved HIV-1 clade B epitopes Pol 894–903, Pol 313–321, and Pol 424–

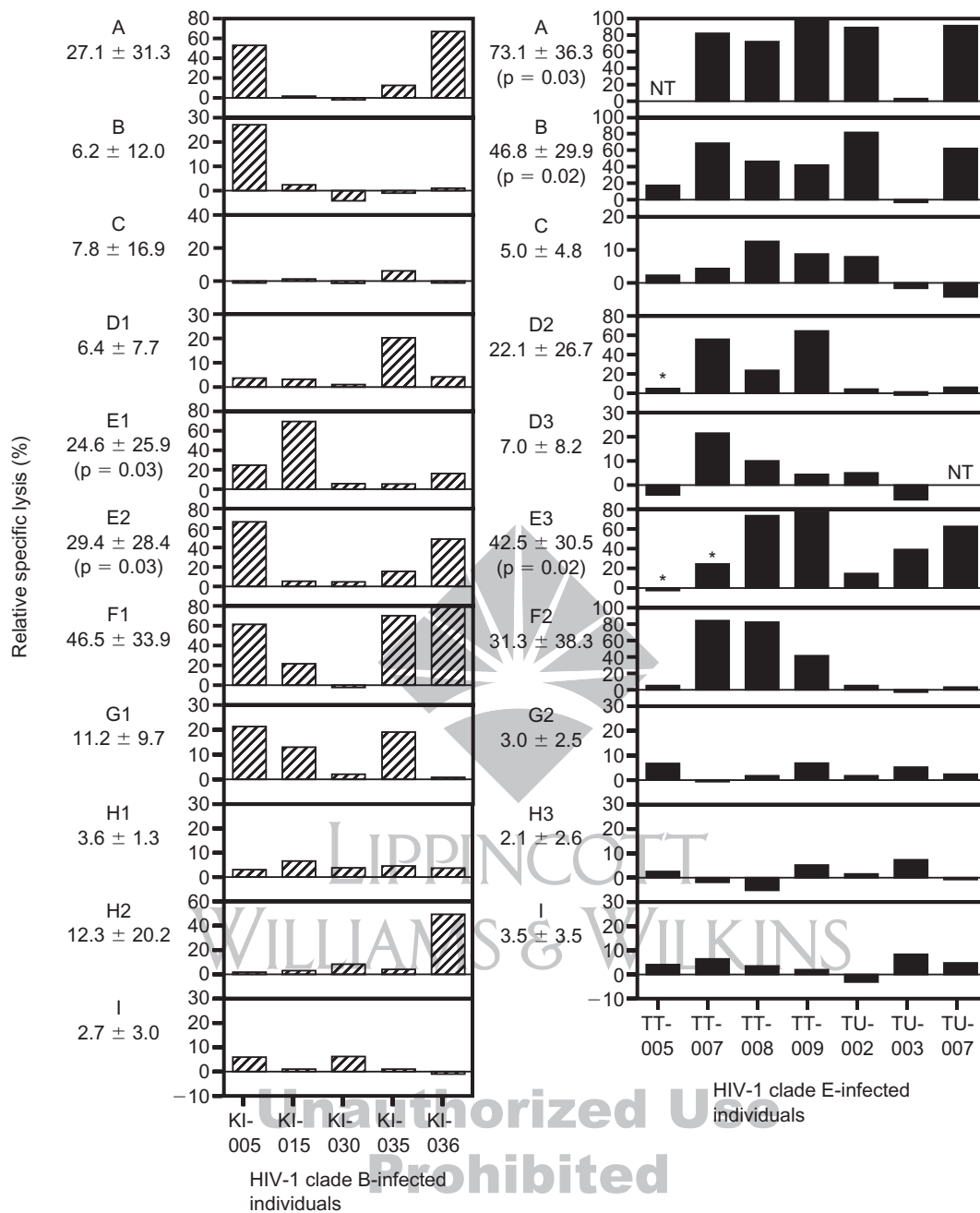
432 are also recognized in HIV-1 clade E-infected individuals, we attempted to induce CTL activity specific for each peptide in PBMC from seven HLA-A\*1101<sup>+</sup> HIV-1 clade E-infected Thai individuals (TT-005, TT-007, TT-008, TT-009, TU-002, TU-003 and TU-007) and five HLA-A\*1101<sup>+</sup> HIV-1 clade B-infected Japanese individuals (KI-005, KI-015, KI-030, KI-035 and KI-036). CTL activities for these three epitopes were observed in PBMC from both HIV-1 clade B-infected and HIV-1 clade E-infected HLA-A\*1101<sup>+</sup> individuals (Fig. 1). CTL activities for Pol 313–321 and Pol 424–432 were more frequently observed in clade E-infected individuals compared to clade B-infected individuals.

There are two major variants of Pol 424–432 in HIV-1 clades A–E: Pol 424–432-9R (QIYAGIKVR) is observed in clades B and E, whereas Pol 424–432-4P9R (QIYPGIKVR) is observed predominantly in clades C and D (Table 1). CTL recognition for these two major variants was investigated using a CTL clone specific for Pol 424–432 (Pol 424–432-34). This clone recognized the Pol 424–432-9R peptide but failed to recognize the Pol 424–432-4P9R peptide (Fig. 2a). This suggests that in addition to Pol 424–432, Pol 424–432-9R is a cross-clade CTL epitope. Recognition for Pol 424–432-9R peptide was about 10 times lower than that for Pol 424–432 peptide (Fig. 2a). The binding affinity of the two variant peptides to HLA-A\*1101 was almost identical, but about 10 times lower than that of Pol 424–432 (Fig. 2b). This indicates that reduced recognition of CTL clone Pol 424–432-34 for the Pol 424–432-9R variant is due to reduced affinity of the peptide to HLA-A\*1101, and that the substitu-

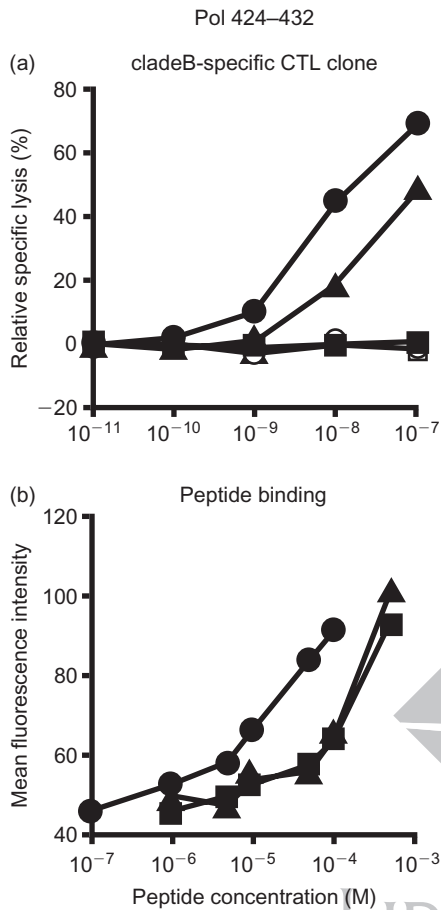
**Table 1.** Natural variants of regions corresponding to HLA-A\*1101-restricted CTL epitopes in HIV-1 clade A–E isolates.

ID <sup>a</sup>	Peptide	Sequence	Frequencies of naturally occurring variants in each clade <sup>b</sup>				
			Clade A	Clade B	Clade C	Clade D	Clade E
A	Pol313–321	AIFQSSMTK	<b>5/9</b>	<b>19/29</b>	<b>6/15</b>	<b>4/5</b>	<b>4/5</b>
B	Pol424–432	QIYAGIKVK	<b>6/9</b>	<b>15/29</b>	1/15	<b>1/5</b>	<b>3/5</b>
	Pol424–432-9R	-----R	1/9	<b>6/29</b>			<b>1/5</b>
	Pol424–432-4P9R	---P---R		2/29	<b>11/15</b>	<b>3/5</b>	
C	Pol894–903	AVFIHNFKRK	<b>8/9</b>	<b>27/29</b>	<b>11/15</b>	<b>4/5</b>	<b>5/5</b>
D1	Pol675–683	QIIEQLIKK		<b>24/29</b>	<b>9/15</b>	<b>5/5</b>	
D2	Pol675–683-5E	----E----		2/29			<b>3/5</b>
D3	Pol675–683-5K8E	----K--E-	<b>4/9</b>				<b>1/5</b>
E1	Nef84–92	AVDLSHFLK	<b>3/12</b>	<b>91/231</b>		<b>2/4</b>	
E2	Nef84–92-2L	-L-----	<b>4/12</b>	<b>75/231</b>		<b>1/4</b>	
E3	Nef84–92-2F6F	-F---F---	<b>3/12</b>	1/231	<b>17/32</b>		<b>20/26</b>
F1	Gag349–359	ACQGVGGPGHK	<b>7/9</b>	<b>27/35</b>	<b>6/15</b>	<b>1/5</b>	<b>1/5</b>
F2	Gag349–359-9S	-----S---	<b>2/9</b>	<b>8/35</b>	<b>8/15</b>	<b>4/5</b>	<b>4/5</b>
G1	Env202–210	SVITQACPK		<b>76/95</b>	1/28		
	Env202–210-2A	-A-----	<b>15/17</b>	1/95	<b>15/28</b>	<b>10/14</b>	
G2	Env202–210-4K	---K-----		2/95			<b>16/20</b>
H1	Gag83–90	TLYCVHQR	<b>5/9</b>	<b>11/35</b>			
H2	Gag83–90-8K	-----K	1/9	<b>8/35</b>	1/15		
H3	Gag83–90-3W	--W-----	1/9				<b>5/5</b>
	Gag83–90-7E	-----E-		1/35		<b>3/5</b>	

<sup>a</sup>The ID described for each peptide is consistent with that used in Fig. 1. <sup>b</sup>Sequences were obtained from the HIV sequence database 1999 (Los Alamos National Laboratory, Los Alamos, New Mexico, USA). Bold type indicates the major variants (> 20% of total isolates) in each clade.



**Fig. 1.** CTL activities in PBMC from HIV-1 clade B- or clade E-infected individuals carrying HLA-A\*1101 after *in vitro* stimulation with HLA-A\*1101-restricted HIV-1 epitope peptides. The cytolytic activity of the bulk PBMC culture for the corresponding peptide was tested at an E:T ratio of 40:1. Asterisks indicate the cytolytic activity of the bulk PBMC culture tested at an E:T ratio of 30:1. PBMC from seven HIV-1 clade E-infected Thai (filled bars) and five clade B-infected Japanese (cross-hatched bars) individuals in each panel were assayed 14 days after stimulation with peptide as follows: A, Pol 313–321 (AIFQSSMTK) peptide; B, Pol 424–432 (QIYAGIKVK) peptide; C, Pol 894–903 (AVFIHNFKRK) peptide; D1, Pol 675–683 (QIIEQLIKK) peptide; D2, Pol 675–683-5E (QIIEELIKK) peptide; D3, Pol 675–683-5K8E (QIIEKLEIK) peptide; E1, Nef 84–92 (AVDLSHFLK) peptide; E2, Nef 84–92-2L (ALDLSHFLK) peptide; E3, Nef 84–92-2F6F (AFDLSFFLK) peptide; F1, Gag 349–359 (ACQGVGGPGHK) peptide; F2, Gag 349–359-9S (ACQGVGGPSHK) peptide; G1, Env 202–210 (SVITQACPK) peptide; G2, Env 202–210-4K (SVIKQACPK) peptide; H1, Gag 83–90 (TLYCVHQK) peptide; H2, Gag 83–90-8K (TLYCVHQK) peptide; H3, Gag 83–90-3W (TLWCVHQK) peptide; I, non-specific peptide (DPNPQEVVL). Relative specific lysis (%) is the percentage of specific lysis of target cells pulsed with peptide minus that of cells not pulsed with peptide. NT, Not tested. The mean relative specific lysis  $\pm$  SD is indicated on the left of each panel. The significant differences ( $P < 0.05$ ) of the values of the relative specific lysis were compared to those for non-specific peptide in the HIV-1 clade B-infected Japanese ( $2.7 \pm 3.0\%$ ) and HIV-1 clade E-infected Thai ( $3.5 \pm 3.5\%$ ) patients, respectively and is indicated on the left of each panel.



**Fig. 2.** Cross-clade HIV-1 epitope recognition by Pol 424-432-specific CTL. (a) Recognition of natural HIV-1 clade B and E variant epitopes by the HLA-A\*1101-restricted, HIV-1 clade B-specific CTL clone Pol 424-432-34. The cytolytic activity of this CTL clone against target cells pulsed with various concentrations of variant peptides was tested at an E:T ratio of 2:1. C1R-A\*1101 cells (filled symbols) and C1R cells (open symbols) were used as target cells. Symbols represent: Pol 424-432 (QIYAGIKVK) peptide (circles); Pol 424-432-9R (QIYAGIKVR) peptide (triangles); Pol 424-432-4P9R (QIYPGIKVR) peptide (squares). Relative specific lysis (%) is the percentage of specific lysis of target cells pulsed with peptide minus that of cells not pulsed with peptide. (b) Binding affinities of natural HIV-1 clade B and E variant peptides to HLA-A\*1101. Symbols are the same as in (a).

tion of Pro for Ala at P4 critically affects T-cell receptor (TCR) recognition by this CTL clone.

### HIV-1 clade B- and clade E-specific CTL epitopes

We next investigated CTL recognition of clade B- and clade E-specific epitopes. The clade B epitopes Pol 675-683, Nef 84-92, and Gag 349-359 induced specific CTL in PBMC from at least one HLA-A\*1101<sup>+</sup> individual infected with HIV-1 clade B (Fig. 1). These results confirmed that these peptides are HLA-A\*1101-restricted CTL epitopes. Clade E-specific variants of

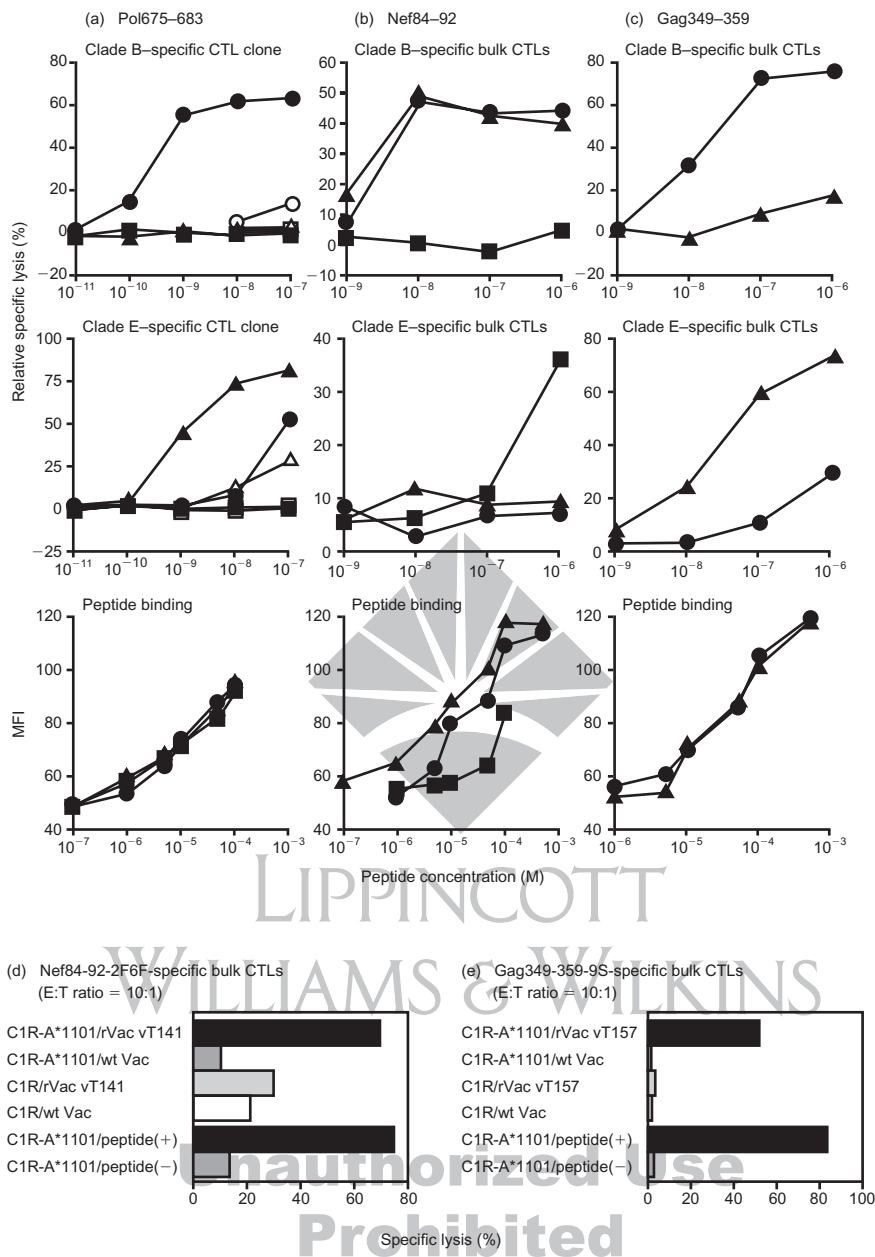
these epitopes induced specific CTL in PBMC from at least two of seven HIV-1 clade E-infected individuals (Fig. 1).

We generated a CTL clone specific for the clade E epitope Pol 675-683-5E from an individual infected with HIV-1 clade E (TT-007). This clone (Pol 675-683-5E-3) and a previously generated Pol 675-683-specific CTL clone (Pol 675-683-133) were used to investigate cross-recognition of clade B- and E-specific Pol 675-683 variants. CTL clone Pol 675-683-133 failed to recognize Pol 675-683-5E and Pol 675-683-5K8E variant peptides (Fig. 3a, upper), whereas CTL clone Pol 675-683-5E-3 showed weak recognition for Pol 675-683 but failed to recognize Pol 675-683-5K8E (Fig. 3a, middle). The binding affinity of these peptides to HLA-A\*1101 was identical (Fig. 3a, lower). These results indicate that the difference at P5 between Pol 675-683 and Pol 675-683-5E is critical for TCR recognition by these CTL clones, and similarly that either one or both difference(s) at P5 and P8 between Pol 675-683-5K8E and Pol 675-683 or Pol 675-683-5E affect(s) TCR recognition by these CTL clones.

The Nef 84-92 variant Nef 84-92-2L is also an HIV-1 clade-B-specific sequence, while the variant Nef 84-92-2F6F is found predominantly in clades C and E (Table 1). Nef 84-92-2L-specific bulk CTL generated from an HIV-1 clade B-infected individual recognized Nef 84-92 peptide, but failed to recognize Nef 84-92-2F6F peptide (Fig. 3b, upper). Conversely, Nef 84-92-2F6F-specific bulk CTL generated from a clade E-infected individual recognized neither Nef 84-92 peptide nor Nef 84-92-2L peptide (Fig. 3b, middle). The binding affinity of Nef 84-92-2F6F peptide to HLA-A\*1101 was 10-50 times lower than that of Nef 84-92 and Nef 84-92-2L peptides (Fig. 3b, lower). These results suggest that the substitutions at P2 and P6 are critical for both peptide binding to HLA-A\*1101 and TCR recognition by specific CTL.

Gag 349-359 is found predominantly in HIV-1 clades A, B, and C whereas Gag 349-359-9S predominates in clades C, D and E (Table 1). Gag 349-359-specific bulk CTL generated from an HIV-1 clade B-infected individual failed to recognize the Gag 349-359-9S variant peptide (Fig. 3c, upper). Similarly, Gag 349-359-9S-specific bulk CTL generated from an HIV-1 clade E-infected individual failed to recognize the Gag 349-359 peptide (Fig. 3c, middle). The binding affinity of these peptides to HLA-A\*1101 was almost identical (Fig. 3c, lower), suggesting that the substitution at P9 affects TCR recognition by specific CTL but not HLA-A\*1101 binding.

Overall, these results for CTL cross-recognition suggest that HIV-1 clade B- and clade E-specific sequences corresponding to Pol 675-683, Nef 84-92, and Gag



**Fig. 3.** (a, b and c) Clade-specific CTL recognition for three HLA-A\*1101-restricted HIV-1 epitopes. Upper and middle panels: recognition of natural HIV-1 clade B and E variant epitopes by HIV-1 clade B-specific and clade E-specific CTL clones (a, Pol 675-683-133 and Pol 675-683-5E-3, respectively), and HIV-1 clade B- and clade E-specific bulk T-cell cultures (b and c, PBMC stimulated twice with HIV-1 clade B- and clade E-specific peptide, respectively). The cytolytic activities of CTL clones and bulk T-cell cultures against target cells pulsed with various peptide concentrations were tested at an E:T ratio of 2:1 (CTL clones) or 10:1 (bulk T-cell cultures). C1R-A\*1101 cells (filled symbols) and C1R cells (open symbols) were used as target cells for the CTL clones, and Tm-EBV cells (filled symbols) were used as target cells for the bulk T-cell cultures. Symbols represent: Pol 675-683 (QIIEQLIKK), Nef 84-92 (AVDLSHFLK) and Gag 349-359 (ACQGVGGPGHK) peptides (circles); Pol 675-683-5E (QIIEELIKK), Nef 84-92-2L (ALDLSHFLK) and Gag 349-359-9S (ACQGVGGPSHK) peptides (triangles); Pol 675-683-5K8E (QIIEKLIEK) and Nef 84-92-2F6F (AFDLSFFLK) peptides (squares). Relative specific lysis (%) is the percentage of specific lysis of target cells pulsed with peptide minus that of cells not pulsed with peptide. Lower panels: binding affinities of natural HIV-1 clade B and E variant peptides to HLA-A\*1101. Symbols are the same as in the upper and middle panels. MFI, Mean fluorescence intensity. (d and e) HIV-1 clade E-specific CTL recognition for target cells infected with HIV-1 recombinant vaccinia virus. The cytolytic activities of Nef 84-92-2F6F-specific (d) and Gag 349-359-9S-specific (e) bulk CTL cultures for target cells infected with HIV-1 recombinant vaccinia virus vT141 (expressing Nef protein) or vT157 (expressing Gag protein), respectively, were tested at an E:T ratio of 10:1.

349–359 are recognized as clade-specific epitopes in HIV-1 clade B-infected and HIV-1 clade E-infected individuals, respectively. To determine whether the clade E-specific sequences are naturally occurring epitopes, we tested the cytolytic activity of clade E-specific CTL for target cells infected with HIV-1 recombinant vaccinia virus containing the Nef 84–92-2F6F or Gag 349–359-9S sequence. Bulk CTL cultures specific for Nef 84–92-2F6F or Gag 349–359-9S effectively lysed HLA-A\*1101<sup>+</sup> target cells infected with the corresponding HIV-1 recombinant vaccinia virus (Figs 3d and 3e). These results indicate that these two peptides are naturally occurring epitopes.

### CTL epitopes unique to HIV-1 clade B

There are three dominant sequences for the Env 202–210 epitope in HIV-1 clades A–E: Env 202–210 is predominantly observed in clade B, Env 202–210-4K in clade E, and Env 202–210-2A in clades A, C and D (Table 1). CTL activity for Env 202–210 was induced in PBMC from HIV-1 clade B-infected HLA-A\*1101<sup>+</sup> individuals. However, CTL activity for Env 202–210-4K was not induced in PBMC from HIV-1 clade E-infected individuals (Fig. 1). These observations suggest that Env 202–210 is a clade B-specific CTL epitope but that Env 202–210-4K does not function as an epitope. Indeed, an Env 202–210-specific CTL clone recognized Env 202–210-2A but not Env 202–210-4K (data not shown). Since the binding affinity of all three peptides to HLA-A\*1101 was almost identical (data not shown), it is likely that the substitution of Lys for Thr is critical for TCR recognition by the CTL clone. Another possibility is that Env 202–210-4K is not recognized by these HIV-1 clade E-infected individuals as this is not an immunodominant epitope.

There are four dominant sequences for the Gag 83–90 epitope in HIV-1 clades A–E: Gag 83–90 and Gag 83–90-8K are predominantly found in clade B while Gag 83–90-3W and Gag 83–90-7E are predominantly observed in clades E and D, respectively (Table 1). CTL activity for Gag 83–90 was not induced in PBMC from five HLA-A\*1101<sup>+</sup> individuals infected with HIV-1 clade B while CTL activity for Gag 83–90-8K was induced in PBMC from only one of these individuals (Fig. 1). CTL activity for the clade E-specific peptide Gag 83–90-3W was not induced in PBMC from any of the HIV-1 clade E-infected individuals (Fig. 1). These results suggest that Gag 83–90-8K is a clade B-specific epitope but is not dominant, and that Gag 83–90-3W is not a clade E-specific epitope or not an immunodominant epitope. CTL induced by Gag 83–90-8K failed to recognize the other three Gag 83–90 variants (data not shown). As these peptides showed similar binding affinity to HLA-A\*1101 (data not shown), it is likely that the substitutions at P3, P7 and P8 are critical for TCR recognition by specific CTL.

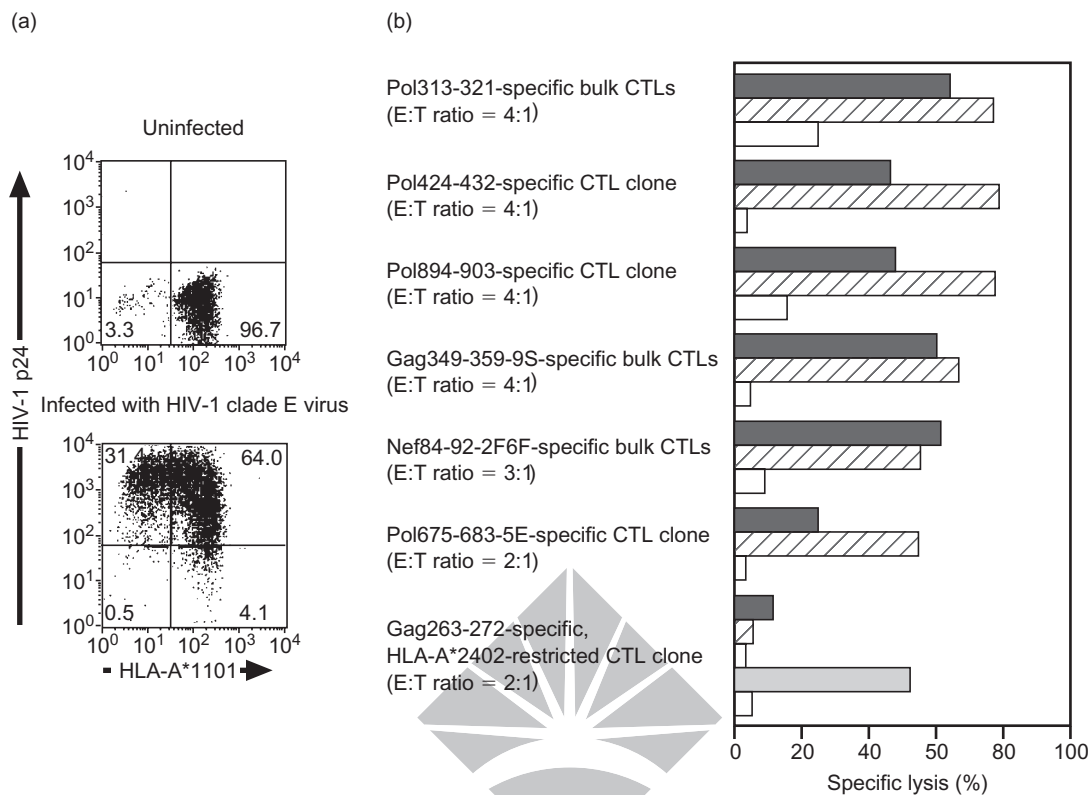
### CTL recognition of target cells infected with HIV-1 clade E

This and previous studies [22,29] showed that the three cross-clade epitopes and two of the clade E-specific epitopes are naturally processed in target cells infected with HIV-1 recombinant vaccinia virus. However, cells infected with HIV-1 recombinant vaccinia virus are not an exact model for individuals infected with HIV-1. It is therefore important to clarify whether T cells specific for these epitopes recognize the epitopes when presented by cells infected with HIV-1. We investigated the cytolytic activity of CTL specific for the three cross-clade and three clade E-specific epitopes for target cells infected with an HIV-1 clade E clone, p93JP-NH1, which has these epitope sequences. We generated a CXCR4 CD4 HLA-A\*1101 cell line, 721.221-CD4-A\*1101, by transfection of HLA class I<sup>-</sup> CXCR4 721.221 cells with HLA-A\*1101 and CD4 genes. The transfected cells were then infected with the HIV-1 clade E clone. After culture for 6–7 days, the cells were tested for infection by flow cytometry every 2 days. When approximately 95% of the cells were stained with anti-p24 mAb, the cells were used as target cells for a CTL assay. Down-regulation of HLA-A\*1101 was observed in approximately 30% of the infected cells (Fig. 4a). An HLA-A\*2402-restricted, Gag 263–272-specific CTL clone failed to lyse infected target cells (Fig. 4b). Pol 424–432-specific and Pol 894–903-specific CTL clones as well as Pol 313–321-specific bulk CTL effectively lysed infected target cells but failed to lyse uninfected 721.221-CD4-A\*1101 cells (Fig. 4b). These results indicate that these cross-clade epitopes are naturally processed and presented in cells infected with HIV-1 clade E clone 93JP-NH1. Pol 675–683-5E-specific CTL clone as well as Nef 84–92-2F6F-specific and Gag 349–359-9S-specific bulk CTL effectively lysed infected target cells but failed to lyse uninfected 721.221-CD4-A\*1101 cells (Fig. 4b). This indicates that these clade E-specific epitopes are also naturally processed and presented in cells infected with the HIV-1 clade E clone p93JP-NH1.

### Discussion

We identified six HLA-A\*1101-restricted CTL epitopes in individuals infected with HIV-1 clade E; three are cross-clade epitopes between clades B and E and three are clade E-specific epitopes. Of the three cross-clade epitopes, statistical analysis suggested that Pol 313–321 is an immunodominant epitope in HIV-1 clade E-infected individuals but not in HIV-1 clade B-infected individuals. As a strong specific CTL activity for this epitope (relative specific lysis, 53.5% and 67.3%) was induced in two out of five clade B-infected individuals, this epitope might be recognized as a subdominant epitope. The sequence of this epitope is





**Fig. 4.** Cytolysis of target cells infected with HIV-1 clade E by three cross-clade epitope-specific CTL and three clade E epitope-specific CTL. (a) Expression of HIV-1 p24 and HLA-A\*1101 antigens in HIV-1 clade E-infected cells. 721.221-CD4-A\*1101 cells were infected with HIV-1 clade E clone p93JP-NH1. Uninfected cells and infected cells were analysed for HLA-A\*1101 expression on target cells by staining with a p24-specific mAb and anti-HLA-A11 mAb. Ninety-five percent of cells were p24-positive and approximately 30% of p24-positive cells showed down-regulation of HLA-A\*1101. (b) Cytolysis of HIV-1 clade E-infected cells by HIV-1-specific CTL. Infected 721.221-CD4-A\*1101 cells (filled bars) and uninfected 721.221-CD4-A\*1101 cells pulsed with (cross-hatched bars) or without (open bars) the corresponding peptide were used as target cells. Cytolytic activities of three cross-clade epitope-specific CTL clones (Pol 424–432-34, Pol 894–903-14 and Pol 675–683-5E-3) and three clade E epitope-specific CTL for these target cells were examined. The Gag 263–272-specific, HLA-A\*2402-restricted CTL clone Gag 263–272-3 was used as a negative control. The specific lysis of this clone for C1R-A\*2402 cells pulsed with (stippled bar) and without (open bar) the corresponding epitope peptide was also examined.

predominantly observed in clade A, B, C, D and E isolates, implying that this epitope is also recognized in individuals infected with HIV-1 clades A, C and D. Similarly, Pol 424–432 is an immunodominant epitope in individuals infected with HIV-1 clade E but not in those infected with clade B, as specific CTL were detected frequently in clade E-infected individuals but rarely in clade B-infected individuals. Pol 424–432-specific CTL can recognize Pol 424–432-9R, which is found in approximately 20% of both clade B and E isolates. Therefore, both sequences are likely to be cross-clade epitopes.

Previous studies suggested the existence of cross-clade CTL epitopes by showing HIV-1-specific CTL activity in PBMC from individuals infected with one HIV-1 clade for target cells infected with recombinant vaccinia virus containing genes derived from another HIV-1 clade [30–35]. These studies demonstrated CTL cross-

reactivity between clades A, B, C, D, F and G. Cross-clade CTL recognition between clades B and E was also shown in North Americans infected with HIV-1 clade B and Thais infected with HIV-1 clade E, although cross-clade epitopes were not defined [36]. Thus, cross-clade CTL are frequently detected in individuals infected with HIV-1. Cross-clade epitopes between clades A, B, and D have been demonstrated [18,19]. We here identified three cross-clade epitopes between clades B and E. Such epitopes are useful for HIV vaccine design.

Of the three clade E-specific epitopes, statistical analysis suggested that Nef 84–92-2F6F is an immunodominant epitope in HIV-1 clade E-infected individuals. On the other hand, Gag 349–359-9S is not indicated as immunodominant epitope by statistical analysis. However, as a strong Gag 349–359-9S-specific CTL activity was induced in three out of seven HIV-1 clade E-

infected individuals, Gag 349–359-9S may be a subdominant epitope in those infected with HIV-1 clade E. Similarly, Pol 675–683-5E may be subdominant as Pol 675–683-5E-specific CTL were induced in three out of seven clade E-infected individuals tested. Further analysis of these epitopes in HIV-1 clade E-infected individuals is expected to characterize them more accurately.

HIV-1 CTL epitopes are generally demonstrated as naturally processed peptides by showing that specific CTL can lyse target cells infected with HIV-1 recombinant vaccinia virus [37–41]. This is useful for qualitative analysis of epitopes; however, as HIV-1-derived proteins are expressed as a part of vaccinia virus in this system, antigen presentation by cells infected with recombinant vaccinia virus does not reflect that by cells infected with HIV-1. It is therefore important to determine whether HIV-1-specific CTL lyse target cells infected with HIV-1. However, as there are only a few human CD4, HIV-1 sensitive T-cell lines whose HLA class I alleles have been well characterized, it is difficult to analyse various HLA class I-restricted CTL recognition for HIV-1-infected cells. Indeed, only a few studies have demonstrated that HIV-1-specific CTL lyse CD4 T-cell lines infected with HIV-1 clade B [42–44]. In the present study we generated a CD4CXCR4 cell line, which expresses HLA-A\*1101 only, for use as target cells in HIV-1-specific CTL activity assays. This cell line (721.221-CD4-A\*1101) was generated by transfection of 721.221 cells with both CD4 and HLA-A\*1101 genes. The cells are sensitive to HIV-1 infection because they express high levels of CD4 and CXCR4. We clearly demonstrated that 721.221-CD4-A\*1101 cells infected with an HIV-1 clade E clone were effectively lysed by six HIV-1 epitope-specific, HLA-A\*1101-restricted CTL. A recent study showed impaired HIV-1 antigen presentation by down-regulation of HLA class I in CD4 T cells infected with HIV-1 [45]. We did observe HLA-A\*1101 down-regulation in HIV-1-infected 721.221-CD4-A\*1101 cells; however, because such cells accounted for only 30% of total HIV-1-infected cells, this down-regulation had a minimal effect on CTL recognition. Thus, 721.221-CD4 cells expressing a single HLA class I allele should be useful for studies of HIV-1-specific CTL recognition for target cells infected with HIV-1.

This study is the first report of HIV-1 clade E-specific CTL epitopes. Furthermore, we showed that the strategy of using known clade B epitopes to identify non-clade B epitopes is very useful. It should be noted, however, that this strategy may not be useful for identifying Env epitopes as Env is more diverse between HIV-1 clades than other HIV-1 proteins. Identification of clade E Env epitopes is now under investigation using reverse immunogenetics, a more

effective strategy for identifying such epitopes. The clade E epitopes identified in the present study will contribute to various studies of HIV-1-specific CTL in Asian countries.

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## References

- Borrow P, Lewicki H, Wei X, *et al.* Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nature Med* 1997, **3**:205–211.
- Goulder PJ, Phillips RE, Colbert RA, *et al.* Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nature Med* 1997, **3**:212–217.
- Koenig S, Conley AJ, Brewah YA, *et al.* Transfer of HIV-1-specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression. *Nature Med* 1995, **1**:330–336.
- Koup RA, Safrit JT, Cao Y, *et al.* Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* 1994, **68**:4650–4655.
- Ogg GS, Jin X, Bonhoeffer S, *et al.* Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 1998, **279**:2103–2106.
- Langlade-Demoyen P, Ngo-Giang-Huong N, Ferchal F, Oksenhendler E. Human immunodeficiency virus (HIV) nef-specific cytotoxic T lymphocytes in noninfected heterosexual contact of HIV-infected patients. *J Clin Invest* 1994, **93**:1293–1297.
- Rowland-Jones S, Sutton J, Ariyoshi K, *et al.* HIV-1 specific cytotoxic T cells in HIV-exposed but uninfected Gambian women. *Nature Med* 1995, **1**:59–64.
- Allen TM, O'Connor DH, Jing P, *et al.* Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. *Nature* 2000, **407**:386–390.
- Evans DT, O'Connor DH, Jing P, *et al.* Virus-specific cytotoxic T-lymphocyte responses select for amino-acid variation in simian immunodeficiency virus Env and Nef. *Nature Med* 1999, **5**:1270–1276.
- Kuroda MJ, Schmitz JE, Charini WA, *et al.* Emergence of CTL coincides with clearance of virus during primary simian immunodeficiency virus infection in rhesus monkeys. *J Immunol* 1999, **162**:5127–5133.
- Schmitz JE, Kuroda MJ, Santra S, *et al.* Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 1999, **283**:857–860.
- Kato K, Shiino T, Kusagawa S, *et al.* Genetic similarity of HIV type 1 subtype E in a recent outbreak among injecting drug users in northern Vietnam to strains in Guangxi Province of southern China. *AIDS Res Hum Retroviruses* 1999, **15**:1157–1168.
- Ou CY, Takebe Y, Weniger BG, *et al.* Independent introduction of two major HIV-1 genotypes into distinct high-risk populations in Thailand. *Lancet* 1993, **341**:1171–1174.
- Weniger BG, Takebe Y, Ou CY, Yamazaki S. The molecular epidemiology of HIV in Asia. *AIDS* 1994, **8**(Suppl 2):S13–S28.
- Chen J, Young NL, Subbarao S, *et al.* HIV type 1 subtypes in

- Guangxi Province, China, 1996. *AIDS Res Hum Retroviruses* 1999, **15**:81–84.
16. Yu XF, Chen J, Shao Y, *et al.* Emerging HIV infections with distinct subtypes of HIV-1 infection among injection drug users from geographically separate locations in Guangxi Province, China. *J Acquir Immune Defic Syndr* 1999, **22**:180–188.
  17. Brander C, Goulder PJ. Recent advances in HIV-1 CTL epitope characterization. HIV Molecular Immunology Database 1999, HIV Sequence Database, Los Alamos National Laboratory, Los Alamos, New Mexico, USA.
  18. Dorrell L, Dong T, Ogg GS, *et al.* Distinct recognition of non-clade B human immunodeficiency virus type 1 epitopes by cytotoxic T lymphocytes generated from donors infected in Africa. *J Virol* 1999, **73**:1708–1714.
  19. Rowland-Jones SL, Dong T, Fowke KR, *et al.* Cytotoxic T cell responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi. *J Clin Invest* 1998, **102**:1758–1765.
  20. Clayton J, Lonjon C, Whittle D. Allele and haplotype frequencies for HLA loci in various ethnic group. In *HLA Volume I Genetic Diversity of HLA Functional and Medical Implication*. Edited by Charron D. Paris: EDK Medical and Scientific International Publishers; 1997:665–820.
  21. Fukada K, Tomiyama H, Chujoh Y, *et al.* HLA-A\*1101-restricted CTL recognition for a novel epitope derived from the HIV-1 Env protein. *AIDS* 1999, **13**:2597–2599.
  22. Fukada K, Chujoh Y, Tomiyama H, *et al.* HLA-A\*1101-restricted CTL recognition of HIV-1 Pol protein. *AIDS* 1999, **13**:1413–1414.
  23. Chujoh Y, Sobao Y, Miwa K, Kaneko Y, Takiguchi M. The role of anchor residues in the binding of peptides to HLA-A\*1101 molecules. *Tissue Antigens* 1998, **52**:501–509.
  24. Yamamoto J, Kariyone A, Akiyama N, Kano K, Takiguchi M. Presentation of human minor histocompatibility antigens by HLA-B35 and HLA-B38 molecules. *Proc Natl Acad Sci U S A* 1990, **87**:2583–2587.
  25. Tanabe M, Sekimata M, Ferrone S, Takiguchi M. Structural and functional analysis of monomorphic determinants recognized by monoclonal antibodies reacting with the HLA class I alpha 3 domain. *J Immunol* 1992, **148**:3202–3209.
  26. Culmann B, Gomard E, Kieny MP, *et al.* Epitopes reacting with human cytotoxic CD8+ T cells in the central region of the HIV-1 Nef protein. *J Immunol* 1991, **146**:1560–1565.
  27. Harrer T, Harrer E, Barbosa P, *et al.* Recognition of two overlapping CTL epitopes in HIV-1 p17 by CTL from a long-term nonprogressing HIV-1-infected individual. *J Immunol* 1998, **161**:4875–4881.
  28. Sipsas NV, Kalams SA, Trocha A, *et al.* Identification of type-specific cytotoxic T lymphocyte responses to homologous viral proteins in laboratory workers accidentally infected with HIV-1. *J Clin Invest* 1997, **99**:752–762.
  29. Threlkeld SC, Wentworth PA, Kalams SA, *et al.* Degenerate and promiscuous recognition by CTL of peptides presented by the MHC class I A3-like superfamily. *J Immunol* 1997, **159**:1648–1657.
  30. Betts MR, Krowka J, Santamaria C, *et al.* Cross-clade human immunodeficiency virus (HIV)-specific cytotoxic T-lymphocyte responses in HIV-infected Zambians. *J Virol* 1997, **71**:8908–8911.
  31. Buseyne F, Chaix ML, Fleury B, *et al.* Cross-clade-specific cytotoxic T lymphocytes in HIV-1-infected children. *Virology* 1998, **250**:316–324.
  32. Cao H, Kanki P, Sankale JL, *et al.* Cytotoxic T-lymphocyte cross-reactivity among different human immunodeficiency virus type 1 clades: implications for vaccine development. *J Virol* 1997, **71**:8615–8623.
  33. Durali D, Morvan J, Letourneur F, *et al.* Cross-reactions between the cytotoxic T-lymphocyte responses of human immunodeficiency virus-infected African and European patients. *J Virol* 1998, **72**:3547–3553.
  34. McAdam S, Kaleebu P, Krausa P, *et al.* Cross-clade recognition of p55 by cytotoxic T lymphocytes in HIV-1 infection. *AIDS* 1998, **12**:571–579.
  35. Wilson SE, Pedersen SL, Kunich JC, *et al.* Cross-clade envelope glycoprotein 160-specific CD8+ cytotoxic T lymphocyte responses in early HIV type 1 clade B infection. *AIDS Res Hum Retroviruses* 1998, **14**:925–937.
  36. Lynch JA, deSouza M, Robb MD, *et al.* Cross-clade cytotoxic T cell response to human immunodeficiency virus type 1 proteins among HLA disparate North Americans and Thais. *J Infect Dis* 1998, **178**:1040–1046.
  37. Ikeda-Moore Y, Tomiyama H, Miwa K, *et al.* Identification and characterization of multiple HLA-A24-restricted HIV-1 CTL epitopes: Strong epitopes are derived from V regions of HIV-1. *J Immunol* 1997, **159**:6242–6252.
  38. Johnson RP, Trocha A, Yang L, *et al.* HIV-1 gag-specific cytotoxic T lymphocytes recognize multiple highly conserved epitopes. *J Immunol* 1991, **147**:1512–1521.
  39. Nixon DF, Townsed ARM, Elvin JG, Rizza CR, Gallwey J, McMichael AJ. HIV-1 gag-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides. *Nature* 1988, **336**:484–487.
  40. Shiga H, Shioda T, Tomiyama H, *et al.* Identification of multiple HIV-1 T cell epitopes presented by HLA-B35 molecules. *AIDS* 1996, **10**:1075–1083.
  41. Tomiyama H, Sakaguchi T, Miwa K, *et al.* Identification of multiple HIV-1 epitopes presented by HLA-B\*5101 molecules. *Hum Immunol* 1999, **60**:177–186.
  42. Tomiyama H, Chujoh Y, Shioda T, *et al.* Cytotoxic T lymphocyte recognition of HLA-B\*5101-restricted HIV-1 Rev epitope which is naturally processed in HIV-1-infected cells. *AIDS* 1999, **13**:861–863.
  43. Yang OO, Tran AC, Kalams SA, Johnson RP, Roberts MR, Walker BD. Lysis of HIV-1-infected cells and inhibition of viral replication by universal receptor T cells. *Proc Natl Acad Sci USA* 1997, **94**:11478–1183.
  44. Yang OO, Kalams SA, Rosenzweig M, *et al.* Efficient lysis of human immunodeficiency virus type 1-infected cells by cytotoxic T lymphocytes. *J Virol* 1996, **70**:5799–5806.
  45. Collins KL, Chen BK, Kalams SA, Walker BD, Baltimore D. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocyte. *Nature* 1998, **391**:397–401.