

Original article

Identification of cross-clade CTL epitopes in HIV-1 clade A/E-infected individuals by using the clade B overlapping peptides

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Received 12 July 2013; accepted 8 August 2013

Available online 19 August 2013

Abstract

Identification of cross-clade T cell epitopes is one of key factors for the development of a widely applicable AIDS vaccine. We here investigated cross-clade CD8⁺ T cell responses between clade B and A/E viruses in chronically HIV-1 clade A/E-infected Japanese individuals. CD8⁺ T cell responses to 11-mer overlapping peptides derived from Nef, Gag, and Pol clade B consensus sequences were at a similar level to those to the same peptides found in clade B-infected individuals. Fifteen cross-clade CTL epitopes were identified from 13 regions where the frequency of responders was high in the clade A/E-infected individuals. The sequences of 6 epitopes were conserved between the clade B and clade A/E viruses whereas 9 epitopes had different amino acid sequences between the 2 viruses. CD8⁺ T cells specific for the 6 conserved epitopes recognized cells infected with the clade A/E virus, whereas those for 8 diverse epitopes recognized both the clade A/E virus-infected and clade B-infected cells. All of the cross-clade CD8⁺ T cells specific for conserved and diverse epitopes were detected in chronically HIV-1 clade A/E-infected individuals. These results show that in addition to conserved regions polymorphic ones across the clades can be targets for cross-clade CTLs.

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Keywords: Cross-clade; CTL; HLA; Epitope; HIV

1. Introduction

The HIV-1 genome is characterized by genetic diversity wherein distinct HIV-1 clades are expanding not only in different geographical regions but also even in the same locality [1]. HIV-1 clade B is the most prevalent virus in Japan accounting for more than 80% of the patients in this country. CRF01_AE (clade A/E) is the second most prevalent virus, accounting for 6.1% (Sugiura W, unpublished report). In contrast, the clade A/E virus is mainly prevalent in south-east

Asian countries including Thailand. An RV144 phase III vaccine trial, which was recently performed in Thailand, demonstrated a partial beneficial effect on HIV-1 infection [2]. In this trial, the recombinant canarypox virus-vectored HIV-1 *gag/pollen* vaccine (ALVAC-HIV) and the recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E) were used for priming and boosting, respectively. These vaccines were generated by using genes from both the clade B and A/E viral strains [2] to cover a wider range of potential challenge strains in Thailand, where approximately 80% and 10% of HIV-1-infected individuals are infected with the clade A/E and clade B viruses, respectively [3]. Recent analyses confirmed CD4⁺ cell-mediated and humoral immune responses in vaccines [3–6]. Thus, this clinical trial also highlighted the importance of the cross-clade immune responses to the clade B and A/E viruses. Although T cell functions in the acute and

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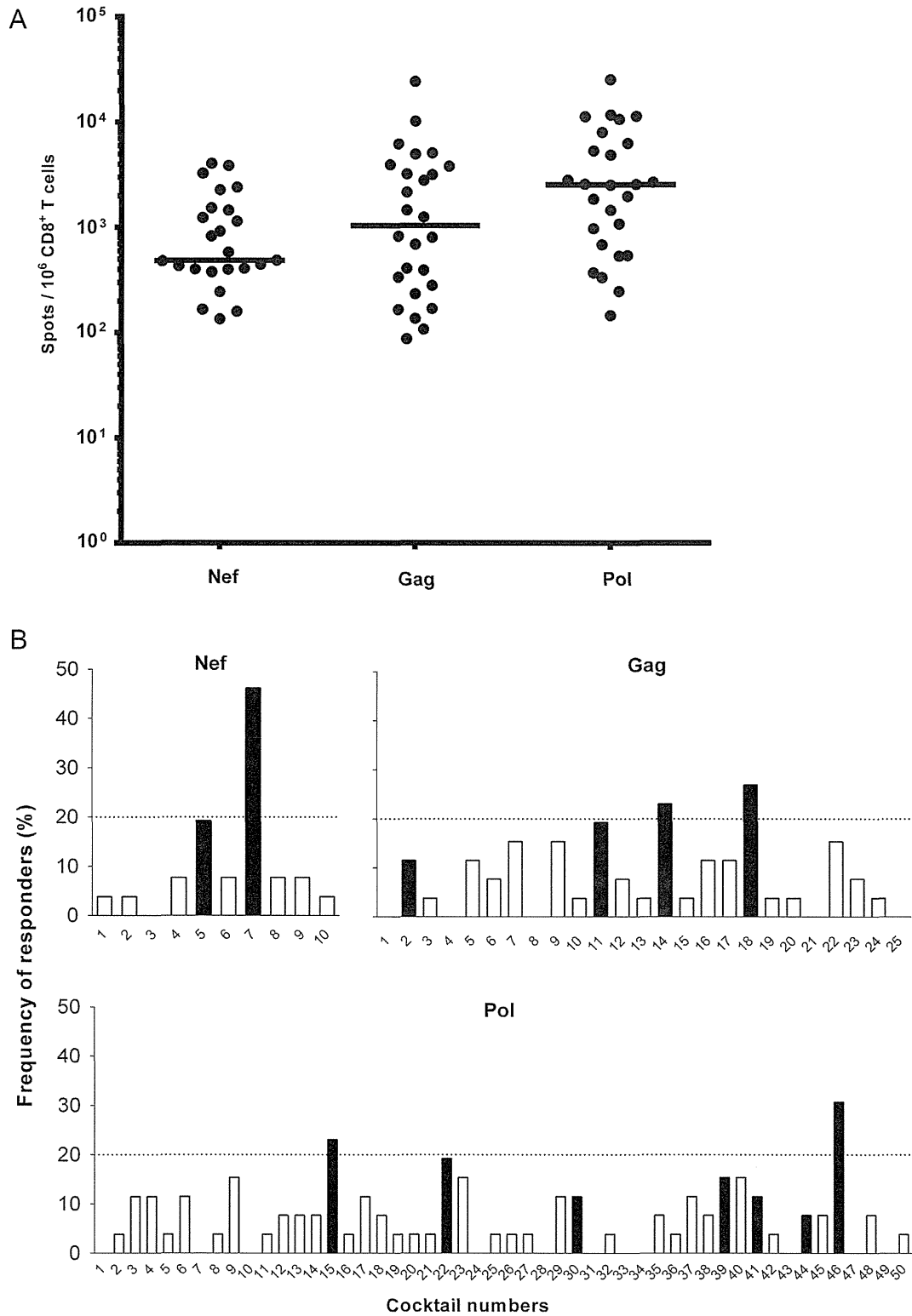
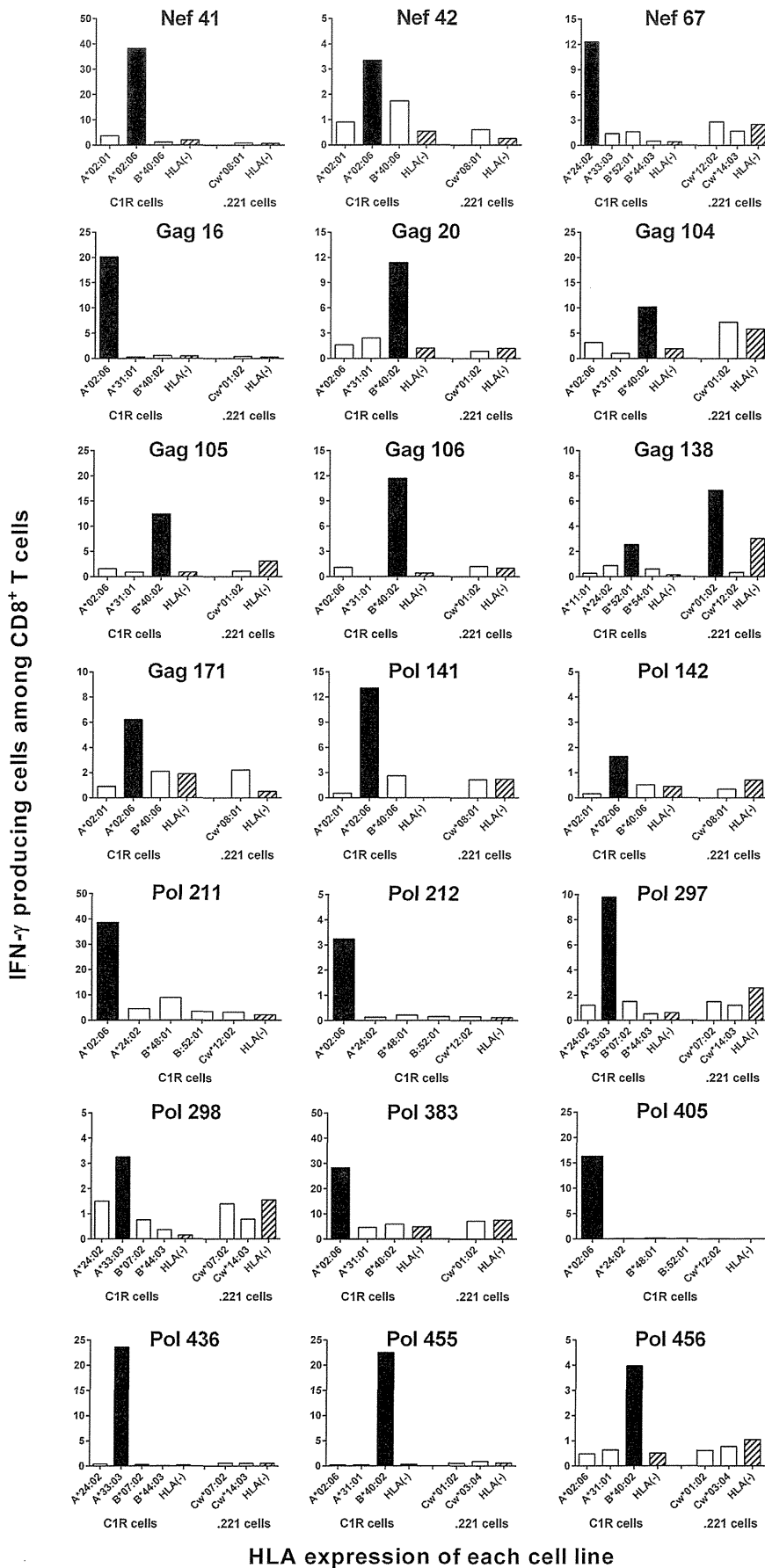


Fig. 1. CD8⁺ T cell responses of clade A/E-infected individuals to HIV-1 clade B-derived overlapping peptides. CD8⁺ T cell responses to peptide cocktails containing clade B consensus overlapping 11-mer peptides were analyzed by performing the ELISPOT assay using CD8⁺ T cells from 26 clade A/E-infected individuals. A. Total magnitude of CD8⁺ T cell responses to the clade B consensus overlapping peptides spanning Nef, Gag and Pol regions. B. Frequency of the responders to each cocktail. Positive response is defined as more than 200 spots. The cocktails for which the frequency of responders was more than 20% or less than 20% are shown as solid bars. In the latter case, at least 1 patient showed a high response (>750 spots).



chronic phases of an HIV-1 infection were well studied in Caucasians infected with the clade B virus and in Africans infected with the clade C virus [7–12], there are only a limited number of studies about the cross-clade reactivity of CTLs [13–17]. However, even in such studies a comprehensive analysis of cross-clade reactivity of the CTLs was not performed.

In the context of HIV vaccine development, it is very important to choose vaccine immunogens capable of eliciting CTLs that can control the variable mutant viruses and exhibit cross-reactivity across the different clade viruses [18,19]. The conserved parts of HIV-1 are good candidates as vaccine antigens [11,12,16,20,21], since they include epitopes conserved among viruses not only in the same clade but also among those clades. Indeed, CTL vaccines containing conserved epitopes have been shown to elicit CTL responses to HIV-1 [22–25]. Although the safety of these consensus CTL vaccines was confirmed in humans [26–32], such vaccines were poorly immunogenic in previous phase I and II trials [27,28,30,31]. Thus more studies on cross-clade effective epitopes will be needed for the development of more potent vaccines.

In the present study, we analyzed cross-clade CD8⁺ T cells between HIV-1 clade B and A/E viruses in chronically HIV-1 clade A/E-infected Japanese individuals. For this analysis, we sought to identify cross-clade CTL epitopes between the clade B and A/E viruses in the Japanese individuals by using 11-mer overlapping peptides derived from the clade B consensus sequence spanning Nef, Gag, and Pol regions. Thereafter, we analyzed cross-clade CD8⁺ T cell recognition for epitope peptides between clade A/E and B as well as CTL recognition for cells infected with the clade B or the A/E virus. This is the first comprehensive study to identify cross-clade CD8⁺ T cells by using overlapping HIV-1 peptides.

2. Materials and methods

2.1. Patient samples

This study was approved by the Ethical Committee of in National Center for Global Health and Medicine and Kumamoto University. Informed consent was obtained from all subjects, according to the Declaration of Helsinki. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from whole blood. HLA types of HIV-infected individuals were determined by standard sequence-based genotyping. HIV-1 subtypes were determined by the sequence results on Pol and Gag, and confirmed by Env sequencing. All samples were collected from the cohort in AIDS Clinical Center, National Center for Global Health and Medicine.

2.2. Sequence of autologous virus

Viral RNA was extracted from plasma samples from HIV-1-infected patients by the use of a QIAamp MinElute virus spin kit (Qiagen). cDNA was synthesized from the RNA by use of the SuperScript III First-Strand Synthesis System for RT-PCR and random hexamers (Invitrogen). Nef, Gag, and Pol regions were amplified by nested PCR using Taq DNA polymerase (Promega). The PCR products were purified by using ExoSAP-IT (GE). All DNA sequencing was performed with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and an ABI 3500 Genetic Analyzer.

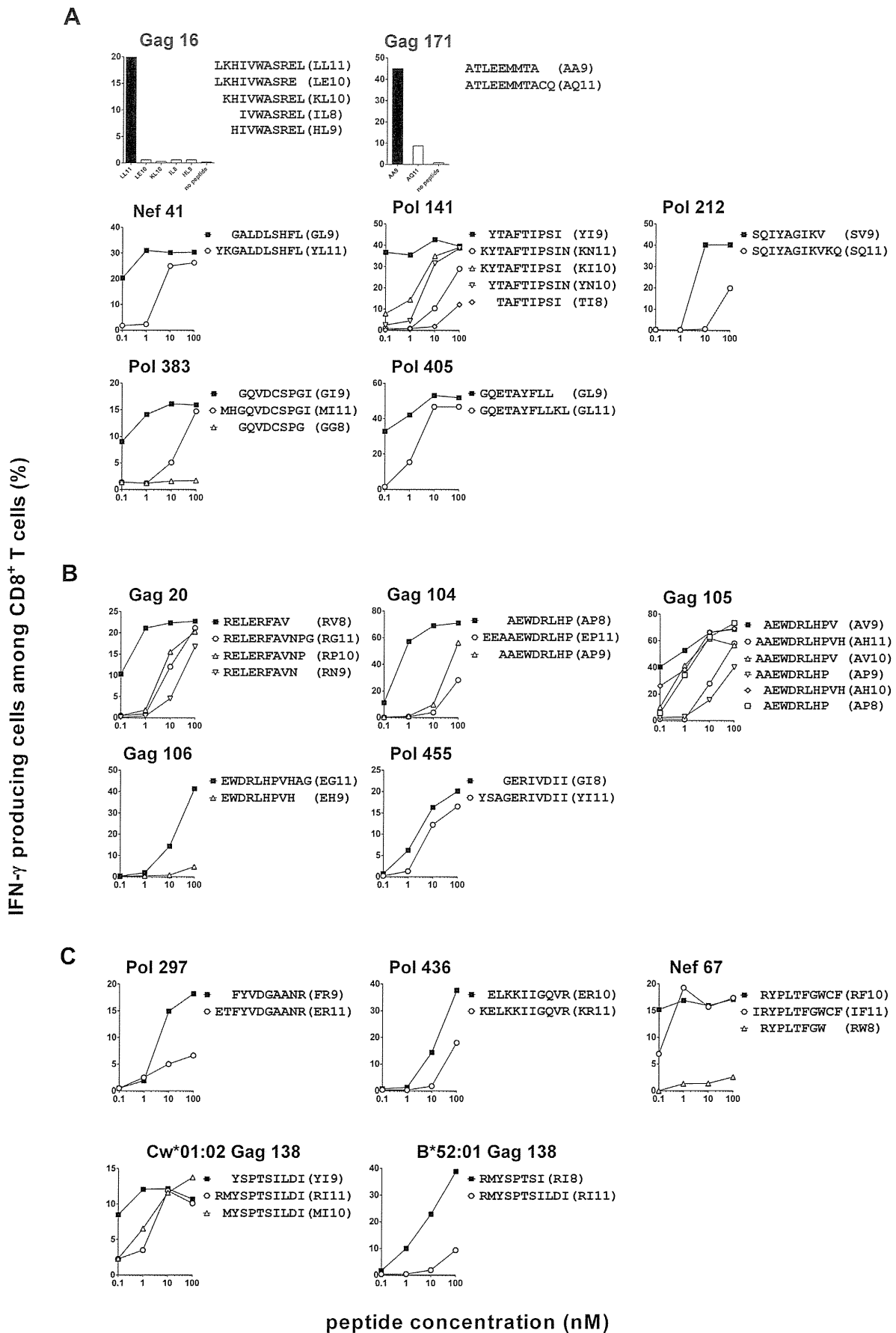
2.3. Synthetic peptides

We previously designed overlapping peptides consisting of 11-mer amino acids and spanning Gag, Pol, and Nef of HIV-1 clade B consensus sequences. Each 11-mer peptide was overlapped by 9 amino acids [33]. These 11-mer peptides and truncated 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.

2.4. ELISPOT assay

CD8⁺ T cells were sorted from cryopreserved PBMCs from 26 chronically HIV-1 clade A/E-infected Japanese individuals by using CD8 magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The sorted cells were plated in 96-well polyvinylidene plates (Millipore, Bedford, MA) that had been pre-coated with 5 mg/mL anti-IFN- γ mAb 1-D1K (Mabtech, Stockholm, Sweden). The appropriate amount of peptide cocktails including 10 overlapping 11-mer peptides were added in a volume of 50 μ L, and then PBMCs were added at 1×10^5 cells/well in a volume of 100 μ L. The plates were incubated for 16 h at 37 °C in 5% CO₂ and then washed with PBS before the addition of biotinylated anti-IFN- γ Mab (Mabtech) at 1 mg/mL. After the plates had been incubated at room temperature for 90 min and then washed with PBS, they were subsequently incubated with streptavidin-conjugated alkaline phosphatase (Mabtech) for 60 min at room temperature. Individual cytokine-producing cells were detected as dark spots after a 20-min. reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium by using an alkaline phosphatase-conjugate substrate (Bio-Rad, Richmond, CA, USA). The spot number was counted by using an Eliphoto-Counter (Minerva Teck, Tokyo,

Fig. 2. Identification of HLA restriction of the responses to each 11-mer peptide. Peptide-specific CD8⁺ bulk T cells were induced from PBMCs of the following 6 responders by stimulating the cells with each single peptide. KI-648 for Nef 41, Nef 42, Gag 171, Pol 141, and Pol 142 peptides, KI-632 for Nef 67, Pol 297, Pol 298, and Pol 436 peptides, KI-388 for Gag 16, Gag 20, Gag 104, Gag 105, Gag 106, and Pol 383 peptides, KI-724 for Gag 138 peptide, KI-964 for Pol 211, Pol 212, and Pol 405 peptides, and KI-837 for Pol 455 and Pol 456 peptides. Induced CD8⁺ bulk T cells were stimulated with the corresponding peptide-pulsed C1R cells or 0.221 cells expressing each HLA-class I allele molecule. IFN- γ production by CD8⁺ T cells was detected by performing the intracellular cytokine staining (ICS) assay.



Japan). The CD8⁺ T cells without peptide stimulation were used as a negative control. The number of spots for each peptide-specific T cell response was calculated by subtracting the number of negative-control spots (the number of spots in wells without peptides). Spots giving a mean of more than + 2 SD of the negative-control spots were defined as positive responses. In order to find cross-clade CD8⁺ T cells in our cohort, we performed the ELISPOT assay by the same method with 11-mer single peptides that were the components of the cocktail peptides where 1) the frequency of responders was more than 20% or 2) the frequency was less than 20% but in which case at least 1 patient showed a high spot count (>750 spots).

2.5. Cells

721.221-CD4 cells expressing HLA-A*02:06, -A*33:03 or Cw*01:02 were generated by transfecting both human CD4 gene and one of these HLA-class I genes into 721.221 cells. These cells were maintained in RPMI medium containing 10% fetal calf serum (FCS) and 0.15 mg/mL hygromycin B. C1R cells expressing HLA-A*02:06 and those expressing HLA-A*33:03 were generated by transfecting C1R cells with HLA-A*02:06 and -A*33:03, respectively; and they were maintained in RPMI medium containing 10% FCS and 0.15 mg/mL hygromycin. C1R and 721.221 cells expressing other HLAs used in this study were previously generated and maintained in RPMI medium with 10% FCS and 0.15 mg/mL hygromycin B or 0.2 mg/mL neomycin [33–36].

2.6. Induction of peptide-specific CTLs from PBMCs

PBMCs from HIV-1-infected individuals who showed the responses to the cocktail peptides in the ELISPOT assay were stimulated with 11-mer single peptide or optimal peptide derived from consensus clade B HIV-1 (100 nM) and then cultured in culture medium (RPMI-1640 containing 10% FCS and 200 U/ml interleukin-2) for 2 weeks. These bulk cultured cells were used for intracellular IFN- γ staining assays.

2.7. HIV clones

The replication-competent molecular clones of p93JP-NH1 [37] and pNL-432 [38] reported previously were used in this study. Viral stocks were generated from plasmid DNA as described elsewhere [15,39].

2.8. HIV-1 infection of .221-CD4 cells expressing HLA molecules or not

.221-CD4 cells expressing HLA molecules or not were exposed to each virus for several days. These infected cells were used as stimulator cells for performing an intracellular cytokine staining assay (ICS) when approximately 30–60% of the cells had been infected, which infection was confirmed by intracellular staining for HIV-1 p24 antigen (KC-57-FITC; Beckman Coulter).

2.9. Intracellular cytokine staining assay (ICS)

After .221 cells or C1R cells had been incubated for 60 min with each peptide (0.01–100 nM), they were washed twice with RPMI-1640 containing 10% FCS. These peptide-pulsed or HIV-1-infected .221-CD4 cells (1×10^5 cells per well) and bulk cultured cells (2×10^4 cells per well) were added to wells of a 96-well round-bottomed plate, and then the cells were incubated for 2 h at 37 °C. Brefeldin A (10 μ g/ml) was then added, after which the cells were incubated for a further 4 h. After having been stained with APC-labeled anti-CD8 mAb (DAKO, Glostrup, Denmark), the cells were fixed with 4% paraformaldehyde and then made permeable with the permeabilizing buffer (0.1% saponin and 5% FCS in PBS). Thereafter the cells were stained with FITC-labeled anti-IFN- γ mAb (BD Bioscience, CA). The percentage of IFN- γ ⁺CD8⁺ cells was analyzed by flow cytometry.

3. Results

3.1. CD8⁺ T cell responses to HIV-1 clade B-derived overlapping peptides by HIV-1 clade A/E-infected individuals

To clarify cross-clade responses of CD8⁺ T cells between the clade B and A/E, we analyzed cross-clade responses of CD8⁺ T cells from 26 clade A/E-infected Japanese individuals to 11-mer overlapping peptides derived from the consensus sequence of HIV-1 clade B Nef, Gag, and Pol regions. We measured the responses of CD8⁺ T cells to cocktails including ten 11-mer overlapping peptides by performing the ELISPOT assay. The median of total magnitudes of the CD8⁺ T cell responses to Nef, Gag, and Pol cocktails were 483, 1037, and 2538, respectively (Fig. 1A). There were no significant differences in total magnitude of the CD8⁺ T cell responses between the clade A/E-infected and 401 clade B-infected Japanese individuals (the median of total magnitude against Nef, Gag and Pol in the clade B-infected individuals were 529,

Fig. 3. Identification of optimal epitopes Truncated peptides were designed based on HLA binding motif, and CD8⁺ bulk T cells were induced from PBMCs of the following 6 responders. KI-648 for Nef 41, Gag 171, and Pol 141 peptides, KI-632 for Nef 67, Pol 297 and Pol 436 peptides, KI-388 for Gag 16, Gag 20, Gag 104, Gag 105, Gag 106, and Pol 383 peptides, KI-724 for Gag 138 peptide, KI-964 for Pol 212 and Pol 405 peptides, and KI-837 for Pol 455 peptide. IFN- γ production by CD8⁺ bulk T cells was measured by performing the ICS assay using the target C1R or .221 cells expressing HLA molecules prepulsed with truncated peptide or 11-mer peptide at a concentration of 100 nM. When the same level of response was seen at 100 nM, the ICS assay was performed again at concentrations from 0.1 to 100 nM. **A.** responses of HLA-A*02:06-restricted CD8⁺ bulk T cells **B.** responses of HLA-B*40:02-restricted CD8⁺ bulk T cells **C.** responses of HLA-A*33:03, A*24:02, Cw*01:02 or B*52:01-restricted CD8⁺ bulk T cells.

1774, and 2300, respectively; H. Murakoshi et al. unpublished observation), although the identities of amino acid sequence in Nef, Gag, and Pol between clade the A/E and the clade B were 80.1, 84.3 and 92.3%, respectively. These results strongly suggest that cross-clade CD8⁺ T cells were frequently elicited in the clade A/E-infected individuals.

3.2. Identification of cross-clade CD8⁺ T cells elicited in HIV-1 clade A/E-infected individuals

To identify cross-clade CD8⁺ T cells in the clade A/E-infected individuals, we focused on the CD8⁺ T cell responses found to be strong or at a high frequency in these individuals (see Materials and methods). We selected the CD8⁺ T cell responses to 13 cocktails including 2 Nef, 4 Gag, and 7 Pol cocktails (solid bars in Fig. 1B). First, to clarify which 11-mer peptides were recognized by the specific CD8⁺ T cells, we selected the clade A/E-infected responders (KI-388, KI-632, KI-648, KI-659, KI-724, KI-837, and KI-964) and measured the CD8⁺ T cell responses to ten 11-mer peptides in each cocktail by using the ELISPOT assay. We found positive responses to three 11-mer Nef peptides (Nef cocktail 5: Nef 41 and 42, Nef cocktail 7: Nef 67), to ten 11-mer Gag peptides (Gag cocktail 2: Gag 11, 16, 19, and 20, Gag cocktail 11: Gag 101, 104, 105, and 106, Gag cocktail 14: Gag 138, Gag cocktail 18: Gag 171), and to eleven 11-mer Pol peptides (Pol cocktail 15: Pol 141 and 142, Pol cocktail 22: Pol 211 and 212, Pol cocktail 30: Pol 297 and 298, Pol cocktail 39: Pol 383, Pol cocktail 41: Pol 405, Pol cocktail 44: Pol 436, Pol cocktail 46: Pol 455 and 456) (data not shown). We next sought to determine HLA restriction molecules in these responses. PBMCs from these responders were stimulated with the 11-mer peptides and then cultured for 14 days. In order to determine the HLA restriction molecules, responses of the cultured cells against the corresponding peptides were analyzed by performing the intracellular cytokine staining (ICS) assay using HLA class I gene-transfected C1R cells or 721.221 cells as stimulators. We found 10 HLA-A*02:06-restricted responses, 6 HLA-B*40:02-restricted responses, 3 A*33:03-restricted responses, 1 HLA-A*24:02-restricted response, 1 HLA-B*52:01-restricted response, and 1 Cw*01:02-restricted response (Fig. 2).

We first analyzed the responses to the 10 HLA-A*02:06-restricted responses. Concerning the responses to overlapping peptides at 3 locations (Nef 41/42, Pol 141/142, and Pol 211/212), we speculated that they would be the same epitope-specific CD8⁺ T cell responses since the responses to these overlapping peptides were restricted by HLA-A*02:06. Therefore we focused on analyzing the response to Nef 41, Pol 141 or Pol 211, which showed higher responses than those to the other overlapping peptides (data not shown). We generated truncated peptides that were speculated based on HLA-A*02:06 binding motif (Ala, Thr or Gln at position 2) [40–43] and then analyzed these CD8⁺ T cell responses to Nef 41, Pol 141, and Pol 212 by using them. As shown in Fig. 3A, we identified 3 optimal epitopes: Nef GL9 (GALDLSHFL), Pol YI9 (YTAFTIPSI), and Pol SV9

(SQIYAGIKV). CD8⁺ T cell responses to Nef GL9 and Pol SV9 were detected among the responses to other overlapping peptides, Nef 42 and Pol 211, respectively (data not shown), indicating that the responses to Nef GL9 and Pol SV9 reflected those to Nef 42 and Pol 212, respectively. In contrast, Pol 142 did not contain Pol YI9. We analyzed the CD8⁺ bulk T cells induced by Pol 142 by using the truncated peptides and identified Pol TI8 (TAFTIPSI) as an optimal epitope. However, the response to Pol TI8 in CD8⁺ bulk T cells induced by Pol 142 (4.24% IFN- γ secretion at 100 nM peptide concentration) was much lower than that to Pol TI8 in CD8⁺ bulk T cells induced by Pol 141 (12.07% at 100 nM; Fig. 3A), suggesting that Pol TI8 may have been a very weak epitope. Similarly, we analyzed the other 4 HLA-A*02:06-restricted responses (Gag16, Gag171, Pol383, and Pol405) by using truncated peptides and identified 4 optimal epitopes; Gag LL11 (LKHIVWASREL), Gag AA9 (ATLEEMMTA), Pol GI9 (GQVDCSPGI), and Pol GL9 (GQETAYFLL; Fig. 3A).

By using the same method identified the HLA-A*02:06-restricted epitopes, we attempted to identify other epitopes. We generated truncated peptides based on HLA binding motif [7,33,40,44–50] and then the responses to these truncated peptides were analyzed using the ICS assay. We finally identified 5 HLA-B*40:02-restricted epitopes (Gag RV8, Gag AP8, Gag AV9, Gag EG11, and Pol GI8; Fig. 3B), 2 HLA-A*33:03-restricted epitopes (Pol FR9 and Pol ER10; Fig. 3C), 1 HLA-A*24:02-restricted epitope (Nef RF10; Fig. 3C), 1 HLA-B*52:01-restricted epitope (Gag RI8; Fig. 3C) and 1 HLA-Cw*01:02-restricted epitope (Gag YI9; Fig. 3C).

Eleven of the above 17 peptides were reported as epitopes in previous studies [7,33,40–43,45,47–50], whereas the other 6 peptides (HLA-A*02:06-restricted Gag LL11, Pol SV9, and Pol GI9, as well as HLA-B*40:02-restricted Gag RV8, Gag AP8, and Gag EG11) had not been previously reported to be epitopes. Therefore, we examined whether the CD8⁺ bulk T cells specific for these 6 epitopes could recognize HIV-1 clade B virus-infected cells. We measured the IFN- γ production from the CD8⁺ bulk T cells for target cells infected with HIV-1 clade B clone, NL4-3. These CD8⁺ bulk T cells effectively produced IFN- γ (data not shown), indicating that these 6 peptides had been naturally processed and presented in cells infected with HIV-1.

3.3. Cross-recognition between the clade B and A/E

We sequenced each epitope region in 26 HIV-1 clade A/E-infected Japanese individuals and then compared these sequences to those from the clade A/E and B viruses reported in the database of the Los Alamos National Library. The results showed that the consensus amino acid sequences of these epitopes in our cohort were the same as those in the database of Los Alamos National Library. The clade B consensus sequences of 6 epitopes (Gag AA9, Pol YI9, Pol SV9, Pol GI9, Pol GL9, and Pol ER10) were identical to the clade A/E consensus ones, whereas other 11 epitopes showed different consensus sequences between the clade B and A/E viruses (Table 1).

Table 1
Frequency of amino-acid sequence for each epitope region of clade A/E viruses from Los Alamos National Library database and our cohort patients.

Epitope	HXB2 region	Sequence	Frequency of amino acid sequence of each clade virus		
			Clade B viruses for Los Alamos database	Clade A/E viruses for Los Alamos database	Clade A/E viruses for our cohort patients
Nef GL9	Nef(83–91)	GALDLSHF ^a	529/1494	0/76	1/26
		—F—F—	12/1494	47/76	16/26
		Others	953/1494	29/76	9/26
Nef RF10	Nef(134–143)	RYPLTFGWCF ^a	800/1494	0/76	2/25
		—C—	94/1494	56/76	10/25
		others	600/1494	20/76	13/25
Gag LL11	p17(31–41)	LKHIVWASREL ^a	1182/1644	18/315	2/26
		M—L—	0/1644	147/315	14/26
		others	462/1644	150/315	10/26
Gag RV8	p17(39–46)	RELERFAV ^a	1299/1644	20/315	4/26
		—L	111/1644	217/315	17/26
		others	234/1644	78/315	5/26
Gag AP8	p24(78–85)	AEWDRLHP ^a	1263/1644	53/315	1/26
		—V—	128/1644	185/315	10/26
		others	253/1644	77/315	15/26
Gag AV9	p24(78–86)	AEWDRLHPV ^a	1135/1644	47/315	1/26
		—V—	121/1644	178/315	8/26
		others	388/1644	90/315	17/26
Gag EG11	p24(79–89)	EWDRLHPVHAG ^a	921/1644	48/315	1/26
		—V—	107/1644	149/315	5/26
		others	616/1644	118/315	20/26
Gag RI8	p24(143–150)	RMYSPTSI ^a	1033/1644	30/315	2/26
		—V—	351/1644	212/315	15/26
		others	260/1644	73/315	9/26
Gag YI9	p24(145–153)	YSPTSILDI ^a	1032/1644	30/315	2/26
		—V—	345/1644	212/315	15/26
		others	267/1644	73/315	9/26
Gag AA9	p24(209–217)	ATLEEMMTA ^a	1468/1644	285/315	25/26
		others	176/1644	30/315	1/26
		others	429/1003	15/59	10/26
Pol YI9	RT(127–135)	YTAFTIPSI ^a	574/1003	44/59	16/26
		others	429/1003	15/59	10/26
		others	565/1003	21/59	8/26
Pol SV9	RT(268–276)	SQIYAGIKV ^a	438/1003	38/59	18/26
		others	565/1003	21/59	8/26
		others	838/1003	9/59	9/26
Pol FR9	RT(440–448)	FYVDGAANR ^a	838/1003	9/59	9/26
		—S—	47/1003	45/59	15/26
		others	118/1003	5/59	2/26
Pol GI9	Integrase(50–60)	GQVDCSPGI ^a	915/1003	55/59	26/26
		others	88/1003	4/59	0/26
		others	519/1003	52/59	19/26
Pol GL9	Integrase(94–102)	GQETAYFLL ^a	519/1003	52/59	19/26
		others	484/1003	7/59	7/26
		others	770/1003	40/59	21/26
Pol ER10	Integrase(157–166)	ELKKIIGQVR ^a	770/1003	40/59	21/26
		others	233/1003	19/59	5/26
		others	535/1003	0/57	0/26
Pol GI8	Integrase(197–204)	GERIVDII ^a	535/1003	0/57	0/26
		—I—	378/1003	56/59	23/26
		others	90/1003	3/59	3/26

^a Amino acid sequence in clade B consensus used in this study.

We investigated the cross-recognition of these 11 epitope peptides by CD8⁺ T cells that had been induced by stimulating PBMCs from clade A/E virus-infected individuals with clade B-derived epitope peptides. The CD8⁺ bulk T cells induced by Nef RF10, Gag RV8, Gag AP8, Gag AV9, or Gag YI9 peptides recognized both clades B and A/E peptides evenly (Fig. 4A). The CD8⁺ bulk T cells induced by Gag EG11, Gag RI8, or Pol FR9 more strongly recognized the clade B-derived peptide than the clade A/E-derived one, whereas those induced by Nef GL9 or Pol GI8 more strongly recognized the clade A/E-derived peptide than the clade B-derived one (Fig. 4A). Interestingly, the CD8⁺ bulk T cells induced by Gag 16 LL11

failed to recognize the clade A/E-derived Gag LL11-1M-4L peptide (Fig. 4A).

Next, we investigated whether CD8⁺ T cells recognizing these 10 clade A/E peptides could recognize clade A/E-infected cells. We measured IFN- γ production from the CD8⁺ bulk T cells for target cells infected with HIV-1 clade A/E clone or for those infected with HIV-1 clade B clone. The CD8⁺ bulk T cells induced by Nef RF10, Gag RV8, Gag AP8, Gag AV9, Gag EG11, Gag YI9, Gag RI8, or Pol GI8 recognized not only the clade B virus-infected cells but also the clade A/E virus-infected ones (Fig. 4B), indicating that these cross-clade epitopes had been naturally processed

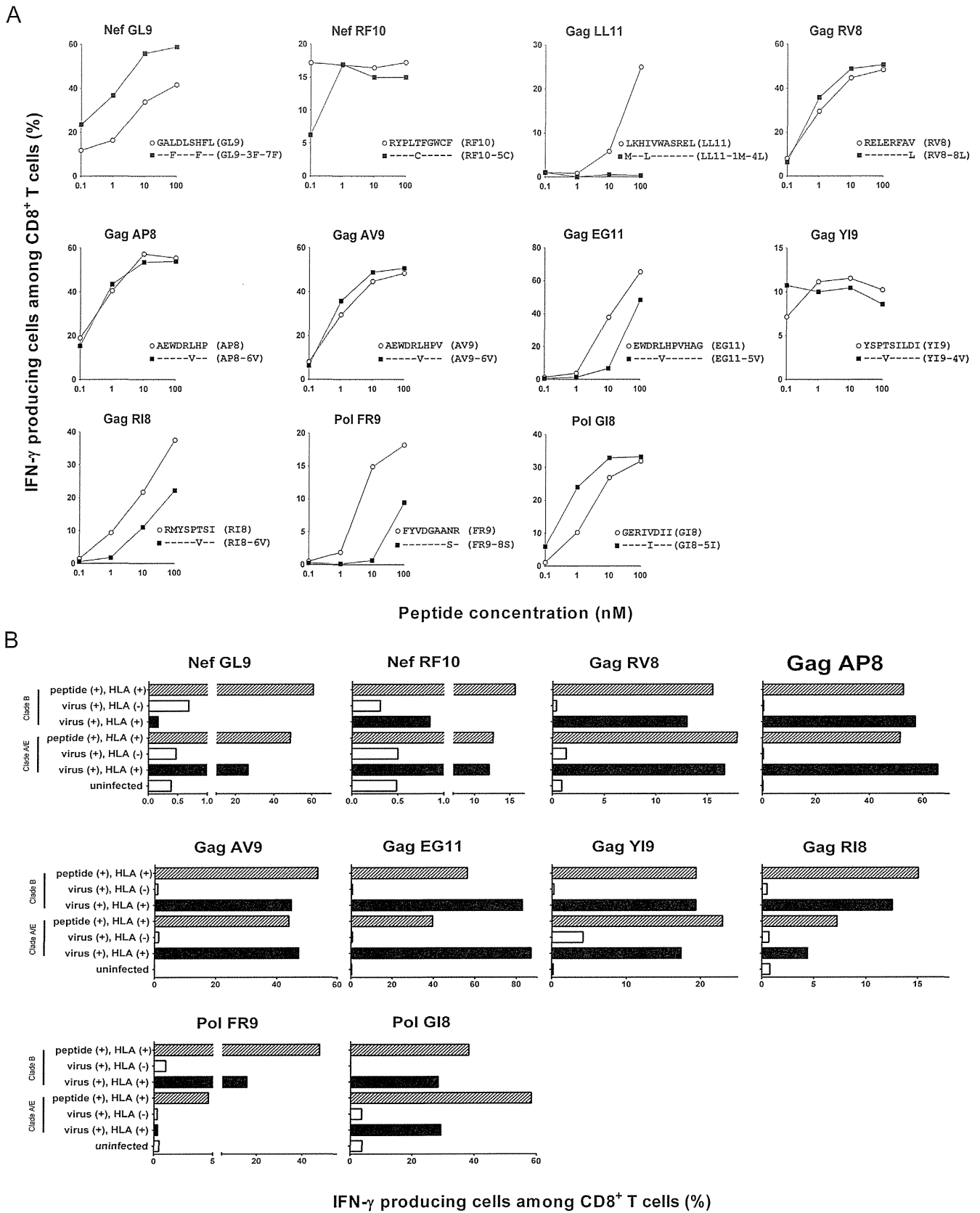


Fig. 4. Cross-recognition by CD8⁺ T cells from HIV-1 clade A/E-infected individuals IFN- γ production by CD8⁺ bulk T cells in response to stimulator cells with optimal epitope peptides and to cells infected with clade B or clade A/E viruses was measured by use of the ICS assay. Nef GL9 specific bulk T cells were induced from PBMCs of KI-648, Nef RF10 and Pol FR9 specific bulk T cells were induced from PBMCs of KI-632, Gag LL11, Gag RV8, Gag AP8, Gag AV9 and Gag EG11 specific bulk T cells were induced from PBMCs of KI-388, Gag RI8 and Gag YI9 specific bulk T cells were induced from KI-724, and Pol GI8 specific bulk

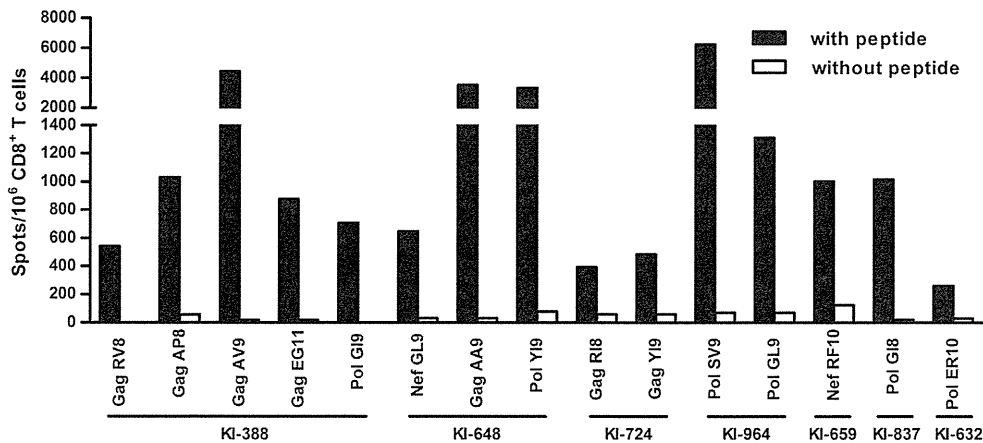


Fig. 5. CD8⁺ T cell responses to clade B-derived epitope peptides in HIV-1 clade A/E-infected Japanese individuals. CD8⁺ T cell responses to 15 clade B-derived epitope peptides were analyzed by performing ELISPOT assay using CD8⁺ T cells from seven clade A/E-infected individuals (KI-388, KI-632, KI-648, KI-659, KI-724, KI-837, and KI-964). >200 spots were evaluated as positive response.

and presented in cells infected with these viruses. On the other hand, CD8⁺ bulk T cells induced by Pol FR9 recognized the clade B virus-infected cells but failed to recognize the clade A/E virus-infected cells (Fig. 4B). This finding is consistent with the low ability of these cells to recognize the clade A/E peptide (Fig. 4A). In contrast, CD8⁺ T cells induced by Nef GL9 recognized the clade A/E virus-infected cells but failed to recognize the clade B virus-infected cells although these T cells could recognize GL9 peptide. This result may be explained by the fact that the amino acid sequence of the clade B consensus peptide is different than that of the clade B clone, NL4-3 (Ala and Val at position 1 and 3, respectively, in Nef GL9 region). Thus, CD8⁺ T cells induced by 8 out of 10 clade B-derived epitope peptides successfully recognized both the clade B virus-infected and clade A/E-infected cells.

3.4. Detection of cross-clade CD8⁺ T cell responses in the clade A/E-infected Japanese individuals

To confirm CD8⁺ T cell responses to the 15 epitopes including Nef GL9, we analyzed CD8⁺ T cell responses to the clade B-derived epitope peptides in clade A/E-infected individuals who had HLA alleles restricting these epitopes. Positive CD8⁺ T cell responses to these 15 clade B-derived epitope peptides were detected in PBMCs from chronically HIV-1 clade A/E-infected individuals (Fig. 5). These results indicate that these cross-clade CTLs are elicited in these individuals.

4. Discussion

Previous studies, which focused on known CTL epitopes for the clade B or C viruses, showed the existence of cross-clade CTLs in HIV-1-infected individuals by demonstrating that CTL clones established by using clade-matched peptides from the clade B-infected or the clade C-infected individuals recognize the cells infected with other clade viruses [13–17]. These studies also showed that conserved epitopes across the clades are more likely recognized by the T cell clones and suggested that conserved epitopes would be a more preferable target for a widely effective CTL vaccine than variable ones. In the present study, we for the first time performed a comprehensive analysis of cross-clade CD8⁺ T cells by using 11-mer overlapping clade B-derived peptides to stimulate CD8⁺ T cells from HIV-1 clade A/E-infected individuals. Interestingly, we found a similar level of CD8⁺ T cell responses to clade B-derived Nef, Gag, and Pol peptides in the clade A/E virus-infected individuals as compared to those to the same peptides in clade B-infected individuals. These results strongly suggested the existence of a high number of cross-clade CTLs in the clade A/E virus-infected individuals. Indeed, we finally identified 15 cross-clade CTL epitopes from only 13 out of 85 overlapping peptide cocktails. These results strongly suggest that a large number of cross-clade CTLs were elicited in the clade A/E virus-infected individuals.

CD8⁺ T cells induced by Pol FR9 recognized to a much lesser extent the clade A/E-derived peptide (FR9-8S) than the clade B-derived peptide and recognized cells infected with

T cells were induced from KI-837. **A.** Cross-recognition of HIV-1 clade B (open circle) and clade A/E (closed square) optimal epitope peptides of the consensus sequence. These analyses were performed at peptide concentrations from 0.1 to 100 nM. **B.** Cross-recognition of cells infected with clade B or clade A/E virus. IFN- γ production by CD8⁺ bulk T cells in response to HLA-positive cells prepulsed with clade B or clade A/E consensus optimal peptide and that in response to HLA-negative cells infected with the virus and to uninfected HLA-positive cells were measured as positive and negative controls, respectively. All epitope sequences derived from the clade B or the clade A/E were identical to the sequences from clone virus (NL4-3 or 93JP-NH1) except for clade B Nef GL9 epitope (GALDLSHFL). NL4-3 has Ala and Val at positions 1 and 3 of this epitope, respectively.

clade B virus but not those infected with A/E viruses, suggesting that PolFR9-8S was not an epitope. Indeed, the HLA-A*33:03⁺ individuals were infected with the clade A/E virus carrying Pol FR9 sequence but not Pol FR9-8S one (data not shown). The CD8⁺ T cells induced by Nef GL9 recognized the clade A/E virus-infected cells, whereas they failed to recognize the clade B-infected ones. These T cells could recognize GL9 peptide, though they recognized more effectively Nef GL9-3F-7F peptides than the Nef GL9 one. However, CD8⁺ T cells specific for both Nef GL9 and Nef GL9-3F-7F were detected in 3 of 7 HLA-A*02:06⁺ individuals (data not shown). These results suggest that Nef GL9-3F-7F had been presented in the clade A/E-infected individuals. Therefore, the failure of the T cells to recognize cells infected with NL4-3 virus may have resulted from a different amino acid sequence of this epitope between the clade B consensus peptide and NL4-3 (Ala and Val at position 1 and 3, respectively, in Nef GL9 region). CD8⁺ bulk T cells induced by 8 other diverse epitopes effectively recognized both the clade B-infected and the clade A/E-infected cells, suggesting that these diverse epitopes could be cross-recognized by the T cells.

We previously reported that Phe at position 2 of Nef RF10 is an escape mutation in the clade B virus [48]. This escape mutation was frequently found in the clade A/E virus, though the consensus sequence was RF10-5C (RYPLCFGWCF; Table 1). Since RF10 and RF10-5C were cross-recognized by the CD8⁺ T cells induced by the RF10 peptide, these T cells would be expected to select 2F mutants in the clade A/E-infected individuals. These results indicate that RF10-5C was an HLA-A*24:02-restricted epitope in the clade A/E-infected individuals and that RF10-5C-specific CD8⁺ T cells could cross-recognize the RF10 epitope.

Since these epitopes were restricted by Asian HLA alleles, vaccine targeting these epitopes can cover Asian countries including south-east Asia and China where clade A/E and clade B viruses are prevalent. An HLA-B*40:02-restricted Nef epitope was known to be presented by world-wise HLA allele HLA-B*40:01 [33]. In addition, a previous study showed that Pol GL9-specific CD8⁺ T cells were elicited in a vaccinated individual carrying world-wise HLA allele, HLA-A*02:01 [42]. These studies together suggest that some of the HLA-B*40:02-restricted and HLA-A*02:06-restricted epitopes identified in this study may be CTL epitopes presented by these world-wise HLA alleles. Thus, vaccine targeting the cross-clade epitopes identified in this study may cover countries in Europe, and northern and southern Americas in addition to Asian countries.

In conclusion, we here performed the first comprehensive study of cross-clade T cell responses and demonstrated that CD8⁺ T cell responses to clade B-derived Nef, Gag, and Pol peptides were successfully induced in the clade A/E virus-infected individuals. We finally identified the 15 cross-clade epitopes which include not only conserved epitopes but also polymorphic epitopes across the different clades. These epitopes can thus be candidate targets of CTL-based vaccines.

Acknowledgments

The authors thank Dr. T. Akahoshi and Rie Maruyama for technical assistance and Sachiko Sakai for her secretarial assistance.

This research was supported by the Global COE program “Global Education and Research Center Aiming at the control of AIDS,” launched as a project commissioned by the Ministry of Education, Science, Sports, and Culture, Japan; and by a Joint Research Grant with the Institute of Tropical Medicine, Nagasaki University.

References

- [1] B.D. Walker, B.T. Korber, Immune control of HIV: the obstacles of HLA and viral diversity, *Nat. Immunol.* 2 (2001) 473–475.
- [2] S. Rerks-Ngarm, P. Pitisuttithum, S. Nitayaphan, J. Kaewkungwal, J. Chiu, R. Paris, N. Premsri, C. Namwat, M. de Souza, E. Adams, M. Benenson, S. Gurnathan, J. Tartaglia, J.G. McNeil, D.P. Francis, D. Stablein, D.L. Birx, S. Chunsuttiwat, C. Khamboonruang, P. Thongcharoen, M.L. Robb, N.L. Michael, P. Kulasol, J.H. Kim, MOPHTAVEG investigators, Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand, *N. Engl. J. Med.* 361 (2009) 2209–2220.
- [3] J. Hemelaar, E. Gouws, P.D. Ghys, S. Osmanov, WHO-UNAIDS Network for HIV isolation and characterisation, global trends in molecular epidemiology of HIV-1 during 2000–2007, *AIDS* 25 (2011) 679–689.
- [4] M.S. de Souza, S. Ratto-Kim, W. Chuenarom, A. Schuetz, S. Chantakulkij, B. Nuntapinit, A. Valencia-Micolta, D. Thelian, S. Nitayaphan, P. Pitisuttithum, R.M. Paris, J. Kaewkungwal, N.L. Michael, S. Rerks-Ngarm, B. Mathieson, M. Marovich, J.R. Currier, J.H. Kim, Ministry of Public Health-Thai AIDS Vaccine Evaluation Group Collaborators, The Thai phase III trial (RV144) vaccine regimen induces T cell responses that preferentially target epitopes within the V2 region of HIV-1 envelope, *J. Immunol.* 188 (2012) 5166–5176.
- [5] M. Bonsignori, J. Pollara, M.A. Moody, M.D. Alpert, X. Chen, K.K. Hwang, P.B. Gilbert, Y. Huang, T.C. Gurley, D.M. Kozink, D.J. Marshall, J.F. Whitesides, C.Y. Tsao, J. Kaewkungwal, S. Nitayaphan, P. Pitisuttithum, S. Rerks-Ngarm, J.H. Kim, N.L. Michael, G.D. Tomaras, D.C. Montefiori, G.K. Lewis, A. DeVico, D.T. Evans, G. Ferrari, H.X. Liao, B.F. Haynes, Antibody-dependent cellular cytotoxicity-mediating antibodies from an HIV-1 vaccine efficacy trial target multiple epitopes and preferentially use the VH1 gene family, *J. Virol.* 86 (2012) 11521–11532.
- [6] B.F. Haynes, P.B. Gilbert, M.J. McElrath, S. Zolla-Pazner, G.D. Tomaras, S.M. Alam, D.T. Evans, D.C. Montefiori, C. Karnasuta, R. Sutthent, H.X. Liao, A.L. DeVico, G.K. Lewis, C. Williams, A. Pinter, Y. Fong, H. Janes, A. DeCamp, Y. Huang, M. Rao, E. Billings, N. Karasavvas, M.L. Robb, V. Ngauy, M.S. de Souza, R. Paris, G. Ferrari, R.T. Bailer, K.A. Soderberg, C. Andrews, P.W. Beraman, N. Frahm, S.C. De Rosa, M.D. Alpert, N.L. Yates, X. Shen, R.A. Koup, P. Pitisuttithum, J. Kaewkungwal, S. Nitayaphan, S. Rerks-Ngarm, N.L. Michael, J.H. Kim, Immune-correlates analysis of an HIV-1 vaccine efficacy trial, *N. Engl. J. Med.* 366 (2012) 1275–1286.
- [7] P. Kiepiela, K. Ngumbela, C. Thobakgale, D. Ramduth, I. Honeyborne, E. Moodley, S. Reddy, C. de Pierres, Z. Mncube, N. Mkhwanazi, K. Bishop, M. van der Stok, K. Nair, N. Khan, H. Crawford, R. Payne, A. Leslie, J. Prado, A. Prendergast, J. Frater, N. McCarthy, C. Brander, G.H. Learn, D. Nickle, C. Rousseau, H. Coovadia, J.I. Mullins, D. Heckerman, B.D. Walker, P. Goulder, CD8⁺ T-cell responses to different HIV proteins have discordant associations with viral load, *Nat. Med.* 13 (2007) 46–53.
- [8] R. Zuniga, A. Lucchetti, P. Galvan, S. Sanchez, C. Sanchez, A. Hernandez, H. Sanchez, N. Frahm, C.H. Linde, H.S. Hewitt,

- W. Hildebrand, M. Altfeld, T.M. Allen, B.D. Walker, B.T. Korber, T. Leitner, J. Sanchez, C. Brander, Relative dominance of Gag p24-specific cytotoxic T lymphocytes is associated with human immunodeficiency virus control, *J. Virol.* 80 (2006) 3122–3125.
- [9] S.A. Migueles, M.S. Sabbaghian, W.L. Shupert, M.P. Bettinotti, F.M. Marincola, L. Martino, C.W. Hallahan, S.M. Selig, D. Schwartz, J. Sullivan, M. Connors, HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors, *Proc. Natl. Acad. Sci. USA* 97 (2000) 2709–2714.
- [10] E.L. Turnbull, A.R. Lopes, N.A. Jones, D. Cornforth, P. Newton, D. Aldam, P. Pellegrino, J. Turner, I. Williams, C.M. Wilson, P.A. Goepfert, M.K. Maini, P. Borrow, HIV-1 epitope-specific CD8+ T cell responses strongly associated with delayed disease progression cross-recognize epitope variants efficiently, *J. Immunol.* 176 (2006) 6130–6146.
- [11] S.L. Rowland-Jones, T. Dong, K.R. Fowke, J. Kimani, P. Krausa, H. Newell, T. Blanchard, K. Ariyoshi, J. Oyugi, E. Ngugi, J. Bwayo, K.S. MacDonald, A.J. McMichael, F.A. Plummer, Cytotoxic T cell responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi, *J. Clin. Invest.* 102 (1998) 1758–1765.
- [12] R.P. Johnson, A. Trocha, L. Yang, G.P. Mazzara, D.L. Panicali, T.M. Buchanan, B.D. Walker, HIV-1 gag-specific cytotoxic T lymphocytes recognize multiple highly conserved epitopes. Fine specificity of the gag-specific response defined by using unstimulated peripheral blood mononuclear cells and cloned effector cells, *J. Immunol.* 147 (1991) 1512–1521.
- [13] D.M. McKinney, R. Skvoretz, B.D. Livingston, C.C. Wilson, M. Anders, R.W. Chesnut, A. Sette, M. Essex, V. Novitsky, M.J. Newman, Recognition of variant HIV-1 epitopes from diverse viral subtypes by vaccine-induced CTL, *J. Immunol.* 173 (2004) 1941–1950.
- [14] M.S. Bennett, H.L. Ng, A. Ali, O.O. Yang, Cross-clade detection of HIV-1-specific cytotoxic T lymphocytes does not reflect cross-clade antiviral activity, *J. Infect. Dis.* 197 (2008) 390–397.
- [15] K. Fukada, H. Tomiyama, C. Wasi, T. Matsuda, S. Kusagawa, H. Sato, S. Oka, Y. Takebe, M. Takiguchi, Cytotoxic T-cell recognition of HIV-1 cross-clade and clade-specific epitopes in HIV-1-infected Thai and Japanese patients, *AIDS* 16 (2002) 701–711.
- [16] H. Cao, P. Kanki, J.L. Sankale, A. Dieng-Sarr, G.P. Mazzara, S.A. Kalams, B. Korber, S. Mboup, B.D. Walker, Cytotoxic T-lymphocyte cross-reactivity among different human immunodeficiency virus type 1 clades: implications for vaccine development, *J. Virol.* 71 (1997) 8615–8623.
- [17] D. Durali, J. Morvan, F. Letourneur, D. Schmitt, N. Guegan, M. Dalod, S. Saragosti, D. Sicard, J.P. Levy, E. Gomard, Cross-reactions between the cytotoxic T-lymphocyte responses of human immunodeficiency virus-infected African and European patients, *J. Virol.* 72 (1998) 3547–3553.
- [18] H. Chen, Z.M. Ndhlovu, D. Liu, L.C. Porter, J.W. Fang, S. Darko, M.A. Brockman, T. Miura, Z.L. Brumme, A. Schneidewind, A. Piechocka-Trocha, K.T. Cesa, J. Sela, T.D. Cung, I. Toth, F. Pereyra, X.G. Yu, D.C. Douek, D.E. Kaufmann, T.M. Allen, B.D. Walker, TCR clonotypes modulate the protective effect of HLA class I molecules in HIV-1 infection, *Nat. Immunol.* 13 (2012) 691–700.
- [19] M.P. Sanou, A.S. De Groot, M. Murphey-Corb, J.A. Levy, J.K. Yamamoto, HIV-1 vaccine trials: evolving concepts and designs, *Open AIDS J.* 6 (2012) 274–288.
- [20] B.T. Korber, N.L. Letvin, B.F. Haynes, T-cell vaccine strategies for human immunodeficiency virus, the virus with a thousand faces, *J. Virol.* 83 (2009) 8300–8314.
- [21] A.M. Masemola, T.N. Mashishi, G. Khoury, H. Bredell, M. Paximadis, T. Mathebula, D. Barkhan, A. Puren, E. Vardas, M. Colvin, L. Zijenah, D. Katzenstein, R. Musonda, S. Allen, N. Kumwenda, T. Taha, G. Gray, J. McIntyre, S.A. Karim, H.W. Sheppard, C.M. Gray, HIVNET 028 Study Team, Novel and promiscuous CTL epitopes in conserved regions of Gag targeted by individuals with early subtype C HIV type 1 infection from southern Africa, *J. Immunol.* 173 (2004) 4607–4617.
- [22] Y. Roshorm, M.G. Cottingham, M.J. Potash, D.J. Volsky, T. Hanke, T cells induced by recombinant chimpanzee adenovirus alone and in prime-boost regimens decrease chimeric EcoHIV/NDK challenge virus load, *Eur. J. Immunol.* 42 (2012) 3243–3255.
- [23] M.L. Knudsen, A. Mbewe-Mvula, M. Rosario, D.X. Johansson, M. Kakoulidou, A. Bridgeman, A. Reyes-Sandoval, A. Nicosia, K. Ljungberg, T. Hanke, P. Liljestrom, Superior induction of T cell responses to conserved HIV-1 regions by electroporated alphavirus replicon DNA compared to that with conventional plasmid DNA vaccine, *J. Virol.* 86 (2012) 4082–4090.
- [24] M. Rosario, N. Borthwick, G.B. Stewart-Jones, A. Mbewe-Mvula, A. Bridgeman, S. Colloca, D. Montefiori, A.J. McMichael, A. Nicosia, E.D. Quakkelaar, J.W. Drijfhout, C.J. Melief, T. Hanke, Prime-boost regimens with adjuvanted synthetic long peptides elicit T cells and antibodies to conserved regions of HIV-1 in macaques, *AIDS* 26 (2012) 275–284.
- [25] Y. Roshorm, J.P. Hong, N. Kobayashi, A.J. McMichael, D.J. Volsky, M.J. Potash, M. Takiguchi, T. Hanke, Novel HIV-1 clade B candidate vaccines designed for HLA-B*5101(+) patients protected mice against chimeric ecotropic HIV-1 challenge, *Eur. J. Immunol.* 39 (2009) 1831–1840.
- [26] I. Cebere, L. Dorrell, H. McShane, A. Simmons, S. McCormack, C. Schmidt, C. Smith, M. Brooks, J.E. Roberts, S.C. Darwin, P.E. Fast, C. Conlon, S. Rowland-Jones, A.J. McMichael, T. Hanke, Phase I clinical trial safety of DNA- and modified virus Ankara-vectored human immunodeficiency virus type 1 (HIV-1) vaccines administered alone and in a prime-boost regime to healthy HIV-1-uninfected volunteers, *Vaccine* 24 (2006) 417–425.
- [27] N. Goonetilleke, S. Moore, L. Dally, N. Winstone, I. Cebere, A. Mahmoud, S. Pinheiro, G. Gillespie, D. Brown, V. Loach, J. Roberts, A. Guimaraes-Walker, P. Hayes, K. Loughran, C. Smith, J. De Bont, C. Verlinde, D. Vooijs, C. Schmidt, M. Boaz, J. Gilmour, P. Fast, L. Dorrell, T. Hanke, A.J. McMichael, Induction of multifunctional human immunodeficiency virus type 1 (HIV-1)-specific T cells capable of proliferation in healthy subjects by using a prime-boost regimen of DNA- and modified vaccinia virus Ankara-vectored vaccines expressing HIV-1 Gag coupled to CD8+ T-cell epitopes, *J. Virol.* 80 (2006) 4717–4728.
- [28] M. Mwau, I. Cebere, J. Sutton, P. Chikoti, N. Winstone, E.G. Wee, T. Beattie, Y.H. Chen, L. Dorrell, H. McShane, C. Schmidt, M. Brooks, S. Patel, J. Roberts, C. Conlon, S.L. Rowland-Jones, J.J. Bwayo, A.J. McMichael, T. Hanke, A human immunodeficiency virus 1 (HIV-1) clade A vaccine in clinical trials: stimulation of HIV-specific T-cell responses by DNA and recombinant modified vaccinia virus Ankara (MVA) vaccines in humans, *J. Gen. Virol.* 85 (2004) 911–919.
- [29] G.J. Gorse, L.R. Baden, M. Wecker, M.J. Newman, G. Ferrari, K.J. Weinhold, B.D. Livingston, T.L. Villafana, H. Li, E. Noonan, N.D. Russell, HIV Vaccine Trial Network, Safety and immunogenicity of cytotoxic T-lymphocyte poly-epitope, DNA plasmid (EP HIV-1090) vaccine in healthy, human immunodeficiency virus type 1 (HIV-1)-uninfected adults, *Vaccine* 26 (2008) 215–223.
- [30] P. Spearman, S. Kalams, M. Elizaga, B. Metch, Y.L. Chiu, M. Allen, K.J. Weinhold, G. Ferrari, S.D. Parker, M.J. McElrath, S.E. Frey, J.D. Fuchs, M.C. Keefer, M.D. Lubeck, M. Egan, R. Braun, J.H. Eldridge, B.F. Haynes, L. Corey, NIAID HIV Vaccine Trials Network, Safety and immunogenicity of a CTL multiepitope peptide vaccine for HIV with or without GM-CSF in a phase I trial, *Vaccine* 27 (2009) 243–249.
- [31] D. Salmon-Ceron, C. Durier, C. Desaint, L. Cuzin, M. Surenaud, N.B. Hamouda, J.D. Lelievre, B. Bonnet, G. Pialoux, I. Poizat-Martin, J.P. Aboulker, Y. Levy, O. Launay, ANRS VAC trial group, immunogenicity and safety of an HIV-1 lipopeptide vaccine in healthy adults: a phase 2 placebo-controlled ANRS trial, *AIDS* 24 (2010) 2211–2223.
- [32] W. Jaoko, F.N. Nakwagala, O. Anzala, G.O. Manyonyi, J. Birungi, A. Nanvubya, F. Bashir, K. Bhatt, H. Ogutu, S. Wakasiaka, L. Matu, W. Waruingi, J. Odada, M. Oyaro, J. Indangasi, J. Ndinya-Achola, C. Konde, E. Mugisha, P. Fast, C. Schmidt, J. Gilmour, T. Tarragona, C. Smith, B. Barin, L. Dally, B. Johnson, A. Muluubya, L. Nielsen, P. Hayes, M. Boaz, P. Hughes, H. Hanke, A. McMichael, J. Bwayo, P. Kaleebu, Safety and immunogenicity of recombinant low-dosage HIV-1 A vaccine candidates vectored by plasmid pThr DNA or modified

- vaccinia virus Ankara (MVA) in humans in East Africa, *Vaccine* 26 (2008) 2788–2795.
- [33] T. Watanabe, H. Murakoshi, H. Gatanaga, M. Koyanagi, S. Oka, M. Takiguchi, Effective recognition of HIV-1-infected cells by HIV-1 integrase-specific HLA-B *4002-restricted T cells, *Microbe. Infect.* 13 (2011) 160–166.
- [34] S. Karaki, A. Kariyone, N. Kato, K. Kano, Y. Iwakura, M. Takiguchi, HLA-B51 transgenic mice as recipients for production of polymorphic HLA-A, B-specific antibodies, *Immunogenetics* 37 (1993) 139–142.
- [35] H. Koizumi, T. Iwatani, J. Tanuma, M. Fujiwara, T. Izumi, S. Oka, M. Takiguchi, Escape mutation selected by Gag28-36-specific cytotoxic T cells in HLA-A*2402-positive HIV-1-infected donors, *Microbe. Infect.* 11 (2009) 198–204.
- [36] Y. Yagita, N. Kuse, K. Kuroki, H. Gatanaga, J.M. Carlson, T. Chikata, Z.L. Brumme, H. Murakoshi, T. Akahoshi, N. Pfeifer, S. Mallal, M. John, T. Ose, H. Matsubara, R. Kanda, Y. Fukunaga, K. Honda, Y. Kawashima, Y. Ariumi, S. Oka, K. Maenaka, M. Takiguchi, Distinct HIV-1 escape patterns selected by cytotoxic T cells with identical epitope specificity, *J. Virol.* 87 (2013) 2253–2263.
- [37] H. Sato, Y. Tomita, K. Ebisawa, A. Hachiya, K. Shibamura, T. Shiino, R. Yang, M. Tatsumi, K. Gushi, H. Umeyama, S. Oka, Y. Takebe, Y. Nagai, Augmentation of human immunodeficiency virus type 1 subtype E (CRF01_AE) multiple-drug resistance by insertion of a foreign 11-amino-acid fragment into the reverse transcriptase, *J. Virol.* 75 (2001) 5604–5613.
- [38] A. Adachi, H.E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, M.A. Martin, Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone, *J. Virol.* 59 (1986) 284–291.
- [39] T. Akahoshi, T. Chikata, Y. Tamura, H. Gatanaga, S. Oka, M. Takiguchi, Selection and accumulation of an HIV-1 escape mutant by three types of HIV-1-specific cytotoxic T lymphocytes recognizing wild-type and/or escape mutant epitopes, *J. Virol.* 86 (2012) 1971–1981.
- [40] A.C. Karlsson, A.K. Iversen, J.M. Chapman, T. de Oliveira, G. Spotts, A.J. McMichael, M.P. Davenport, F.M. Hecht, D.F. Nixon, Sequential broadening of CTL responses in early HIV-1 infection is associated with viral escape, *PLoS One* 2 (2007) e225.
- [41] W. Kantakamalakul, M. de Souza, S. Bejrachandra, S. Ampol, J. Cox, R. Sutthent, Identification of a novel HIV type 1 CRF01_AE cytotoxic T lymphocyte (CTL) epitope restricted by an HLA-Cw0602 allele and a novel HLA-A0206/peptide restriction, *AIDS Res. Hum. Retroviruses* 22 (2006) 1271–1282.
- [42] F. Li, A.C. Finnefrock, S.A. Dubey, B.T. Korber, J. Szinger, S. Cole, M.J. McElrath, J.W. Shiver, D.R. Casimiro, L. Corey, S.G. Self, Mapping HIV-1 vaccine induced T-cell responses: bias towards less-conserved regions and potential impact on vaccine efficacy in the Step study, *PLoS One* 6 (2011) e20479.
- [43] S. Sabbaj, A. Bansal, G.D. Ritter, C. Perkins, B.H. Edwards, E. Gough, J. Tang, J.J. Szinger, B. Korber, C.M. Wilson, R.A. Kaslow, M.J. Mulligan, P.A. Goepfert, Cross-reactive CD8+ T cell epitopes identified in US adolescent minorities, *J. Acquir. Immune Defic. Syndr.* 33 (2003) 426–438.
- [44] M. Altfeld, M.M. Addo, R.L. Eldridge, X.G. Yu, S. Thomas, A. Khatri, D. Strick, M.N. Phillips, G.B. Cohen, S.A. Islam, S.A. Kalams, C. Brander, P.J. Goulder, E.S. Rosenberg, B.D. Walker, HIV Study Collaboration, Vpr is preferentially targeted by CTL during HIV-1 infection, *J. Immunol.* 167 (2001) 2743–2752.
- [45] M.S. Hossain, H. Tomiyama, T. Inagawa, S. Ida, S. Oka, M. Takiguchi, Identification and characterization of HLA-A*3303-restricted, HIV type 1 Pol- and Gag-derived cytotoxic T cell epitopes, *AIDS Res. Hum. Retroviruses* 19 (2003) 503–510.
- [46] K. Falk, O. Rotzschke, M. Takiguchi, B. Grahovac, V. Gnau, S. Stevanovic, G. Jung, H.G. Rammensee, Peptide motifs of HLA-A1, -A11, -A31, and -A33 molecules, *Immunogenetics* 40 (1994) 238–241.
- [47] Y. Ikeda-Moore, H. Tomiyama, K. Miwa, S. Oka, A. Iwamoto, Y. Kaneko, M. Takiguchi, Identification and characterization of multiple HLA-A24-restricted HIV-1 CTL epitopes: strong epitopes are derived from V regions of HIV-1, *J. Immunol.* 159 (1997) 6242–6252.
- [48] M. Fujiwara, J. Tanuma, H. Koizumi, Y. Kawashima, K. Honda, S. Mastuoka-Aizawa, S. Dohki, S. Oka, M. Takiguchi, Different abilities of escape mutant-specific cytotoxic T cells to suppress replication of escape mutant and wild-type human immunodeficiency virus type 1 in new hosts, *J. Virol.* 82 (2008) 138–147.
- [49] C. Brander, O.O. Yang, N.G. Jones, Y. Lee, P. Goulder, R.P. Johnson, A. Trocha, D. Colbert, C. Hay, S. Buchbinder, C.C. Bergmann, H.J. Zweerink, S. Wolinsky, W.A. Blattner, S.A. Kalams, B.D. Walker, Efficient processing of the immunodominant, HLA-A*0201-restricted human immunodeficiency virus type 1 cytotoxic T-lymphocyte epitope despite multiple variations in the epitope flanking sequences, *J. Virol.* 73 (1999) 10191–10198.
- [50] S. Buranapraditkun, U. Hempel, P. Pitakpolrat, R.L. Allgaier, P. Thantivorasit, S.I. Lorenzen, S. Sirivichayakul, W.H. Hildebrand, M. Altfeld, C. Brander, B.D. Walker, P. Phanuphak, P. Hansasuta, S.L. Rowland-Jones, T.M. Allen, K. Ruxrungtham, A novel immunodominant CD8+ T cell response restricted by a common HLA-C allele targets a conserved region of Gag HIV-1 clade CRF01_AE infected Thais, *PLoS One* 6 (2011) e23603.

High Prevalence of Illicit Drug Use in Men Who Have Sex with Men with HIV-1 Infection in Japan

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Abstract

Objective: To examine the prevalence of illicit drug use among men who have sex with men (MSM) with HIV-1 infection in Japan, where the life-time prevalence of illicit drug use in the general population is only 2.9%.

Design: A single-center cross-sectional study at a large HIV clinic in Tokyo, which treats approximately 15% of HIV-1 infected patients in Japan.

Methods: The prevalence of illicit drug use and the association of characteristics and social demographics of the patients with illicit drug use were examined. Patients who visited the clinic for the first time from 2005 to 2010 were enrolled. Relevant variables were collected using a structured interview and from the medical records. Multivariate logistic regression analyses were applied to estimate the odds of association of MSM over non-MSM HIV-infected patients with illicit drug use.

Results: 1,196 patients were enrolled. They were mostly Japanese men of relatively young age. Illicit drug use (including injection drugs) was reported by 35% of the patients (by 40% of MSM), and 4% were IDU while 5% were on methamphetamine. 2% of the population was arrested due to illicit drugs. MSM was significantly associated with illicit drug use (adjusted OR = 4.60; 95% CI, 2.88–7.36; $p < 0.01$). Subgroup analysis of the patients stratified by three age groups (≤ 30 , 31 to 40, and > 40) showed that the odds of association of MSM with illicit drug use was the strongest in the youngest age group (≤ 30 years: adjusted OR = 7.56; 95% CI, 2.86–20.0; $p < 0.01$), followed by the oldest (> 40 years: adjusted OR = 6.15; 95% CI, 2.40–15.8; $p < 0.01$), and the weakest in the group aged 31 to 40 (adjusted OR = 3.39; 95% CI, 1.73–6.63; $p < 0.01$).

Conclusions: The prevalence of illicit drug use is high among MSM patients with HIV-1 infection in Japan. Effective intervention for illicit drug use in this population is warranted.

Citation: Nishijima T, Gatanaga H, Komatsu H, Takano M, Ogane M, et al. (2013) High Prevalence of Illicit Drug Use in Men Who Have Sex with Men with HIV-1 Infection in Japan. PLoS ONE 8(12): e81960. doi:10.1371/journal.pone.0081960

Editor: Wenzhe Ho, Temple University School of Medicine, United States of America

Received: May 8, 2013; **Accepted:** October 18, 2013; **Published:** December 10, 2013

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Funding: This work was supported by Grants-in Aid for AIDS research from the Japanese Ministry of Health, Labour, and Welfare (H23-AIDS-001). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Illicit drug users, especially injection drug users (IDU), are at high risk of infection with HIV-1 [1,2]. They are one of the “difficult to reach” populations, especially with regard obtaining accurate prevalence data [3]. In Japan, the prevalence of illicit drug use in the general population is only 2.9% according to the 2009 Nationwide General Population Survey on Drug Use and Abuse [4,5] (<http://www.ncnp.go.jp/nimh/pdf/h21.pdf>, in Japanese) (<http://www.mhlw.go.jp/bunya/iyakuhin/yakubuturanyou/torikumi/dl/index-04.pdf>, in Japanese). To our knowledge, however, no study has examined the prevalence of illicit drug use among patients with HIV-1 infection in Japan.

Among patients with HIV-1 infection, illicit drug use is associated with lower antiretroviral therapy (ART) uptake and inferior adherence [6–9], which leads to suboptimal treatment outcome, compared with patients with other risk categories [10–12]. The aim of the present study was to examine the prevalence

of illicit drug use in patients with HIV-1 infection and its association with characteristics of the patients in Japan, in order to establish effective intervention strategies.

Methods

Ethics Statement

This study was approved by the Human Research Ethics Committee of National Center for Global Health and Medicine, Tokyo, Japan. The Committee waived a written informed consent, because this study only used data of patients from routine clinical practice. However, at our clinic each patient provided a written informed consent for the clinical and laboratory data to be used and published for research purposes [13]. We conducted this study according to the principles expressed in the Declaration of Helsinki.

Study design

This study was designed and reported according to the recommendations of Strengthening the Reporting of Observational studies in Epidemiology (STROBE) statement [14]. We performed a single center cross-sectional study of patients with HIV-1 infection to examine the prevalence of illicit drug according to patient characteristics including sexual orientation, primarily focusing on men who have sex with men (MSM). Illicit drugs were defined as legally prohibited substances in Japan; They included amyl nitrite and 5-methoxy-diisopropyltryptamine, which became prohibited by law in 2006 and 2005, respectively, in Japan [15]. This study was conducted at the AIDS Clinical Center, Tokyo. Our facility is one of the largest clinics for HIV care in Japan with more than 3,300 registered patients [13]. Considering that the total reported number of patients with HIV-1 infection is 21,415 by the end of 2011, this clinic treats approximately 15% of the HIV-1 infected patients in Japan (http://api-net.jfap.or.jp/status/2011/11nenpo/hyo_02.pdf. in Japanese).

Study Subjects

The study population comprised patients with HIV-1 infection, aged >17 years, who visited our clinic for the first time from January 1, 2005 to August 31, 2010. The following exclusion criteria were applied; 1) those who visited the clinic for a second opinion, 2) those referred to other facilities on their first or second visit. These patients were excluded because the structured interview on social demographics was often not conducted in these patients, 3) patients infected through contaminated blood products (e.g. hemophiliacs) and mother to child transmission, and 4) patients who refused to be included in the study.

Measurements

Variables were collected through a structured interview conducted at the first visit as part of routine clinical practice by the nurses specializing at the HIV outpatient care. The interview by these “coordinator nurses” included the following variables: history of illicit drug use and injection drug use (and their types if available), perceived route of transmission, sexual orientation (men were asked whether they have sex with men), history of gay bathhouse use (if MSM), working status, and living status (alone or with someone else) [16]. Because interviews could potentially underestimate the prevalence of illicit drug use, we also searched the medical records for information on illicit drug use and related variables covering the period from the first visit to December 2012. Data of age, sex, ethnicity, current treatment status for HIV infection, and history of AIDS (defined as history of or concurrent 23 AIDS-defining diseases set by the Japanese Ministry of Health, Labour and Welfare) were obtained from the medical records (<http://www.haart-support.jp/pdf/guideline2012.pdf> in Japanese). The laboratory data of CD4 cell count, HIV-1 viral load, hepatitis C antibody on the first visit were also collected, and when these tests were not conducted on that day, data within three months from the first visit were used.

Statistical analysis

Patients’ characteristics and social demographics were compared between MSM and non-MSM groups by the Student’s t-test for continuous variables and by either the χ^2 test or Fisher’s exact test for categorical variables. Logistic regression analysis was used to estimate the odds of association of MSM, relative to non-MSM, with illicit drug use. The odds of association of each basic demographics, baseline laboratory data, and other medical conditions listed above was also estimated with univariate analysis.

To estimate the odds of association of MSM over non-MSM with illicit drug use, we conducted multivariate logistic regression analysis adjusted by age and ethnicity. Age and ethnicity (Japanese) were selected among four variables with p value <0.05 in univariate analysis, because age is a basic demographic and the literature had reported that population/ethnicity can affect the prevalence of illicit drug use [17]. The two variables; “ART” and “history of AIDS” were not included because they were not considered to be related to illicit drug use.

To estimate the odds of association of different age categories with illicit drug use, we divided the group into three age subgroups: ≤ 30 , 31 to 40, and >40 years. Then, the above-mentioned multivariate analysis was conducted for each subgroup.

Statistical significance was defined at two-sided p value of <0.05. We used odds ratios (ORs) and 95% confidence intervals (95% CIs) to estimate the odds of association of each variable with illicit drug use. All statistical analyses were performed with The Statistical Package for Social Sciences ver. 20.0 (SPSS, Chicago, IL).

Results

During the study period, 1,366 patients with HIV-1 infection visited the AIDS Clinical Center for the first time, and 170 patients were excluded from the analysis based on the above-mentioned exclusion criteria (Figure 1). For the 1,196 patients included in the study, the perceived route of transmission was male-to-male sexual contact in 948 (79%), heterosexual contact in 173 (14%), IDU in 22 (2%), and unknown in 53 (4%). The majority of the study patients were relatively young Japanese men with a median age of 36 years. Most patients were ART-naïve, with a median CD4 count of 245/ μ l (Table 1).

Among the 1,196 patients, 415 (35%) had used or were illicit drug users, and 53 (4%) were IDUs while 63 (5%) reported using methamphetamine. With regard to social history, 27 (2%) had been detained or arrested due to possession or use of illicit drugs (Table 1). Among the illicit drugs used, amyl nitrite and 5-methoxy-diisopropyltryptamine were the most commonly named by the patients. 3,4-methylenedioxymethamphetamine, cannabis, heroin, cocaine, and opium were also mentioned (numbers not counted except for methamphetamine).

Of the 1,196 patients, 973 (81%) were MSM regardless of the perceived route of transmission (e.g., if a patient considered to have been infected with HIV-1 through injection drug use and was MSM, he was classified as MSM in Table 1). Compared with non-MSM patients, MSM were significantly younger and more likely to be Japanese. MSM patients were more likely to have experienced illicit drugs [392 (40%)] than non-MSM [23 (10%), $p < 0.01$], and have used methamphetamine [57 (6%) versus 6 (3%), $p = 0.07$], and to have been arrested/detained due to illicit drug use/possession [(26 (3%) versus 1 (0.4%), $p = 0.04$) (Table 1). There was no difference in the percentage of IDUs among the MSM and non-MSM groups [44 (5%) versus 9 (4%), $p = 0.73$]. The CD4 count of MSM patients tended to be higher, and MSM were less likely to present with AIDS than non-MSM, although HIV viral load of MSM was significantly higher than that of non-MSM. MSM were more likely to have a job and be living alone. Further analysis showed that 47% of MSM patients used a gay bathhouse, and among them, the prevalence of illicit drug use was higher (49%) than all MSM (40%). The prevalence of illicit drug use was even higher in MSM aged ≤ 30 years (52%).

Univariate analysis showed a significant relationship between MSM and illicit drug use (OR = 5.87; 95% CI, 3.74–9.20; $p < 0.01$) (Table 2, Model 1). Furthermore, younger age, being

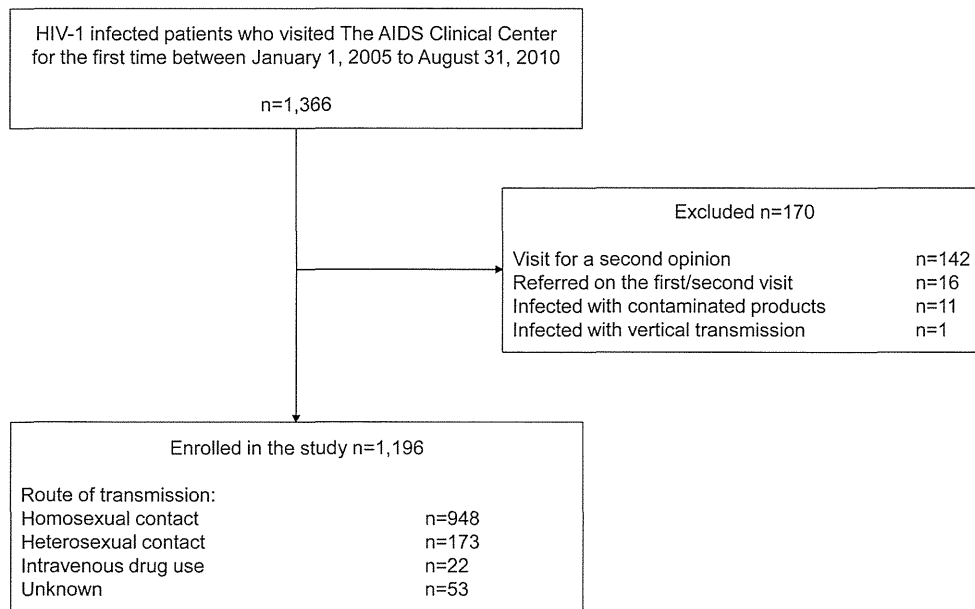


Figure 1. Patient enrollment.
doi:10.1371/journal.pone.0081960.g001

Table 1. Baseline characteristics of total study patients, MSM, and non-MSM.

	Total (n = 1,196)	MSM (n = 973)	Non-MSM (n = 223)	P value
Sex (male), n (%)	1,114 (93)	973 (100)	152 (63)	<0.01
Age (years) [†]	36 (29–43)	35 (29–42)	38 (31–47)	<0.01
History of illicit drug use, n (%)	415 (35)	392 (40)	23 (10)	<0.01
Injection drug use, n (%)	53 (4)	44 (5)	9 (4)	0.73
Methamphetamine use, n (%)	63 (5)	57 (6)	6 (3)	0.07
Arrested due to illicit drugs, n (%)	27 (2)	26 (3)	1 (0.4)	0.04
History of gay bathhouse use, n (%)	Not applicable	461 (47)		
Ethnicity, n (%) ^a				
Japanese	1058 (89)	906 (93)	152 (68)	<0.01
Asian	70 (6)	29 (3)	41 (18)	
White	27 (2)	26 (3)	1 (0.4)	
Black	26 (2)	2 (0.2)	24 (11)	
Latino	12 (1)	7 (1)	5 (2)	
Working status, n (%) ^b				
Without job	226 (19)	163 (17)	63 (28)	<0.01
Working	902 (75)	763 (78)	139 (63)	
Student	56 (5)	47 (5)	9 (4)	
Housewife	11 (1)	0	11 (5)	
Living alone, n (%) ^c	530 (44)	475 (49)	55 (25)	<0.01
CD4 count (/μl) ^{†d}	245 (101–379)	252 (114–380)	207 (50–379)	0.08
HIV-1 viral load (log ₁₀ /ml) ^{†e}	4.60 (3.91–5.20)	4.64 (3.94–5.20)	4.43 (3.26–5.08)	<0.01
On antiretroviral therapy, n (%)	120 (10)	85 (9)	35 (16)	<0.01
History of AIDS, n (%)	321 (27)	247 (25)	74 (33)	0.02
Positive HCV antibody, n (%)	38 (3)	19 (2)	19 (9)	<0.01

[†]median (interquartile range).
Data of ^athree, ^bone, ^cfifteen, ^dtwo, and ^efour patients were not available (missing).
doi:10.1371/journal.pone.0081960.t001

Japanese, on ART, and history of AIDS were associated with illicit drug use. On the other hand, without a job, living alone, and positive HCV antibody were not associated with illicit drug use. Multivariate analysis identified MSM to be significantly associated with illicit drug use after adjustment for age and Japanese (adjusted OR = 4.60; 95% CI, 2.88–7.36; p<0.01) (Table 2, Model 2).

Subgroup analysis of the patients stratified by three age groups (≤30, 31 to 40, and >40) showed that the odds of association of MSM with illicit drug use was the strongest in the youngest age group (≤30 years: adjusted OR = 7.56; 95% CI, 2.86–20.0; p<0.01), followed by the oldest (>40 years: adjusted OR = 6.15; 95% CI, 2.40–15.8; p<0.01), and the weakest in the group aged 31 to 40 (adjusted OR = 3.39; 95% CI, 1.73–6.63; p<0.01) (Table 3).

Discussion

The prevalence of illicit drug use among patients with HIV-1 infection in this large urban HIV clinic in Tokyo, which treats approximately 15% of patients with HIV-1 infection in Japan, was high at 35%. The prevalence was higher among HIV-1 infected MSM (40%), especially among young MSM aged ≤30 years (52%). Furthermore, HIV-1 infected MSM were more likely to use methamphetamine and to be arrested due to illicit drugs, compared with non-MSM. It should be emphasized that these numbers are likely to be underreported, since some patients would not admit illicit drug use to the interviewers on their first visit.

To our knowledge, this is the first study on the prevalence of illicit drug use among patients with HIV-1 infection in Japan. Although the prevalence of illicit drug use is considered extremely low among the general population in Japan with lifetime prevalence of 2.9% in 2009, high prevalence of illicit drug use in patients with HIV-1 infection, especially among HIV-1 infected MSM, was demonstrated [4,5] (<http://www.ncnp.go.jp/nimh/pdf/h21.pdf>. in Japanese) (<http://www.mhlw.go.jp/bunya/iyakuhin/yakubuturanyou/torikumi/dl/index-04.pdf>. in Japanese). The prevalence of methamphetamine use and incarceration due to illicit drug was also high, suggesting a substantial impact of illicit drugs, not only on the well-being of this population in terms of both medical and social perspectives, but also on public health perspectives [11,12].

In Japan, the number of illicit drug users arrested in 2010 was 14,965. Among these, 12,200 used methamphetamine, followed by cannabis (2,367), while only several hundred at most used other drugs (<http://www.mhlw.go.jp/bunya/iyakuhin/yakubuturanyou/torikumi/dl/index-01.pdf> in Japanese). Of note, the number of arrestees due to other injectable drugs, such as heroin and cocaine, was small (22 and 112, respectively). Thus, most injection drug users in Japan are methamphetamine users. Majority of the patients identified as IDU in this study were considered to be methamphetamine users as well.

Table 3. Results of multivariate analysis of the association of MSM over non-MSM for illicit drug use according to age.

	Adjusted OR	95% CI	P value
Age ≤30 years (n = 369)			
MSM vs. non-MSM	7.56	2.86–20.0	<0.01
Age 31 to 40 years (n = 473)			
MSM vs. non-MSM	3.39	1.73–6.63	<0.01
Age >40 years (n = 354)			
MSM vs. non-MSM	6.15	2.40–15.8	<0.01

MSM was adjusted with the same variables as Model 2, Table 2.

MSM: men who have sex with men.

doi:10.1371/journal.pone.0081960.t003

By the end of 2011, of 19,976 patients (excluding those infected with contaminated blood products) reported to be infected with HIV-1, 108 (0.5%) were reported to be infected through injection drug use according to the surveillance conducted by the AIDS Surveillance Committee of the Japanese Ministry of Health, Labour and Welfare (http://api-net.jfap.or.jp/status/2011/11nenpo/hyo_02.pdf in Japanese). The prevalence of IDUs in this study is substantially higher; 53 (4%) of the 1,196 were IDUs, suggesting a considerable underreporting of IDU in the surveillance data. It is well known that for IDUs, prognosis is much worse than non-injecting drug users, as one multicenter study conducted in Europe and North America reported that IDUs experienced approximately five times higher mortality rates than patients infected through sexual intercourse [18]. Although the prevalence of IDUs among patients with HIV-1 infection in Japan is still much lower than that in neighboring countries, such as Taiwan (27.6%) and China (24.3%), there is an urgent need to develop effective prevention programs for HIV-1 infected illicit drug users [19] (<http://www.unaids.org.cn/download/2009%20China%20Estimation%20Report-En.pdf>) (<http://www.cdc.gov.tw/english/list.aspx?treecid=00ED75D6C887BB27&nowtreeid=334C2073091C8677>).

Although the prognosis of injection drug users is reported to be worse than that of non-injection drug users [20], this study primarily focused on illicit drug use as a whole, rather than injection drug use. This is because only a few studies focused on illicit drug use among HIV-1 infected patients, although a large number of studies focused on injection drugs [21–25]. Illicit drug use in patients with HIV-1 infection is an important issue, because not only illicit drug use lead to inferior treatment outcome compared with non users [10–12], but also non injection drug users are prone to practice high risk sexual behaviors, which might lead to transmission of HIV and other infectious diseases [8,26]. Studies from the US reported that especially MSM who use illicit

Table 2. Results of multivariate analysis of the association of MSM over non-MSM for illicit drug use.

	Model 1 Crude n = 1,196		Model 2 Adjusted n = 1,196	
	OR	95% CI	OR	95% CI
Men who have sex with men [†]	5.87	3.74–9.20	4.60	2.88–7.36
Age per 1 year [†]			0.95	0.94–0.97
Japanese [†]			1.74	1.07–2.82

[†]p<0.05.

doi:10.1371/journal.pone.0081960.t002

drugs are at high risk for HIV and sexual transmitted infections due to close associations between risky sexual behaviors and illicit drug use [27,28] Furthermore, illicit drug use, especially opioid use, can be a trajectory into injection drug use [29,30].

Several limitations need to be acknowledged. First, due to the nature of single-center study, this is a convenience sample and the results of this study do not necessarily represent the prevalence of illicit drug use in all patients with HIV-1 infection in Japan. However, as mentioned above, our clinic treats approximately 15% of the total HIV patients in Japan, and furthermore, most HIV-1 infected patients reside in urban areas such as Tokyo metropolitan area (http://api-net.jfap.or.jp/status/2011/11nenpo/hyo_02.pdf in Japanese). Thus, the discrepancy in the prevalence of illicit drug use between the study patients and all HIV patients in Japan should not be too large. Second, the structured interview method to collect data cannot avoid underreporting of illicit drug usage. Thus, the prevalence of illicit drug use in this population is very likely to be higher than what is reported here. However, underreporting to a certain degree is unavoidable with regard to issues such as illicit drug use [3].

In conclusion, the prevalence of illicit drug use in patients with HIV-1 infection in this large HIV clinic in Tokyo was high at 35%, and was higher in HIV-1 infected MSM (40%). Despite the low prevalence of IDUs (0.5%) among HIV-infected patients reported by the AIDS Surveillance Committee, 5% of patients in this study were IDUs. All relevant parties to the issue of illicit drug

use in patients with HIV-1 infection need to recognize that illicit drug use is a huge burden in care and well-being of this population even in Japan, a country with very low prevalence of illicit drug use in the general population. Appropriate measures for prevention and intervention of illicit drug use are urgently needed to ensure proper treatment and prevention of spread of HIV infection.

Acknowledgments

The authors thank Dr. Kiyoshi Wada, Department of Drug Dependence Research, National Institute of Mental Health, National Center of Neurology and Psychiatry, and Dr. Keishiro Yajima, AIDS Medical Center, National Hospital Organization Osaka National Hospital, for valuable comments for the manuscript. The authors also thank “coordinator nurses” who conducted the structured interviews (Ruiko Yakuwa, Beni Ito, Yuko Sugino, Miki Koyama, Kenji Takeda, Megumi Shimada, Jongmi Seo, Yuki Yamada, Kyoko Ishigaki), and all other clinical staff at the AIDS Clinical Center, for their help in the completion of this study.

Author Contributions

Conceived and designed the experiments: TN HG HK MT SO. Performed the experiments: MO KI. Analyzed the data: TN HK HG MT SO. Contributed reagents/materials/analysis tools: MO KI SO. Wrote the paper: TN HG MT SO.

References

- Lehman JS, Allen DM, Green TA, Onorato IM (1994) HIV infection among non-injecting drug users entering drug treatment, United States, 1989–1992. *Field Services Branch. AIDS* 8: 1465–1469.
- Hahn RA, Onorato IM, Jones TS, Dougherty J (1989) Prevalence of HIV infection among intravenous drug users in the United States. *JAMA* 261: 2677–2684.
- Magnani R, Sabin K, Saidel T, Heckathorn D (2005) Review of sampling hard-to-reach and hidden populations for HIV surveillance. *AIDS* 19 Suppl 2: S67–72.
- Wada K (2011) The history and current state of drug abuse in Japan. *Ann N Y Acad Sci* 1216: 62–72.
- Tominaga M, Kawakami N, Ono Y, Nakane Y, Nakamura Y, et al. (2009) Prevalence and correlates of illicit and non-medical use of psychotropic drugs in Japan: findings from the World Mental Health Japan Survey 2002–2004. *Soc Psychiatry Psychiatr Epidemiol* 44: 777–783.
- Wood E, Montaner JS, Tyndall MW, Schechter MT, O’Shaughnessy MV, et al. (2003) Prevalence and correlates of untreated human immunodeficiency virus type 1 infection among persons who have died in the era of modern antiretroviral therapy. *J Infect Dis* 188: 1164–1170.
- Strathdee SA, Palepu A, Cornelisse PG, Yip B, O’Shaughnessy MV, et al. (1998) Barriers to use of free antiretroviral therapy in injection drug users. *JAMA* 280: 547–549.
- Malta M, Magnanini MM, Strathdee SA, Bastos FI (2010) Adherence to antiretroviral therapy among HIV-infected drug users: a meta-analysis. *AIDS Behav* 14: 731–747.
- Horstmann E, Brown J, Islam F, Buck J, Agins BD (2010) Retaining HIV-infected patients in care: Where are we? Where do we go from here? *Clin Infect Dis* 50: 752–761.
- Weber R, Huber M, Rickenbach M, Furrer H, Elzi L, et al. (2009) Uptake of and virological response to antiretroviral therapy among HIV-infected former and current injecting drug users and persons in an opiate substitution treatment programme: the Swiss HIV Cohort Study. *HIV Med* 10: 407–416.
- Milloy MJ, Marshall BD, Kerr T, Buxton J, Rhodes T, et al. (2012) Social and structural factors associated with HIV disease progression among illicit drug users: a systematic review. *AIDS* 26: 1049–1063.
- Porter K, Babiker A, Bhaskaran K, Darbyshire J, Pezzotti P, et al. (2003) Determinants of survival following HIV-1 seroconversion after the introduction of HAART. *Lancet* 362: 1267–1274.
- Nishijima T, Gatanaga H, Komatsu H, Tsukada K, Shimbo T, et al. (2012) Renal function declines more in tenofovir- than abacavir-based antiretroviral therapy in low-body weight treatment-naive patients with HIV infection. *PLoS One* 7: e29977.
- Vandenbroucke JP, von Elm E, Altman DG, Gotsche PC, Mulrow CD, et al. (2007) Strengthening the Reporting of Observational Studies in Epidemiology (STROBE): explanation and elaboration. *Epidemiology* 18: 805–835.
- Hidaka Y, Ichikawa S, Koyano J, Urao M, Yasuo T, et al. (2006) Substance use and sexual behaviours of Japanese men who have sex with men: a nationwide internet survey conducted in Japan. *BMC Public Health* 6: 239.
- Nishijima T, Gatanaga H, Komatsu H, Takano M, Ogane M, et al. (2013) Illicit Drug Use Is a Significant Risk Factor for Loss to Follow Up in Patients with HIV-1 Infection at a Large Urban HIV Clinic in Tokyo. *PLoS One* 8: e72310.
- Millett GA, Peterson JL, Wolitski RJ, Stall R (2006) Greater risk for HIV infection of black men who have sex with men: a critical literature review. *Am J Public Health* 96: 1007–1019.
- Zwahlen M, Harris R, May M, Hogg R, Costagliola D, et al. (2009) Mortality of HIV-infected patients starting potent antiretroviral therapy: comparison with the general population in nine industrialized countries. *Int J Epidemiol* 38: 1624–1633.
- Chen YM, Kuo SH (2007) HIV-1 in Taiwan. *Lancet* 369: 623–625.
- Qian HZ, Stimmette SE, Rebeiro PF, Kipp AM, Shepherd BE, et al. (2011) The relationship between injection and noninjection drug use and HIV disease progression. *J Subst Abuse Treat* 41: 14–20.
- Giordano TP, Hartman C, Gifford AL, Backus LI, Morgan RO (2009) Predictors of retention in HIV care among a national cohort of US veterans. *HIV Clin Trials* 10: 299–305.
- Rice BD, Delpech VC, Chadborn TR, Elford J (2011) Loss to follow-up among adults attending human immunodeficiency virus services in England, Wales, and Northern Ireland. *Sex Transm Dis* 38: 685–690.
- Ndiaye B, Ould-Kaci K, Salleron J, Bonnevie F, et al. (2009) Incidence rate and risk factors for loss to follow-up in HIV-infected patients from five French clinical centres in Northern France - January 1997 to December 2006. *Antivir Ther* 14: 567–575.
- Mocroft A, Kirk O, Aldins P, Chies A, Blaxhult A, et al. (2008) Loss to follow-up in an international, multicentre observational study. *HIV Med* 9: 261–269.
- Lebouche B, Yazdanpanah Y, Gerard Y, Sissoko D, Ajana F, et al. (2006) Incidence rate and risk factors for loss to follow-up in a French clinical cohort of HIV-infected patients from January 1985 to January 1998. *HIV Med* 7: 140–145.
- Latkin CA, Knowlton AR, Sherman S (2001) Routes of drug administration, differential affiliation, and lifestyle stability among cocaine and opiate users: implications to HIV prevention. *J Subst Abuse* 13: 89–102.
- Forrest DW, Metsch LR, LaLota M, Cardenas G, Beck DW, et al. (2010) Crystal methamphetamine use and sexual risk behaviors among HIV-positive and HIV-negative men who have sex with men in South Florida. *J Urban Health* 87: 480–485.
- Mansergh G, Shouse RL, Marks G, Guzman R, Rader M, et al. (2006) Methamphetamine and sildenafil (Viagra) use are linked to unprotected receptive and insertive anal sex, respectively, in a sample of men who have sex with men. *Sex Transm Infect* 82: 131–134.

29. Lankenau SE, Teti M, Silva K, Jackson Bloom J, Harocopos A, et al. (2012) Initiation into prescription opioid misuse amongst young injection drug users. *Int J Drug Policy* 23: 37–44.
30. Pollini RA, Banta-Green CJ, Cuevas-Mota J, Metzner M, Teshale E, et al. (2011) Problematic use of prescription-type opioids prior to heroin use among young heroin injectors. *Subst Abuse Rehabil* 2: 173–180.

WHO Antiretroviral Therapy Guidelines 2010 and Impact of Tenofovir on Chronic Kidney Disease in Vietnamese HIV-Infected Patients

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Abstract

Objective: The 2010 WHO antiretroviral therapy (ART) guidelines have resulted in increased tenofovir use. Little is known about tenofovir-induced chronic kidney disease (CKD) in HIV-infected Vietnamese with mean body weight of 55 kg. We evaluated the prevalence and risk factors of CKD in this country.

Design: Cross-sectional study was performed.

Methods: Clinical data on HIV-infected Vietnamese cohort were collected twice a year. To evaluate the prevalence of CKD, serum creatinine was measured in 771 patients in October 2011 and April 2012. CKD was defined as creatinine clearance less than 60 ml/min at both time points. Multivariate logistic regression was used to determine the factors associated with CKD

Results: Tenofovir use increased in Vietnam from 11.9% in April 2011 to 40.3% in April 2012. CKD was diagnosed in 7.3%, of which 7% was considered moderate and 0.3% was severe. Multivariate analysis of October-2011 data identified age per year-increase (OR: 1.229, 95%CI, 1.170-1.291), body weight per 1 kg-decrement (1.286, 1.193-1.386), and tenofovir use (2.715, 1.028-7.168) as risk factors for CKD.

Conclusions: Older age, low body weight and tenofovir use were independent risk factors for CKD in Vietnam. Further longitudinal study is required to evaluate the impact of TDF on renal function in Vietnam and other countries with small-body weight patients.

Citation: Mizushima D, Tanuma J, Kanaya F, Nishijima T, Gatanaga H, et al. (2013) WHO Antiretroviral Therapy Guidelines 2010 and Impact of Tenofovir on Chronic Kidney Disease in Vietnamese HIV-Infected Patients. PLoS ONE 8(11): e79885. doi:10.1371/journal.pone.0079885

Editor: Michael Alan Polis, National Institute of Allergy and Infectious Diseases, United States of America

Received: June 11, 2013; **Accepted:** September 25, 2013; **Published:** November 6, 2013

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Funding: This study was supported by Japan Initiative for Global Research Network on Infectious Diseases (J-GRID). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Advances in antiretroviral therapy (ART) had turned HIV/AIDS into a chronic disease [1-5]. As a consequence of living longer, chronic kidney disease (CKD) has become an important cause of morbidity and mortality in HIV-infected patients [1,3-5]. Several studies have reported increased prevalence of CKD, ranging from 4.9% to 8.4% in such patients [6-9]. In addition to the established risk factors, such as aging, diabetes mellitus (DM) and hypertension [2,10], other factors related to the virus itself and to the treatment [e.g., exposure to tenofovir (TDF), a commonly used antiretroviral (ARV)], are thought to be related to nephrotoxicity in HIV-infected patients [2,11,12].

To date, the benefit of TDF first line treatment is considered to outweigh the risk of TDF-induced nephrotoxicity. A recent meta-analysis study has reported that the use of TDF is associated with a statistically significant though only modest renal dysfunction, and recommended no restriction of TDF use when regular monitoring of renal function and serum phosphate levels is impractical [13]. Furthermore, the 2010 WHO guidelines for ART in adults and adolescents recommended TDF as part of the first line regimens (URL: http://whqlibdoc.who.int/publications/2010/9789241599764_eng.pdf).

However, several studies have reported that low body weight is an independent risk factor for TDF-associated nephrotoxicity and might lead to potentially higher risk for larger drug exposure and thus, more severe toxicity [14-17]. Under such