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Clinical Control of HIV-1 by Cytotoxic T Cells Specific for Multiple Conserved Epitopes

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ABSTRACT

Identification and characterization of CD8⁺ T cells effectively controlling HIV-1 variants are necessary for the development of AIDS vaccines and for studies of AIDS pathogenesis, although such CD8⁺ T cells have been only partially identified. In this study, we sought to identify CD8⁺ T cells controlling HIV-1 variants in 401 Japanese individuals chronically infected with HIV-1 subtype B, in which protective alleles HLA-B*57 and HLA-B*27 are very rare, by using comprehensive and exhaustive methods. We identified 13 epitope-specific CD8⁺ T cells controlling HIV-1 in Japanese individuals, though 9 of these epitopes were not previously reported. The breadths of the T cell responses to the 13 epitopes were inversely associated with plasma viral load ($P = 2.2 \times 10^{-11}$) and positively associated with CD4 count ($P = 1.2 \times 10^{-11}$), indicating strong synergistic effects of these T cells on HIV-1 control *in vivo*. Nine of these epitopes were conserved among HIV-1 subtype B-infected individuals, whereas three out of four nonconserved epitopes were cross-recognized by the specific T cells. These findings indicate that these 12 epitopes are strong candidates for antigens for an AIDS vaccine. The present study highlighted a strategy to identify CD8⁺ T cells controlling HIV-1 and demonstrated effective control of HIV-1 by those specific for 12 conserved or cross-reactive epitopes.

IMPORTANCE

HLA-B*27-restricted and HLA-B*57-restricted cytotoxic T lymphocytes (CTLs) play a key role in controlling HIV-1 in Caucasians and Africans, whereas it is unclear which CTLs control HIV-1 in Asian countries, where HLA-B*57 and HLA-B*27 are very rare. A recent study showed that HLA-B*67:01 and HLA-B*52:01-C*12:02 haplotypes were protective alleles in Japanese individuals, but it is unknown whether CTLs restricted by these alleles control HIV-1. In this study, we identified 13 CTLs controlling HIV-1 in Japan by using comprehensive and exhaustive methods. They included 5 HLA-B*52:01-restricted and 3 HLA-B*67:01-restricted CTLs, suggesting that these CTLs play a predominant role in HIV-1 control. The 13 CTLs showed synergistic effects on HIV-1 control. Twelve out of these 13 epitopes were recognized as conserved or cross-recognized ones. These findings strongly suggest that these 12 epitopes are candidates for antigens for AIDS vaccines.

Development of effective vaccines against HIV-1 is of importance for controlling the HIV-1 epidemic. Several extensive clinical trials have been performed, but only the RV144 vaccine, tested in a trial in Thailand, showed weak protection against HIV-1, most likely through generation of nonneutralizing antibodies (1, 2). A recent clinical trial showed no protection against HIV-1 acquisition, although vaccine-induced HIV-1-specific CD8⁺ T cell responses were detected in 64% of the vaccinees (3). This result implied that induction of low-quality HIV-1-specific CD8⁺ T cells may not be adequate for protection against HIV-1 infection. Therefore, vaccines stimulating the production of high-quality CD8⁺ T cells might be worth exploring. The quality of CD8⁺ T cells has been assessed in terms of several indicators, such as polyfunctionality (4), antigen sensitivity (5), proliferative capacity (6), immunoregulation (7), and properties of the interaction among the T cell receptor (TCR), viral peptide, and major histocompatibility complex (MHC) (8, 9). The ability of CD8⁺ T cells to suppress HIV-1 replication *in vitro* may be a better indicator of CD8⁺ T cell efficacy than the above indicators, since this ability in HIV-1 controllers is significantly higher than that in noncontrollers (10, 11).

It is well known that HLA-B*27-restricted and HLA-B*57-restricted cytotoxic T lymphocytes (CTLs) play a key role in the

control of HIV-1 in Caucasians and Africans carrying these alleles (12–14). However, the emergence of the R264K mutation within an HLA-B*27-restricted KK10 immunodominant epitope (KRW IILGLNK) leads to increased viral replication and progression to AIDS in HLA-B*27-positive HIV-1-infected individuals (13), suggesting that the emergence of the mutation allowing escape from CTLs results in the loss of HIV-1 control *in vivo*. In contrast, HLA-B*57-positive individuals still have a low plasma viral load (pVL) after the emergence of the T242N escape mutant selected by

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TW10-specific CTLs, since this mutant has a high viral fitness cost (15). These individuals eventually show an increased pVL due to rescue from the reduction in the replication capacity by compensatory mutations (16, 17). HLA-B*27 and HLA-B*57 are common alleles in Caucasians and Africans but very rare ones in Japan and other Asian countries, indicating that HIV-1 is not controlled by these immunodominant epitope-specific CTLs in HIV-1-infected individuals in Japan and some Asian countries. So far, there has been no report of epitope-specific CTLs controlling HIV-1 in countries where HLA-B*57 and HLA-B*27 are absent or very rare, such as in Japan. Studies to identify such CTLs controlling HIV-1 will contribute to vaccine development in countries where HLA-B*27 and HLA-B*57 are rare and even in countries where these alleles are frequently found.

In the present study, we sought to identify HIV-1-specific CD8⁺ T cells controlling HIV-1 in chronically HIV-1-infected Japanese individuals by employing exhaustive and comprehensive strategies. We first analyzed CD8⁺ T cell responses to 842 11-mer overlapping HIV-1 Gag, Pol, and Nef peptides in 401 chronically HIV-1 clade B-infected, antiretroviral therapy (ART)-naive Japanese individuals. We then selected the candidates for CD8⁺ T cell responses controlling HIV-1. Following reevaluation for the role of the identified specific CTLs in the control of HIV-1, we characterized the cross-reactivity of their escape mutants.

MATERIALS AND METHODS

Subjects. Four hundred one treatment-naive Japanese individuals with chronic HIV-1 clade B infection were enrolled in the National Center for Global Health and Medicine from 2008 to 2011. To minimize the influence of time lag postinfection among recruited patients on pVL and CD4 count, blood samples were collected from the recruited individuals at the first visit. In addition, patients with clinical AIDS were excluded. Informed consent was obtained from all individuals according to the Declaration of Helsinki. This study was approved by the ethics committees of the National Center for Global Health and Medicine and Kumamoto University. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood. HLA types of HIV-infected individuals were determined by standard sequence-based genotyping. The median virus load and CD4 count were 25,000 copies/ml (interquartile range [IQR], 6,700 to 94,000 copies/ml) and 324 cells/ μ l (IQR, 195 to 443 cells/ μ l), respectively.

Peptides. We previously designed overlapping peptides consisting of 11-mer amino acids, spanning Gag, Pol, and Nef of HIV-1 clade B consensus sequences (18). Each 11-mer peptide was overlapped by 9 amino acids. 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.

Enzyme-linked immunosorbent spot (ELISPOT) assay. CD8⁺ T cells were sorted from cryopreserved PBMCs from chronically HIV-1 clade B-infected Japanese individuals by using CD8 magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Peptide cocktails including 10 11-mer overlapping peptides at a concentration of 1 μ M and sorted cells at 1×10^5 cells/well were added to 96-well polyvinylidene plates (Millipore, Bedford, MA) that had been precoated with 5 mg/ml of anti-gamma interferon (anti-IFN- γ) monoclonal antibody (MAb) 1-D1K (Mabtech, Stockholm, Sweden). The plates were then incubated for 16 h at 37°C in 5% CO₂ and subsequently washed with phosphate-buffered saline (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, they were washed with PBS and then incubated with streptavidin-conjugated alkaline phosphatase (Mabtech) for 60 min at room temperature. After a washing with PBS, individual cytokine-pro-

ducing cells were detected as dark spots after a 20-min reaction with 5-bromo-4-chloro-3-iodolylphosphate and nitroblue tetrazolium by using an alkaline phosphatase-conjugated substrate (Bio-Rad, Richmond, CA). The spots were counted with an Eliphoto-Counter (Minerva Teck, Tokyo, Japan). The number of spots was standardized to that of spots/10⁶ CD8⁺ T cells by measuring the frequency of CD8⁺ T cells using flow cytometry. A mean + 3 standard deviations (SDs) of the spot number of samples from 13 HIV-1 naive individuals for these peptides was 162 spots/10⁶ CD8⁺ T cells. Therefore, we defined >200 spots/10⁶ CD8⁺ T cells as a positive response.

Cells. 721.221-CD4 cells expressing HLA-B*40:06, -B*67:01, -C*03:04, -C*04:01, -C*07:02, or -C*08:01 were generated by transfecting both the 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 of hygromycin B or 0.2 mg/ml of neomycin. C1R cells expressing HLA-B*40:06 and those expressing HLA-B*67:01 were generated by transfecting C1R cells with HLA-B*40:06 and -B*67:01, respectively, and they were maintained in RPMI medium containing 10% FCS and 0.2 mg/ml of neomycin. C1R and 721.221 cells expressing other HLAs used in this study were previously generated (18–25) and maintained in RPMI medium with 10% FCS and 0.15 mg/ml of hygromycin B or 0.2 mg/ml of neomycin.

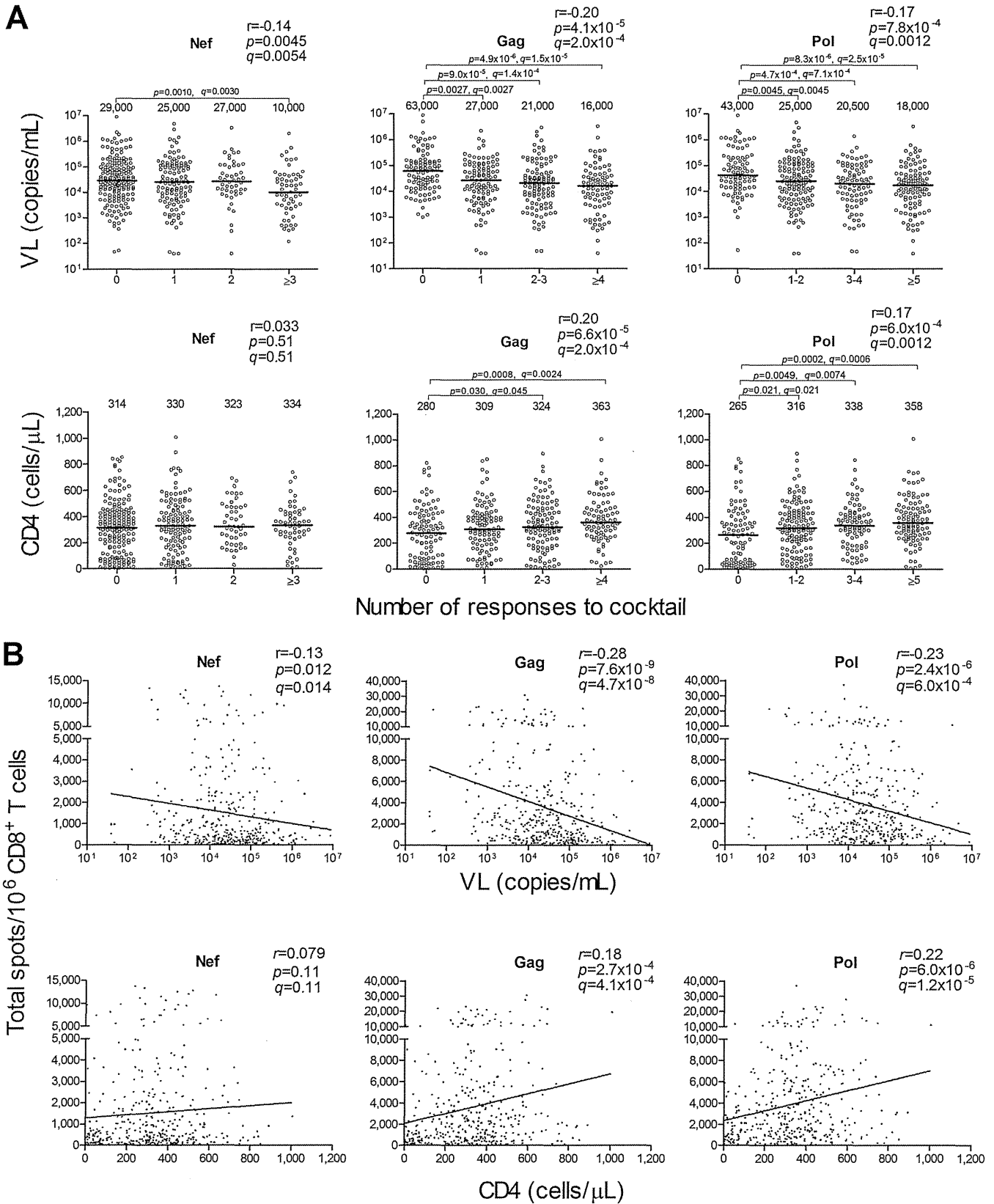
Generation of epitope-specific CTL clones. Epitope-specific CTL clones were generated from epitope-specific bulk T cells by limiting dilution in 96-U plates, together with 200 μ l of cloning mixture (5×10^5 irradiated allogeneic PBMCs from healthy donors, 1×10^5 irradiated C1R cells expressing each HLA molecule, and epitope peptides at a concentration of 100 nM in RPMI medium containing FCS, 200 U/ml of recombinant interleukin 2 [rIL-2], and 2.5% phytohemagglutinin [PHA]).

Intracellular cytokine staining (ICS) assay. After 721.221 cells or C1R cells had been incubated for 60 min with each peptide, they were washed twice with RPMI medium containing 10% FCS. These peptide-pulsed 721.221 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 subsequently added, after which the cells were incubated for a further 4 h. After having been stained with allophycocyanin (APC)-labeled anti-CD8 MAb (Dako, Glostrup, Denmark), the cells were fixed with 4% paraformaldehyde and then made permeable with permeabilizing buffer (0.1% saponin and 5% FCS in PBS). Thereafter the cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-IFN- γ MAb (BD Bioscience, CA). The percentage of IFN- γ ⁺ CD8⁺ cells was determined by flow cytometry.

HIV-1 mutant clones. NL4-3 mutants (NL4-3_{GagRI8-6S}, NL4-3_{GagRI8-6V}, and NL4-3_{GagRI8-6A}) were generated by introducing the respective Gag-T280S, -T280V, and -T280A mutations into NL4-3 by use of a site-directed mutagenesis system (Invitrogen).

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 using the SuperScript III first-strand synthesis system for reverse transcription-PCR (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 with 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.

Determination of HLA alleles associated with a low pVL and high CD4 count in response to each cocktail. We statistically analyzed differences in pVL or CD4 count between responders to each cocktail in individuals carrying a given HLA and the other individuals by using the two-tailed Mann-Whitney test. We then selected HLA alleles associated with a low pVL and high CD4 count in the responders to each cocktail according to the following 2 criteria: (i) the HLA alleles were associated with both a low pVL and a high CD4 count (*P* values for pVL and CD4 count were less than 0.1 and 0.05, respectively, or less than 0.05 and 0.1, respectively) or



associated with a low pVL ($P < 0.005$), and (ii) the frequency of responders was more than 2% (more than 9 out of 401 subjects).

Statistical analysis. For comparison of two groups in this study, two-tailed Mann-Whitney test was performed. Correlations between the breadths or the magnitudes and pVL or CD4 count were statistically analyzed using Pearson's correlation coefficient test and Spearman rank test, respectively. The frequency of the mutation between HLA⁺ and HLA⁻ individuals was statistically analyzed using Fisher's exact test. P values of <0.05 were considered to be statistically significant.

RESULTS

Control of HIV-1 by Gag- or Pol-specific CD8⁺ T cells. We recruited 401 chronically HIV-1-infected treatment-naive Japanese individuals from April 2008 to May 2011. We tested the CD8⁺ T cell responses to 10 Nef, 25 Gag, and 50 Pol peptide cocktails (one cocktail included 10 11-mer single overlapping HIV-1 clade B peptides [total of 842 peptides]) in these individuals and then analyzed the correlations between the CD8⁺ T cell responses to Nef, Gag, or Pol peptide cocktails and HIV-1 plasma viral load (pVL) or CD4 count. The breadths of the CTL responses to Gag and Pol peptides correlated positively with CD4 count ($r = 0.20$ and $P = 6.6 \times 10^{-5}$ and $r = 0.17$ and $P = 6.0 \times 10^{-4}$, respectively) and inversely with pVL ($r = -0.20$ and $P = 4.1 \times 10^{-5}$ and $r = -0.17$ and 7.8×10^{-4} , respectively), whereas those to Nef peptides very weakly correlated negatively with pVL ($r = -0.14$ and $P = 0.0045$) but not with CD4 count (Fig. 1A). In addition, total magnitudes of the CD8⁺ T cell responses to Gag and Pol peptides correlated positively with CD4 count ($r = 0.18$ and $P = 2.7 \times 10^{-4}$ and $r = 0.22$ and $P = 6.0 \times 10^{-6}$, respectively) and inversely with pVL ($r = -0.28$ and $P = 7.6 \times 10^{-9}$ and $r = -0.23$ and $P = 2.4 \times 10^{-6}$, respectively [Fig. 1B]). Those to Nef peptides weakly correlated negatively with pVL ($r = -0.13$ and $P = 0.012$) but not with CD4 count. These results together indicate that CTL responses to both Gag and Pol epitopes played an important role in controlling HIV-1 replication in these chronically HIV-1-infected Japanese individuals.

Identification of HIV-1-specific CTL responses associated with low pVL and high CD4 count. We sought to identify HIV-1-specific CTL responses associated with low pVL and high CD4 count in this cohort as follows. First, we determined HLA alleles significantly associated with a low pVL and high CD4 count in response to each peptide cocktail (see Materials and Methods). Second, we identified single 11-mer peptide-specific CD8⁺ T cell responses restricted by these HLA alleles in the responses. Finally, we determined optimal peptides recognized by the specific CD8⁺ T cells.

We found 14 HLA alleles significantly associated with a low pVL and high CD4 count in the responses to 23 peptide cocktails (see Table S1 in the supplemental material) and then sought to identify the responses to single 11-mer peptides restricted by these HLA alleles, except the HLA-B*40:02-restricted T cell responses in Pol cocktail 46, since we had previously identified PolGI8 and PolTL8 epitopes by using this cocktail (20). We identified T cell

responses to 53 11-mer single peptides from those to 22 peptide cocktails by using the ELISPOT assay or ICS assay (see Table S2). HLA restrictions of these 53 responses were determined by analyzing HLA restriction of the bulk CD8⁺ T cell response to each 11-mer peptide by using C1R or 721.221 cells expressing a given HLA allele. We found that CD8⁺ T cell responses to 23 11-mer peptides were restricted by 8 HLA alleles significantly associated with a low pVL and high CD4 count (see Table S3), but the responses to the other 11-mer peptides were restricted by other HLA alleles not significantly associated with a low pVL and high CD4 count (see Table S4). Finally, to identify optimal epitopes included in these 11-mer peptides, we analyzed the responses to truncated peptides by using the ICS assay. We identified 17 optimal epitopes restricted by the 8 HLA alleles, though 6 of these epitopes were included in 2 overlapping peptides (Table 1; see also Fig. S1 in the supplemental material). Thus, CD8⁺ T cell responses to these 17 epitopes and HLA-B*40:02-restricted PolGI8 and PolTL8 may control HIV-1 in the Japanese individuals.

Effective control of HIV-1 by HIV-1-specific CD8⁺ T cells specific for 13 epitope peptides. To clarify the role of the CD8⁺ T cell responses to the 19 identified epitopes in HIV-1 control, we investigated the responses to these epitope peptides in our cohort (393 individuals) by using the ELISPOT assay. The responders to 10 epitopes had a significantly lower pVL and higher CD4 count than nonresponders, whereas those to 4 other epitopes had a significantly lower pVL or higher CD4 counts than the nonresponders (bold type in Table 1). To select HIV-1-specific CD8⁺ T cells having a strong effect in control of HIV-1 from those 19 specific epitopes, we used the following criteria: (i) both the pVLs and CD4 counts of the responders had to be significantly lower and higher than those of nonresponders, respectively ($P < 0.05$), (ii) the pVLs of the responders had to be much lower than those of the nonresponders ($P < 0.01$), or (iii) CD4 counts of the responders needed to be much higher than those of the nonresponders ($P < 0.01$). The responders to 13 epitope peptides satisfied one of these criteria (Table 1).

To investigate the effect of CD8⁺ T cells specific for these 13 epitopes on the control of HIV-1, we analyzed correlations between the total magnitude or the breadth of CD8⁺ T cell responses to the epitopes and pVL or CD4 count in 235 HIV-1-infected individuals carrying at least one of the restricting HLA alleles. The breadth and the total magnitude of these responses correlated inversely with pVL (breadth, $r = -0.42$ and $P = 2.2 \times 10^{-11}$; total magnitude, $r = -0.37$ and $P = 7.2 \times 10^{-9}$) and positively with CD4 count (breadth, $r = 0.42$ and $P = 1.2 \times 10^{-11}$; total magnitude, $r = 0.42$ and $P = 3.2 \times 10^{-11}$) in these individuals (Fig. 2). They also showed strong inverse and positive correlation with pVL (breadth, $r = -0.34$ and $P = 5.7 \times 10^{-12}$; total magnitude, $r = -0.28$ and $P = 1.5 \times 10^{-8}$) and CD4 count (breadth, $r = 0.40$ and $P = 4.0 \times 10^{-16}$; total magnitude, $r = 0.35$ and $P = 8.9 \times 10^{-13}$), respectively, in all 393 individuals tested (see Fig. S2 in the supplemental material). These findings taken together indicated strong

and CD4 count (top and bottom, respectively). Statistical analysis was performed by use of Pearson's correlation coefficient test. Differences in pVL or CD4 count between nonresponders and responders were statistically analyzed by using the Mann-Whitney test. (B) Correlation between total magnitudes of the responses to the cocktails and pVL or CD4 count. The total magnitude was evaluated by calculating total spot numbers in the responses to Nef, Gag, or Pol peptide cocktails. Correlation coefficients (r) and P values were determined by using the Spearman rank correlation test. The line is the regression line. Multiple tests were performed by using the q value, a measure of significance in terms of the false-discovery rate (46). A significance threshold for q of <0.2 was employed. Each dot represents 1 individual. The limit of detection for pVL in this study is <40 .

TABLE 1 Association of CD8⁺ T cell responses to 19 epitopes with pVL and CD4 count in chronically HIV-1-infected Japanese individuals

Epitope	Sequence	Location	HLA restriction	Frequency		Median pVL (copies/ml)		Median CD4 (cells/ μ l)		P value ^e		q value ^f	
				Responders	Nonresponders	Responders	Nonresponders	Responders	Nonresponders	pVL	CD4	pVL	CD4
NefY11	TQGFDPWQNY	Nef117-127	B*15:01	3	390	3,400	25,000	386	321	0.22	0.38	0.25	0.4
GagEM11	EGATPQDLNTM	Gag177-187	B*67:01	7	386	2,200	25,000	440	321	0.011	0.025	0.024	0.044
			C*08:01	2	391	29,000	25,000	451	322	0.86	0.36	0.86	0.4
GagTL9	TPQDLNTML	Gag180-188	B*67:01	15	378	8,900	25,000	440	319	0.11	6.5 \times 10 ⁻³	0.14	0.015
GagMI8	MQMLKETI	Gag198-205	B*52:01	42	351	5,350	27,000	437	313	3.3 \times 10 ⁻⁴	4.9 \times 10 ⁻⁴	1.6 \times 10 ⁻³	2.3 \times 10 ⁻³
GagQA11	QMLKETTINBEA	Gag199-209	B*52:01	11	382	3,100	26,500	432	321	4.2 \times 10 ⁻⁵	0.044	2.7 \times 10 ⁻⁴	0.07
GagRI8	RMYSPTSII	Gag275-282	B*52:01	60	333	10,650	27,000	446	309	2.1 \times 10 ⁻³	3.2 \times 10 ⁻⁷	6.6 \times 10 ⁻³	6.1 \times 10 ⁻⁶
GagYL9	YVDRYKTL	Gag296-304	C*03:04	2	391	2,885	25,000	303	322	0.079	0.79	0.12	0.79
GagWV8	WMTEITLV	Gag316-323	B*52:01	43	350	4,700	28,000	446	309	1.0 \times 10 ⁻⁶	8.7 \times 10 ⁻⁷	1.9 \times 10 ⁻⁵	8.3 \times 10 ⁻⁶
GagNL11	NPDCITLTKAL	Gag327-337	B*67:01	9	384	2,200	25,500	440	321	2.4 \times 10 ⁻³	0.02	6.6 \times 10 ⁻³	0.038
GagGM9	GPAATLEEM	Gag338-346	B*67:01	1	392	780	25,000	693	322	0.14	0.16	0.16	0.15
GagAA9	ATLEEMMTA	Gag341-349	A*02:06	30	363	8,650	27,000	437	314	0.019	6.6 \times 10 ⁻⁴	0.036	2.5 \times 10 ⁻³
GagKL9	KELYPLASL	Gag481-489	B*40:02	12	381	20,000	25,000	480	321	0.68	0.017	0.72	0.035
PolSV9	SQIYAGHKV	Pol423-431	A*02:06	48	345	15,000	27,000	392	313	0.04	4.6 \times 10 ⁻³	0.064	0.015
PolS18	SOYALGII	Pol654-661	B*52:01	43	350	5,000	28,000	456	310	1.3 \times 10 ⁻⁵	2.5 \times 10 ⁻⁶	1.3 \times 10 ⁻⁴	1.6 \times 10 ⁻⁵
PolLA9	LEGKILVA	Pol783-791	B*40:06	16	377	4,150	27,000	437	321	5.6 \times 10 ⁻⁴	0.078	2.1 \times 10 ⁻³	0.11
PolIT10	IEAEVIPAET	Pol799-808	B*40:06	25	368	13,000	26,500	353	321	4.5 \times 10 ⁻³	0.19	0.011	0.22
PolGI8	GERVDII	Pol912-919	B*40:02	39	354	14,000	28,000	405	317	0.021	5.5 \times 10 ⁻³	0.036	0.015
PolTL8	TDIQTIKEL	Pol921-928	B*40:02	5	388	7,200	25,000	478	322	0.11	0.17	0.14	0.22

^a The statistical analyses of differences in pVL or CD4 count between responders to each epitope and nonresponders were conducted by using the two-tailed Mann-Whitney test. Bold indicates that differences were statistically significant.

^b Multiple tests were performed by using the q value, a measure of significance in terms of the false-discovery rate (46). A significance threshold for q of <0.2 was employed.

synergistic effects of CD8⁺ T cells specific for these 13 epitopes on HIV-1 control.

HLA-B*52:01 and HLA-B*67:01 were significantly associated with a low pVL and high CD4 count in chronically HIV-1-infected treatment-naive Japanese individuals (26). We therefore speculated that HLA-B*52:01-restricted and HLA-B*67:01-restricted CD8⁺ T cells would have stronger abilities to control HIV-1 than other CTLs. Indeed, the breadths of the HLA-B*52:01-restricted and the HLA-B*67:01-restricted CD8⁺ T cell responses were inversely correlated with pVL ($r = -0.44$ and $P = 5.2 \times 10^{-6}$ and $r = -0.76$ and $P = 7.1 \times 10^{-4}$, respectively [Fig. 3A]). In addition, the total magnitudes of the HLA-B*52:01-restricted and HLA-B*67:01-restricted CD8⁺ T cell responses were inversely correlated with pVL ($r = -0.34$ and $P = 5.3 \times 10^{-4}$ and $r = -0.81$ and $P = 1.3 \times 10^{-4}$, respectively [Fig. 3B]). On the other hand, the breadths of 2 HLA-A*02:06-restricted and 2 HLA-B*40:06-restricted CD8⁺ T cell responses showed a weak inverse correlation with pVL ($r = -0.27$ and $P = 0.018$ and $r = -0.43$ and $P = 0.013$, respectively [Fig. 3A]), whereas the total magnitude of HLA-A*02:06-restricted T cell responses showed a weak negative association with pVL ($r = -0.30$ and $P = 7.4 \times 10^{-3}$ [Fig. 3B]). These results together indicate that the HLA-B*52:01-restricted and HLA-B*67:01-restricted CD8⁺ T cells played a predominant role in the control of HIV-1 in these chronically HIV-1-infected Japanese individuals and support the previous finding that HLA-B*52:01 or HLA-B*67:01 are significantly associated with good clinical outcomes in Japanese individuals (26).

T cell recognition of 12 conserved or cross-reactive epitopes.

We analyzed the sequences of the 13 epitopes in this cohort. The data for reported clade B sequences in the Los Alamos database and our cohort are shown in Fig. 4. Nine of the 13 epitopes were conserved among approximately 85% or more of HIV-1 clade B-infected individuals, whereas 3 Pol epitopes (PolSV9, PolLA9, and PolGI8) and 1 Gag one (GagRI8) had some substitutions in approximately 20 to 60% of the individuals. We analyzed HLA-associated polymorphism in these epitopes to clarify the accumulation of CTL escape mutations as previously shown (27). Only 2 HLA-associated polymorphisms were found, at position 6 in GagRI8 and at position 9 in PolSV9 (Fig. 5A; see also Table S5 in the supplemental material). Therefore, we next analyzed cross-recognition of specific CTLs for these mutants. GagRI8-specific CTL clones failed to recognize GagRI8-6S, -6V, and -6A mutant peptides (Fig. 5B; see also Fig. S3 in the supplemental material), suggesting that these mutations had been selected by GagRI8-specific CTLs. Indeed, T cells specific for these 3 mutants were not detected in 3 HLA-B*52:01⁺ individuals (Fig. 5C). These mutations reduced viral fitness (Fig. 5D), suggesting that the emergence of these mutations may have resulted in a low pVL. On the other hand, PolSV9-5P and SV9-5P9I were cross-recognized in 3 of 4 HLA-A*02:06⁺ individuals (Fig. 5C), though these patients had 5P or 5S mutations (see Table S6). These results together suggest that these mutations were selected by PolSV9-specific CTLs and that CTLs cross-recognizing the 5P mutant were elicited after the emergence of the mutant.

Regarding PolLA9 and GI8, these CTL clones cross-recognized LA9-5V and GI8-5I mutant peptides, respectively (Fig. 5B; see also Fig. S3 in the supplemental material). In addition, PolLA9-5V and GI8-5I were cross-recognized in 4 HLA-B*40:06⁺ and 3 HLA B*40:02⁺ individuals, respectively (Fig. 5C). These results indicate that PolLA9 and PolGI8 were functionally cross-recognized

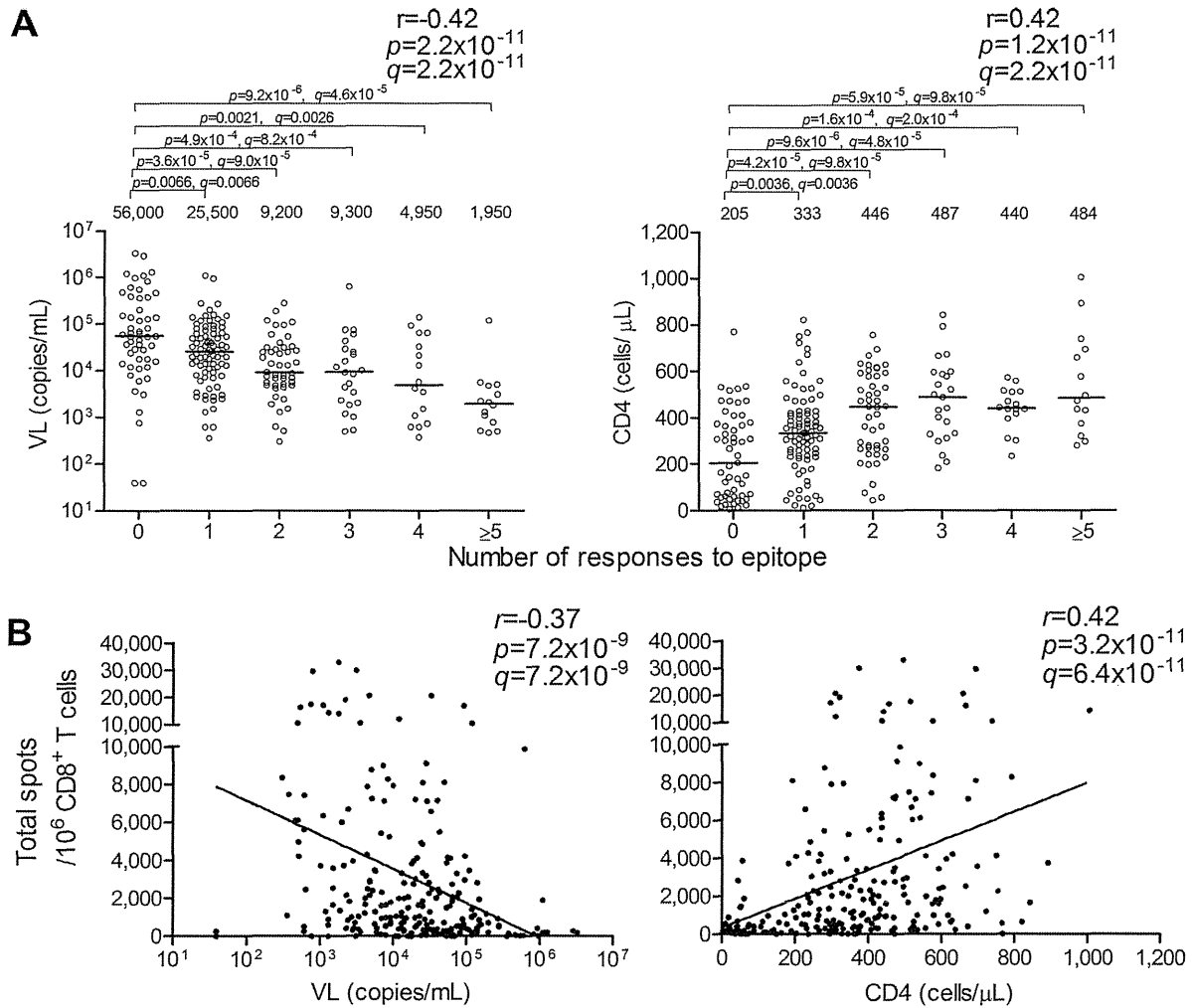


FIG 2 Correlation between multiple CD8⁺ T cell responses and pVL or CD4 count. Epitope-specific CD8⁺ T cell responses at a peptide concentration of 100 nM were analyzed by using the ELISPOT assay. (A) Correlation between the breadth of 13 epitope-specific CD8⁺ T cell responses and pVL or CD4 count in Japanese individuals carrying at least one of the restricting HLA alleles ($n = 235$). The values and the lines in each graph represent medians of pVL and CD4 counts (left and right graphs, respectively). Statistical analysis was performed by use of Pearson's correlation coefficient test. Differences in pVL or CD4 count between nonresponders and responders were statistically analyzed by using the Mann-Whitney test. (B) Correlation between the total magnitude of these responses and pVL or CD4 count in the Japanese individuals. The lines are regression lines. Correlation coefficients and P values were determined by using the Spearman rank correlation test. Multiple tests were performed by using the q value. A significance threshold for q of <0.2 was employed.

by specific CTLs. Thus, 12 epitopes were well recognized by specific CD8⁺ T cells, whereas escape mutants did not accumulate in 11 of 13 epitopes. Moreover, the breadth and the total magnitude of the T cell responses to these 12 epitopes correlated inversely with pVL (breadth, $r = -0.44$ and $P = 1.8 \times 10^{-12}$; total magnitude, $r = -0.38$ and $P = 2.3 \times 10^{-9}$) and positively with CD4 count (breadth, $r = 0.39$ and $P = 3.7 \times 10^{-10}$; total magnitude, $r = 0.41$ and $P = 5.8 \times 10^{-11}$) in the Japanese individuals (see Fig. S4A and B). These findings suggest that these T cells controlled HIV-1 in Japanese individuals, in whom protective alleles HLA-B*57 and B*27 are absent.

DISCUSSION

Analysis of clade C-infected Africans at a large population level demonstrated that the breadth of responses to Gag peptides is inversely associated with pVL, but not in the case of that to peptides in other protein regions (12), indicating that Gag-specific

CTLs predominantly control HIV-1 in African individuals. On the other hand, only small-scale analyses of the CTL responses in clade B-infected individuals have been performed, and they show controversial results (28–32). The present study demonstrated that the breadth and total magnitude of CTL responses to Gag peptides were inversely associated with pVL and positively associated with CD4 count in approximately 400 clade B-infected individuals. In addition, we showed that those of CTL responses to Pol peptides were significantly associated with a low pVL and high CD4 count. These findings together indicated that both Gag and Pol epitope-specific CTLs played a critical role in the control of HIV-1 in the Japanese population studied. Indeed, we identified T cell responses to 5 Pol and 8 Gag epitopes significantly associated with a low pVL and high CD4 count. Thus, Pol-specific CTLs also play a critical role in HIV-1 control in Japanese individuals. A recent phase I clinical trial vaccine study using a conserved immunogen showed that CD8⁺ T cells specific for Pol peptides had a

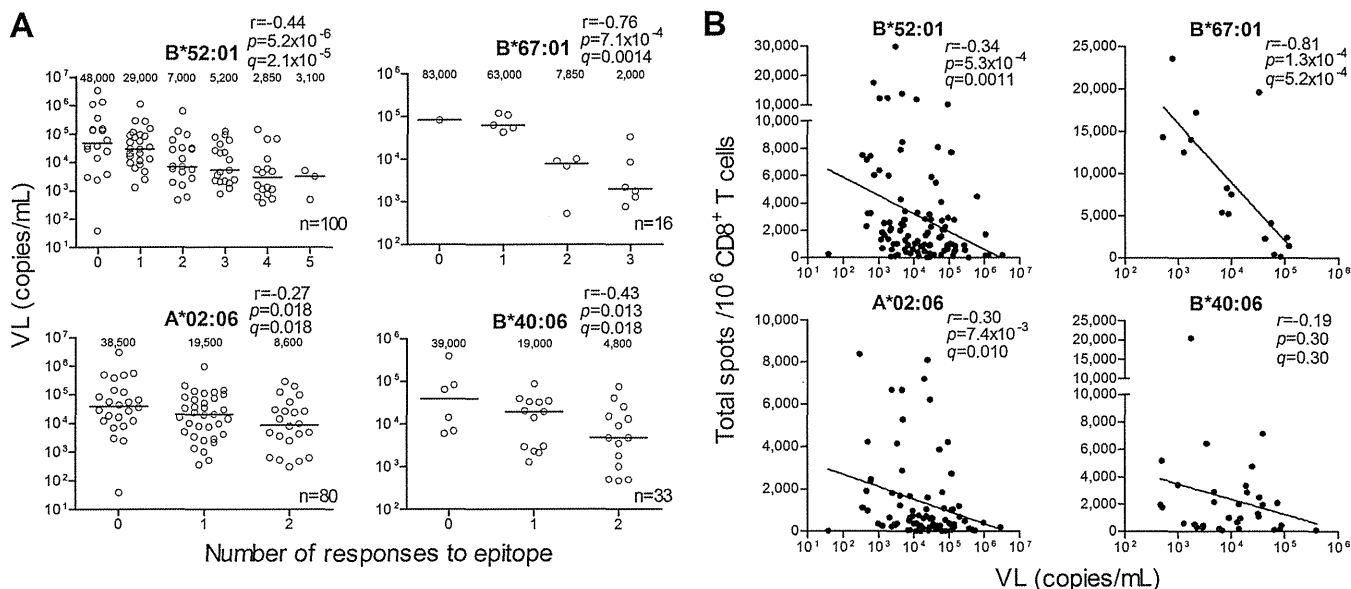


FIG 3 Correlation between CD8⁺ T cell responses restricted by each HLA and pVL or CD4 count. Epitope-specific CD8⁺ T cell responses at a peptide concentration of 100 nM were analyzed by using the ELISPOT assay. (A) Correlations between breadths of HLA-B*52:01-, HLA-B*67:01-, HLA-A*02:06-, or HLA-B*40:06-restricted CD8⁺ T cell responses and pVL in the individuals carrying each HLA. Five HLA-B*52:01-restricted (GagMI8/QA11/RI8/WV8/PolSI8), 3 HLA-B*67:01-restricted (GagEM11/TL9/NL11), 2 HLA-A*02:06-restricted (GagAA9/PolSV9), and 2 HLA-B*40:06-restricted (PolLA9/IT10) CD8⁺ T cell responses were analyzed. The values in each graph represent medians of pVL. (B) Correlations between total magnitudes of these HLA allele-restricted CD8⁺ T cell responses and pVL in individuals carrying the corresponding HLA. Correlations between the breadths or the magnitudes and pVL or CD4 count were statistically analyzed using Pearson's correlation coefficient test and the Spearman rank correlation test, respectively. Multiple tests were performed by using the *q* value. A significance threshold for *q* of <0.2 was employed.

stronger ability to suppress HIV-1 replication *in vitro* than those specific for Gag, Env, and Vif peptides in healthy volunteers immunized with the vaccine (33), suggesting that Pol epitope-specific CTLs can effectively suppress HIV-1 replication *in vivo*. Thus,

both studies suggest that Pol epitopes are also strong candidates as antigens for an AIDS vaccine.

Many previous studies investigated CD8⁺ T cell responses to overlapping HIV-1 peptides in order to clarify the correlation between the T cell responses and clinical outcome (12, 28–32, 34–36). However, they did not identify T cells effectively suppressing HIV-1 replication *in vivo*. A previous analysis of T cell responses to 18-mer overlapping peptides in the individuals infected with clade B or C demonstrated that the responses to the approximately 50 overlapping peptides were significantly associated with low pVL and the specific CTLs had strong antiviral activities *in vitro* (37). However, this study did not show minimum lengths of epitopes. The use of the 18-mer peptides may have a disadvantage in detection of specific CTL responses since such longer peptides hardly induce the specific CTLs. It is therefore essential to identify minimal epitopes to precisely clarify the ability of HIV-1-specific CTLs to control of HIV-1 *in vivo*. In the present study, we employed an exhaustive strategy involving the analysis of T cell responses to overlapping peptides significantly associated with low pVL and high CD4 count followed by identification of the responses to single peptides and HLA restrictions to them with subsequent determination of the optimal epitopes. Thereafter, we re-analyzed the responses to these optimal epitope peptides in our cohort and then reevaluated the correlation between these epitope-specific CTLs and the clinical outcome. By using this strategy, we could finally identify 8 Gag and 5 Pol epitope-specific CTLs controlling HIV-1. In addition, we analyzed the sequences for the epitopes in our cohort and then demonstrated that the CTLs specific for 12 conserved or cross-reactive epitopes controlled HIV-1. Thus, the present study using this exhaustive and comprehensive strategy was shown to be greatly advantageous for

HLA	Epitope	Sequence	Frequency (percentage)	
			Clade B ^a	Our cohort
B*52:01	Gag MI8	MQLKETT	1741/1931 (90.2)	328/352 (93.2)
	Gag QA11	QMLKETINEEA	1662/1931 (86.1)	301/340 (88.5)
	Gag RI8	RMYSPTSI	1222/1931 (63.3)	236/346 (68.2)
		----V--	382/1931 (19.8)	44/346 (12.7)
		----S--	96/1931 (5.0)	30/346 (8.7)
		----A--	41/1931 (2.1)	16/346 (4.6)
----I--		67/1931 (3.5)	5/346 (1.4)	
Gag WV8	WMTEITLLV	1845/1931 (95.6)	356/367 (97.0)	
Pol SI8	SOYALGII	914/999 (91.5)	306/330 (92.7)	
B*67:01	Gag EM11	EGATPQDLNTM	1779/1931 (92.1)	337/352 (95.7)
	Gag TL9	TPQDLNTML	1794/1931 (92.9)	337/351 (96.0)
	Gag NL11	NPDCRTILKAL	1694/1931 (87.7)	334/341 (97.9)
A*02:06	Gag AA9	ATLEEMMTA	1715/1931 (88.8)	317/354 (89.5)
	Pol SV9	SOYVAGIKV	438/999 (43.8)	64/310 (20.6)
		----P----	375/999 (37.5)	148/310 (47.7)
		----P--I	24/999 (2.4)	46/310 (14.8)
		----S----	37/999 (3.7)	29/310 (9.4)
B*40:06	Pol LA9	LEGKILLLVA	506/999 (50.7)	217/294 (73.8)
	----V----	356/999 (35.6)	45/294 (15.3)	
B*40:02	Pol IT10	IEAEVIPAET	844/999 (84.5)	261/306 (85.3)
	Pol GI8	GERIVDII	533/999 (53.4)	126/327 (38.5)
		----I----	377/999 (37.7)	143/327 (43.7)
		----M----	24/999 (2.4)	27/327 (8.3)

^a with reference to the Los Alamos database

FIG 4 Frequencies of amino acid sequences for the 13 epitope regions with reference to clade B. The sequences of the 13 epitopes in this cohort were analyzed. The data for reported clade B sequences in the Los Alamos database and our cohort are shown. Pink shading and boldface indicate nonconserved epitopes.

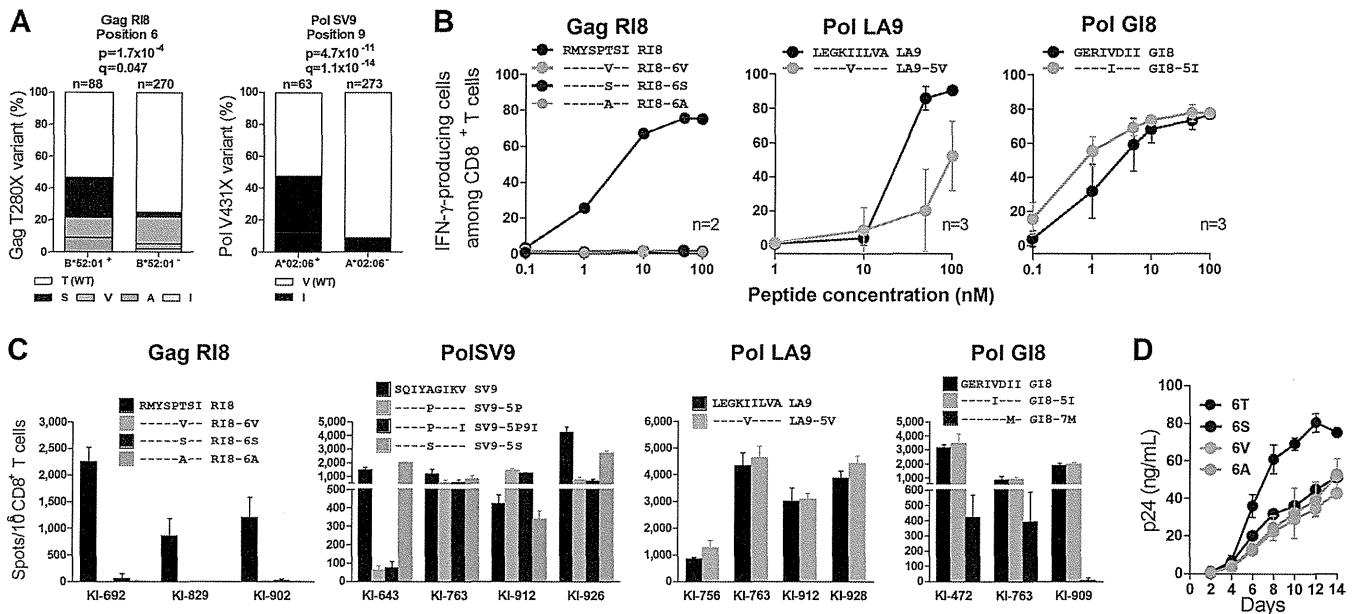


FIG 5 Recognition of conserved and cross-reactive epitopes. (A) Association of HLA-B*52:01 or HLA-A*02:06 with the GagT280X or the PolV431X mutation, respectively, in our cohort. The frequency of the mutation between HLA⁺ and HLA⁻ individuals was statistically analyzed by using Fisher's exact test. Multiple tests were performed by using the q value, a measure of significance in terms of the false-discovery rate (46). In the analyses identifying HLA-associated polymorphisms, a significance threshold for q of <0.2 was employed. (B) Recognition of mutant peptides or wild-type peptide by epitope-specific CD8⁺ T cells. The epitope-specific CTL clones were stimulated with mutant or wild-type peptide-prepulsed C1R cells expressing the corresponding HLA allele, and then IFN- γ production from these CTL clones was detected by performing the ICS assay. The results are shown as means and SDs ($n = 2$ or 3). (C) Recognition of the mutant peptides or the wild-type peptide by specific T cells in HIV-1-infected individuals carrying the corresponding HLA. Peptide-specific CD8⁺ T cell responses at a peptide concentration of 100 nM were analyzed by using the ELISPOT assay. The results are shown as means and SDs ($n = 3$). (D) Fitness of the 3 GagR18 mutant or wild-type viruses. CD4⁺ T cells from an HLA-B*52:01⁺ donor were infected with wild-type virus (NL4-3) or one of the 3 mutants (NL4-3_{GagRI8-6S}, NL4-3_{GagRI8-6V}, or NL4-3_{GagRI8-6A}). The concentration of p24 antigen in the culture supernatant was determined by using an enzyme immunoassay. The results are presented as means and SDs ($n = 3$).

identifying HIV-1 epitope-specific CTLs clinically controlling HIV-1 *in vivo*.

The breadths of the HLA-B*52:01-restricted or HLA-B*67:01-restricted CD8⁺ T cell responses showed significant inverse associations with pVL. In addition, the breadths of these HLA-restricted T cell responses showed stronger effects on pVL than those of HLA-A*02:06-restricted or HLA-B*40:06-restricted T cell responses. These findings indicate that the responses of HLA-B*52:01-restricted or HLA-B*67:01-restricted CD8⁺ T cells controlling HIV-1 are critical factors for HIV-1 control *in vivo*.

A recent study investigated CD8⁺ T cell responses to 286 defined epitopes in 620 mainly Caucasian individuals with primary HIV-1 infection and showed that the specificity of the initial HIV-specific CD8 T cell response is a critical determinant of antiviral function rather than the restricting HLA class I molecule alone (38). In contrast, we here demonstrated that the breadths and the total magnitude of the T cell responses restricted by HLA-B*52:01 or HLA-B*67:01 were negatively correlated with pVL, suggesting that the multiple CTLs restricted by 2 protective alleles synergistically controlled HIV-1 in the Japanese individuals. Another recent study of 341 HIV-1-infected individuals in North America demonstrated that CD8⁺ T cell responses to 8 reported epitopes, including 3 well-established HLA-B*57-restricted ones, were associated with HIV-1 control (39). Since this study analyzed the T cell responses to only reported epitopes, it may identify only a part of the T cells controlling HIV-1.

T cells specific for 9 conserved and 3 cross-reactive epitopes controlled HIV-1 in the Japanese individuals examined in the

present study. Previous studies demonstrated that conserved regions of HIV-1 are strong candidates for vaccine antigens (33, 40–45). Studies on HIV conserved vaccine demonstrated that polyfunctional and broad CTL responses were detected in macaques and humans that had received the vaccine (33, 40, 43–45). Since 6 out of the 12 conserved epitopes in this study (GagEM11/TL9/MI8/QA11/PolLA9/IT10) were included in the HIV conserved vaccine, vaccines may contribute to HIV-1 control in individuals carrying the corresponding HLAs. The addition of other epitopes identified in this study may improve the effect of the vaccine in clinical trials. Further analysis of these epitopes in vaccinated individuals will clarify the role of T cells specific for these epitopes in HIV-1 vaccine.

In the present study, we identified 8 Gag and 5 Pol epitope-specific CTLs controlling HIV-1 in HIV-1-infected Japanese individuals, in whom HLA-B*57 and HLA-B*27 are very rare. Twelve out of these 13 epitopes were recognized by CD8⁺ T cells as conserved or cross-reactive epitopes, suggesting that AIDS vaccines inducing CTLs specific for these 12 epitopes would be effective for protection against HIV-1. The comprehensive and exhaustive analysis of the CTLs shown here has been demonstrated to be a very useful strategy for identification of CTLs controlling HIV-1, and such analysis provides new insights into the studies of AIDS pathogenesis and the development of effective AIDS vaccines. This is the first study that identified HIV-1-specific T cells clinically controlling HIV-1 in the population, in whom HLA-B*57 and HLA-B*27 are very rare.

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RESEARCH ARTICLE

High Proportion of HIV Serodiscordance among HIV-Affected Married Couples in Northern Vietnam

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Abstract

Introduction

Little is known about the state of HIV transmission among married couples in Vietnam. This study aims to clarify HIV serostatus in this group and elucidate risk factors for intra-marital HIV transmission.

Methods

In 2012, we enrolled a group of HIV-positive married men registered at the HIV outpatient clinic of a referral hospital in northern Vietnam, along with their wives. Sociodemographic, behavioural and clinical data were collected from men and wives. HIV serodiscordant couples were followed until March 2014 to determine seroconversion rate. A phylogenetic analysis was performed based on *env* V3 sequence to detail cluster formation among men.

Results

Of the 163 HIV-positive men enrolled in the study, 101 (62.0%) had wives testing HIV-negative. Half of men reported injecting drug use (IDU) as a likely transmission route. Couples reported a high incidence of unprotected sexual intercourse prior to diagnosis; the median (interquartile range) was 4 (4–8) times per month. Only 17 couples (10.4%) reported using condoms during at least half these instances. Multivariable analysis revealed IDU history among men was independently associated with HIV-negative wives (adjusted OR 0.31; 95% CI 0.10–0.95, $p=0.041$). Phylogenetic analysis of 80 samples indicated CRF01_AE. Of these, 69 (86.3%) clustered with IDU-associated viruses from Vietnam. No HIV seroconversion was identified during a follow-up of 61 serodiscordant couples, with 126.5 person-years of observation during which HIV-infected men were on antiretroviral drug therapy (ART).

Conclusion

High HIV serodiscordance was observed among HIV-affected married couples in northern Vietnam. A large number of at-risk wives therefore remain HIV-negative and can be protected with measures including proper use of ART if couples are made aware of the serodiscordance through screening.

Introduction

In Vietnam, the HIV epidemic first surfaced among injecting drug users (IDUs) and female sex workers (FSWs) in the 1990s, after the virus was introduced to southern Vietnam through heterosexual contact [1,2]. In 2012, the epidemic reached an estimated prevalence among the adult population of 0.47%, up from 0.20% in 2000 [3]. Since IDUs in Vietnam are almost exclusively male, the epidemic initially exhibited male-indexed characteristics; more than 80% of reported HIV cases in the 1990s were among men [1,4]. HIV spread rapidly in the IDU population, particularly in northern Vietnam, where estimated HIV prevalence among IDUs in 2009 was in the range of 21% to 56% [5]. A series of HIV CRF01_AE epidemics consisting of three clusters have been reported: a heterosexual-transmission associated cluster in southern Vietnam, an IDU associated cluster in southern Vietnam, and an IDU associated cluster in northern Vietnam, spreading in that order [2].

Of note, the spread of HIV to the general population was much slower than in Thailand and Cambodia in the 1990s [6]. In Thailand, a large bridging population of male sex-worker clients contributed to a rapid spread to the general population. As evidence, HIV prevalence among pregnant women in Thailand quadrupled within the first five years of the epidemic [6,7].

Conversely, the spread of HIV to women in the general population of Vietnam was surprisingly slow. In 2012, a decade after the start of the epidemic, the male-to-female infection ratio remained as high as 2.5, only slightly lower than the ratio of 3.2 in 2000 [3,6]. Most HIV-positive women in Vietnam are neither FSWs nor IDUs; instead they contracted HIV from a male sex partner, himself either an IDU or FSW client [8,9]. This intimate partner transmission has been attributed to a lack of awareness of infection risks, low condom adherence among fixed partners, and male dominance in sexual decision making [9–11]. As such, the threat of HIV spread into the general population in Vietnam remains.

To further control the HIV epidemic in Vietnam, intimate partner transmission from HIV-positive men to wives must be better understood. However, there have been no studies comprehensively investigating HIV-affected couples in Vietnam. Most research on HIV prevention has focused on high-risk groups such as IDUs, FSWs, or men who have sex with men (MSM). The objective of this study is to reveal the sociodemographic and clinical characteristics of HIV-affected married couples in which the husband is HIV-positive, to elucidate factors determining HIV status among wives and to determine incidence of HIV transmission after HIV diagnosis.

Materials and Methods

Study population

A hospital-based cohort study was conducted at the Department of Infectious Diseases of Bach Mai Hospital (BMH) in Hanoi, the largest referral hospital in northern Vietnam. The study team evaluated patient records to facilitate approaches to HIV-positive married men registered at the clinic between 26 September 2009 and 31 December 2011, along with these patients'

wives. This includes legally married men and men considered married in the Vietnamese social context. The following cases were excluded from the analysis: patients who did not agree to bring their wives for HIV screening; patients who rejected participation; patients who had been married after their HIV diagnosis; and patients whose clinical information at the time of diagnosis was unavailable.

Data collection

From 1 January to 31 December 2012, all eligible couples were independently invited to answer a standardized questionnaire written in Vietnamese. The questionnaire included questions on age, occupation, educational attainment, marital status, number of children, desire to conceive, sexual behaviours before and after HIV diagnosis, possible risks of HIV transmission, history of contact with FSWs, and history of genital ulcers. Those reporting an IDU history received further questions regarding use of injection equipment (sharing) and the age at which they both initiated and (if applicable) terminated drug use. A trained nurse was assigned to assist participants having trouble with the questionnaire. Clinical information collected from medical records at the initial visit included date of HIV diagnosis, baseline CD4+ T cell count (cells/ μ l), baseline viral load (\log_{10} copies/ml), anti-hepatitis C virus antibody (HCV Ab), hepatitis B surface antigen (HBsAg), date of antiretroviral treatment (ART) initiation, and clinical stage at the time of HIV diagnosis. Evaluation of clinical stage was based on Vietnam's national guidelines for HIV/AIDS diagnosis and treatment [12]. Later, between 1 January and 31 March 2014, all eligible HIV serodiscordant couples in the study were invited to be tested for HIV serology and asked to complete a follow-up questionnaire regarding their sexual behaviour in 2013.

HIV screening

Wives previously testing HIV-negative as well as wives not yet tested for HIV serology were tested to determine initial HIV serostatus at the time of enrolment. The tests were performed at the Microbiology Department at BMH. An HIV-positive diagnosis was made when serum tested positive in the screening test (Alere Determine HIV-1/2, Alere Medical Japan, Japan) and two different anti-HIV antibody enzyme-linked immunosorbent assays following the official diagnostic protocol of the Ministry of Health of Vietnam [12]. Either of the two following kits were used: Genscree ULTRA HIV Ag-Ab, Bio-Rad Laboratories, France; Murex HIV Ag/Ab Combination, DiaSorin Dartford, UK; Serodia HIV-1/2, Fujirebio Inc., Japan; and Roche Elecsys HIV Combi, Roch Diagnostics, Germany.

Sequence and phylogenetic analyses

Approximately 6 millilitres of blood was collected from HIV-positive men using BD Vacutainer CPT Tube (BD, USA) at the HIV clinic of BMH. Buffy coats were separated using centrifugation, stored at -80°C at the Microbiology Department and then transported to the Department of Immunology and Molecular Biology at the National Institute of Hygiene and Epidemiology in Hanoi for follow-up analysis.

To conduct the phylogenetic analysis of HIV-positive men, a 763 bp region of gp120 (HXB2:6891–7654) was sequenced as follows. DNA was extracted from buffy-coats using a QIAamp DNA Mini Kit (Qiagen, Japan). The V3 region was PCR-amplified in accordance with a previously published primer and protocol [13]. The PCR products were further sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI Genetic Analyzer 3130 (Applied Biosystems, USA). The obtained sequence data were then analysed at the Infectious Diseases Surveillance Center of the National Institute of Infectious Diseases in Tokyo, Japan. Nucleotide sequences were aligned with 33 CRF01_AE

sequences from Vietnam (GenBank accession numbers: FJ185228–FJ185260) analyzed in the previously cited report [2], 102 CRF01_AE sequences and 39 subtype reference sequences retrieved from the Los Alamos HIV Sequence Database (<http://www.hiv.lanl.gov/>) by MUSCLE [14]. Phylogenetic trees were inferred using the maximum likelihood (ML) method with an interior branch test by MEGA 6 [15]. For the ML tree inference, the study applied a general time reversible model with Gamma distributed and invariant sites to a base substitution model, based on model selection under the hierarchical likelihood test in MEGA 6.

HIV-1 co-receptor tropism was further predicted using the Geno2Pheno[co-receptor] (G2P) (<http://coreceptor.bioinf.mpi-inf.mpg.de>) based on the V3 sequence of HIV-1 *env*. To classify the tropism, 5.75% false-positive rates (FPR) were used as cut-offs, referencing European guidelines for tropism testing. Viruses with FPR below the cut-off were classified as cysteine-X-cysteine-chemokine receptor 4 usage (CXCR4, X4) or dual/mixed (DM) and viruses with FPR above the cut-off were classified as cysteine-cysteine-chemokine receptor 5 (CCR5, R5) usage [16].

Data management and statistical analysis

Differences in men's baseline sociodemographic characteristics, risk behaviours and clinical characteristics as regards their wives' HIV status were evaluated using Pearson's chi-squared test for categorical variables and the Wilcoxon rank-sum test for continuous variables. Reported sexual behaviours of husbands were compared to corresponding accounts given by their wives using Spearman's correlation coefficient. To examine factors relating to wives' HIV-positive status, univariate and multivariable logistic regression analyses were performed. Factors with potential correlation in univariate analysis ($p < 0.10$) and clinically vital factors were included in the multivariable logistic regression model. The data was managed by EpiData 3.1 (EpiData Association, Denmark) and analysed by Stata version 12.0 (Stata Corporation, College Station, USA).

Ethics

Standard HIV education and counselling was provided to all study participants. The study was approved by the BMH Ethical Committee and the Ethical Committee of the Institute of Tropical Medicine, Nagasaki University. Among eligible participants, only those who provided informed written consent were enrolled.

Results

Study participants

Of the 380 married men registered at the HIV clinic, 33 had died by the time of study enrolment, 45 had been referred to other hospitals, and 14 had gotten divorced. The remaining 288 men were invited to participate in the study. After providing informed consent and undergoing screening based on enrolment criteria, 163 men were enrolled in the study.

Characteristics of HIV-1 positive married men

The study identified 101 men with HIV-negative wives (62%). Characteristics of HIV-positive married men are summarized in Table 1 and laid out according to HIV status of wives. Age at initial diagnosis did not significantly differ between men with HIV-positive wives and men with HIV-negative wives. Most couples from both groups had a substantial duration of marriage prior to HIV diagnosis, with a median duration of seven years. The duration of marriage prior to HIV diagnosis was longer among men with HIV-negative wives ($p = 0.050$). A total of

Table 1. Characteristics of HIV-positive married men.

		All		Men with HIV-positive wives		Men with HIV-negative wives		p-value	
		N = 163		N = 62		N = 101			
Age at HIV diagnosis (year)		34	[30–39]	33.5	[29–36]	34	[31–39]	0.075	
Marital duration before HIV diagnosis (years)		7.1	[3.7–13.1]	5.8	[2.1–10.4]	7.7	[4.5–13.7]	0.050	
Possible risk of HIV transmission	Heterosexual contact	80	(49.1)	38	(61.3)	42	(41.6)		
	IDU	38	(23.3)	8	(12.9)	30	(29.7)		
	Both	39	(23.9)	15	(24.2)	24	(23.8)		
	No answer	6	(3.7)	1	(1.6)	5	(5.0)	0.031	
Frequency of sex (times per month)	Before HIV diagnosis	4	[4–8]	4	[4–8]	4.5	[4–8]	0.645	
	After HIV diagnosis	2	[1–4]	3	[1–4]	2	[1–4]	0.684	
Frequency of unprotected sex (times per month)	Before HIV diagnosis	4	[3–8]	4	[3–8]	4	[3–8]	0.320	
	After HIV diagnosis	0	[0–0]	0	[0–1]	0	[0–0]	< 0.001	
History of genital ulcer	Men	28	(17.2)	14	(22.6)	14	(14.9)	0.152	
	Their wives	48	(29.5)	27	(43.6)	21	(20.8)	<0.001	
Clinical status at baseline	CD4+ T cell count (μl)	62	[29–155]	90.5	[28–239]	55	[30–138]	0.240	
	Viral load (log ₁₀ cp/ml)	5.18	[4.73–5.52]	5.00	[4.83–5.38]	5.23	[4.70–5.55]	0.481	
	Clinical symptoms	Asymptomatic	25	(15.3)	17	(27.4)	8	(7.9)	
		Symptomatic	138	(84.7)	45	(72.6)	93	(92.1)	0.001
	HCV Ab	92	(57.5)	29	(47.5)	63	(63.6)	0.045	
	HBsAg	31	(19.1)	16	(25.8)	15	(15.0)	0.089	
ART status at enrolment	ART naïve	6	(3.7)	4	(6.5)	2	(2.0)	0.149	
	Duration of ART (years)	0.9	[0–2.3]	0.9	[0–2.3]	0.8	[0–2.1]	0.990	

Values are given as actual count (%) for categorical variables or median [inter-quartile] for continuous variables.

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76% of men reported high-risk heterosexual behaviour such as sexual contact with FSWs as a possible transmission route. Half of men reported risky heterosexual contact as the only logical route, while one quarter reported both risky heterosexual contact and an IDU history. Another quarter of men reported IDU history as the only logical route. None of the men reported MSM as a logical route of transmission.

Intriguingly, an IDU history was significantly more common among men with HIV-negative wives than among men with HIV-positive wives (Table 1). Specifically, 70.1% of men with an IDU history had HIV-negative wives versus 52.5% of men without an IDU history ($p = 0.042$). Among men with an IDU history, behaviours such as sharing equipment, age at drug use initiation, duration of drug use, and current drug use practices did not appear to correlate with wives' HIV status. Other factors such as men's education and employment history also did not significantly correlate with wives' HIV status (data not shown).

Unprotected sexual intercourse before HIV diagnosis was equally frequent among men with HIV positive wives and men with HIV negative wives. Almost 80% of men reported no condom use at all during sexual intercourse with their wives prior to HIV diagnosis. After HIV status was diagnosed and disclosed to wives, the proportion of men reporting condom use increased dramatically to 94.5%, though as can be expected the proportion was higher among couples learning of HIV-negative wives than among couples learning of HIV positive

wives through screening (98.9% versus 87.7%, $p < 0.001$). Crucially, there was no reported difference in the median [inter-quartile (IQR)] of the frequency of unprotected intercourse per month between men with an IDU history and men without, both before and after HIV diagnosis (4 [3–8] versus 4 [3–8], $p = 0.537$ and 0 [0–0] versus 0 [0–0], $p = 0.559$) as well as for the frequency of protected intercourse (4 [4–8] versus 4 [3–8], $p = 0.664$ and 3 [1–4] versus 2 [1–4], $p = 0.297$). Likewise, men with AIDS at the screening and men with asymptomatic HIV infection reported a similar frequency of unprotected intercourse per month before the diagnosis (4 [3–8] and 4 [4–6], $p = 0.953$). The group reporting the most frequent instance of unprotected intercourse before diagnosis was men with AIDS at screening and with HIV positive wives, 8 [3–10], although the difference was not significant ($p = 0.953$). Intercourse frequency reported by men in both groups was in line with corresponding accounts given by their wives. Reported condom use was also in line. For all couples, the Spearman correlation coefficient was 0.789 ($p < 0.001$) and 0.875 ($p < 0.001$) for frequency of reported unprotected sexual intercourse per month before and after HIV disclosure. The study also analysed sexual behaviour between couples with and without a desire to conceive. Men with the desire to conceive reported more frequent unprotected sexual intercourse than men without such a desire (6 [4–8] versus 4 [2–6], $p = 0.003$). Men with HIV-negative wives reported less desire to conceive than did men with HIV-positive wives (38.4% versus 56.5%; $p = 0.025$) and also had more existing children ($p = 0.04$).

A history of genital ulcers was more common among men with HIV-positive wives than among men with HIV-negative wives, though the difference was not significant. HCV infection is strongly associated with having an IDU history; HCV prevalence among IDU men was 93.4%, compared with 25% among non-IDU men ($p < 0.001$). When HCV prevalence is used as a biomarker for the possible risk of parenteral transmission [17]; wives' HIV status set against HCV prevalence among husbands and wives' HIV status set against IDU history among husbands yielded similar proportions. Notably, the prevalence of HBsAg tended to be higher among men with HIV-positive wives than men without. HIV viral load data before ART initiation was available for 82 men (50.3%) and no difference was noted between men with HIV-negative wives and men with HIV-positive wives. Half the men in the study had progressed to AIDS at the first visit. Men with HIV-negative wives were significantly more likely to be symptomatic ($p = 0.003$). Lastly, there was no noteworthy difference in ART coverage and duration at time of enrolment between men in both groups (data not shown).

Characteristics of wives

Of the wives of the 163 men enrolled in the study, 136 wives (58 HIV positive and 78 HIV negative wives) completed the questionnaire, while the remaining two HIV positive wives and 25 HIV negative wives did not. The majority of HIV positive wives (77.0%) were diagnosed within a month of their husbands' diagnosis (median duration from husband to wife diagnosis of 4 days [IQR 0–29]). Median age at study enrolment was 31.3 years [IQR 28.3–36.8] with no significant difference in age between HIV-positive and HIV-negative wives ($p = 0.18$). Median age at HIV diagnosis was 28.4 years [IQR 25.6–36.2]. No HIV-negative wives reported an IDU or FSW history. Additionally, no HIV positive wives reported an IDU history, though one HIV-positive wife did report an FSW history. Twice as many HIV positive wives as HIV negative wives experienced genital ulcers, though this was not the case for men, where there was no significant difference between the two groups. Among HIV positive wives, there were three HCV Ab positive wives, three HBsAg positive wives, and one wife positive for HCV Ab and HBsAg. Most HIV positive wives had visited the hospital in asymptomatic stage (39, 69.6%). Median CD4+ T cell count was 307 cells/ μ l [IQR 212–419].

Table 2. Risk factor analysis for men having HIV-positive wives.

		Crude OR (95%CI, P-value)	Adjusted OR (95%CI, P-value)
Age at HIV diagnosis (every 10 years)		0.76 (0.50–1.15, 0.190)	0.64 (0.36–1.11, 0.110)
Duration of ART before enrolment (year)		1.09 (0.69–1.71, 0.726)	1.44 (0.82–2.52, 0.200)
Duration from marriage to HIV diagnosis (year)		0.97 (0.92–1.01, 0.103)	
Monthly frequency of unprotected sex before HIV diagnosis of men		1.05 (0.97–1.15, 0.231)	1.11 (0.99–1.24, 0.083)
History of genital ulcers	Husband	1.82 (0.80–4.12, 0.155)	
	Wife	2.14 (1.05–4.38, 0.037)	2.17 (0.91–5.22, 0.082)
Risks of HIV transmission	Heterosexual contact	1.0	1.0
	IDU	0.29 (0.12–0.72, 0.007)	0.31 (0.10–0.95, 0.041)
	Both	0.69 (0.32–1.50, 0.353)	0.69 (0.25–1.85, 0.454)
CD4+ T cell count at baseline (every 100/μl)		1.14 (0.92–1.42, 0.224)	
Clinical symptoms at baseline	Asymptomatic	1.0	1.0
	Symptomatic	0.23 (0.09–0.57, 0.001)	0.20 (0.06–0.75, 0.016)
Positive HBsAg		1.97 (0.89–4.35, 0.092)	3.12 (1.08–9.00, 0.035)
Positive HCV Ab		0.52 (0.27–0.99, 0.047)	

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Factors associated with having HIV-positive wives

Logistic regression analyses were performed to examine factors associated with men having HIV-positive wives (Table 2). The unadjusted analysis revealed the frequency of unprotected intercourse before HIV diagnosis of men did not significantly associate with having HIV-positive wives. As mentioned, men with an IDU history and men in the advanced stages of HIV infection were less likely to have HIV-positive wives. By contraries, a history of genital ulcers in wives positively associated with men having HIV-positive wives. No association was found with ART duration and CD4+ T cell count.

In the multiple analysis, the model was adjusted for age at HIV diagnosis, duration of ART before study enrolment, couple’s frequency of unprotected intercourse before the HIV diagnosis of the man, history of genital ulcers in wives, risk of HIV transmission, existence of clinical symptoms, and serostatus of HBsAg. HIV viral load was not added to the model due to the limited nature of available data. Negative associations with IDU history (adjusted OR 0.31, $p = 0.041$) and advanced stage of infection (adjusted OR 0.20, $p = 0.035$) as regards having HIV-positive wives remained significant, even after adjustments. The frequency of unprotected intercourse before the diagnosis of men remained insignificant and a history of genital ulcers in wives turned out to be insignificant in the multiple analysis. Interestingly, the association between the HBsAg positivity and having HIV-positive wives became significant after adjusting for the multiple model. Due to the strong link between HCV Ab and IDU history, the model includes only IDU history. However it is worth mentioning similar results were obtained when using HCV Ab as an indicator for IDU history; each can often be considered a proxy for the other. When the analysis was conducted with clinical symptoms categorized into the three groups (asymptomatic, symptomatic non-AIDS, AIDS), the model displayed similar results (data not shown).

Sequence analysis

Of the 163 buffy-coat samples, the V3 sequence from 80 samples was successfully amplified and identified as CRF01_AE (accession number: KP401976–HP401985, KP401987–KP402056). Further phylogenetic analysis (S1 Fig) revealed an evolutionary relationship