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Multilayered Defense in HLA-B51–Associated HIV Viral Control

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Polymorphism in the HLA region of a chromosome is the major source of host genetic variability in HIV-1 outcome, but there is limited understanding of the mechanisms underlying the beneficial effect of protective class I alleles such as HLA-B57, -B27, and -B51. Taking advantage of a unique cohort infected with clade B' HIV-1 through contaminated blood, in which many variables such as the length of infection, the infecting viral strain, and host genetic background are controlled, we performed a comprehensive study to understand HLA-B51–associated HIV-1 control. We focused on the T cell responses against three dominant HLA-B51–restricted epitopes: Gag327-345(NI9) NANPDCKTI, Pol743-751(LI9) LPPVVAKEI, and Pol283-289(TI8) TAFTIPSI. Mutations in all three dominant epitopes were significantly associated with HLA-B51 in the cohort. A clear hierarchy in selection of epitope mutations was observed through epitope sequencing. L743I in position 1 of epitope LI9 was seen in most B51$^+$ individuals, followed by V289X in position 8 of the TI8, and then, A328S, in position 2 of the NI9 epitope, was also seen in some B51$^+$ individuals. Good control of viral load and higher CD4$^+$ counts were significantly associated with at least one detectable T cell response to unmutated epitopes, whereas lower CD4$^+$ counts and higher viral loads were observed in patients who had developed escape mutations in all three epitopes or who lacked T cell responses specific to these epitope(s). We propose that patients with HLA-B51 benefit from having multiple layers of effective defense against the development of immune escape mutations. ***The Journal of Immunology*****, 2011, 187: 684–691.**

Polymorphism in the HLA class I region on chromosome 6 has been consistently shown to play the major role in host genetic influences on HIV-1 disease outcome, confirmed in several recent genome-wide association studies (1, 2). Strikingly, the recently reported genome-wide association studies of HIV-1–infected subjects with viral control demonstrated a major influence of HLA class I on good clinical outcome, with viral control mapping to three polymorphic positions in the peptide-binding groove of the HLA class I molecule (2). However, the mechanisms underlying this relationship are not entirely clear. Although HLA class I molecules present viral epitopes to Ag-specific T cells, and therefore, certain class I alleles could be important because of the nature of the viral peptides they select, only a few distinct T cell epitopes associated with good clinical outcome have so far been defined for HIV-1. However, multiple T cell responses toward HIV-1 gag are linked with lower viral loads (3). HLA class I molecules are also ligands for receptors on NK cells: the association of certain killer Ig receptor genotypes in combination with their HLA ligands with good clinical outcome (4, 5) could suggest a more important role for NK cell responses in HIV-1 infection. This question is important to resolve to gain a better understanding of protective immunity against HIV-1 infection, which is essential for rational HIV vaccine design.

HIV-specific T cell responses constitute a great pressure on HIV-1 to mutate, enabling T cell epitopes to escape from recognition by virus-specific immune cells (6, 7). Thus, beneficial class I molecules could confer their protective effect by presentation of conserved regions of the virus, particularly in Gag, in which escape mutation is constrained by adverse consequences for the virus in terms of replicative fitness. However, the contribution of individual T cell epitope responses to disease control and the role of viral escape in disease progression still remain controversial (8): in the individual patient, the outcome probably depends on the interaction between that individual's HLA molecules and the infecting viral strain (7, 9). This may be modified by multiple factors, such as the complexities of multiple T cell responses within the individual, the diversity of genetic background, the

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Abbreviations used in this article: SFU, spot-forming unit; WT, wild-type.

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fitness cost to the mutated virus, the kinetics of T cell responses, the selection of escape mutants during the acute phase of infection, and the efficacy of epitope processing and presentation (10, 11).

Several HLA class I B molecules have been repeatedly shown to be associated with delayed disease progression, notably HLA-B27 and B57 in whites (12) and HLA-B51 in Asian populations (13). For HLA-B27, there is one immunodominant epitope (Gag KK10), and escape mutation in this epitope is associated with clinical decline, suggesting that T cell responses through HLA-B27 are important in protection (9). Escape mutation and loss of immune control appears to be delayed because the critical escape mutation in the epitope requires two other compensation mutations to occur, one being outside the epitope (14). Similarly, it has been argued that the reason why HLA-B57 is associated with good T cell control of HIV is because the escape mutations that occur in the three to four immunodominant epitopes all impose a fitness cost on the virus (13, 15).

HLA-B51 is also known to be associated with low viral loads (16), but the mechanism that underlies this protection is still unclear. Indeed, a recent study of a cohort in Japan showed that this association is no longer present in Japan, because the circulating HIV strain has acquired a major escape mutation in one of the most dominant HLA-B51–restricted epitopes (Pol 283-289) in the Japanese population (7). In contrast, this association was observed in a cohort of hemophiliacs who had been infected in early 1980s with a nonmutated virus (7). These observations also could be interpreted as implicating HLA-B51–restricted T cells in control of viral replication. HLA-B51 is a common HLA allele in China and much of Asia, and understanding the mechanism of HLA-B51 association with viral control therefore holds particular importance in the study of the pathogenesis of HIV infection in the Far East.

In most cohort studies, the analysis is complicated by the presence of individuals with different infecting viruses of different pathogenic potential for different lengths of time. In this study, we were able to access a unique cohort in China where individuals had been infected with HIV through contaminated plasma in the early 1990s. All members of this cohort were infected at a similar time and by the same infection route and have subsequently progressed to diverse disease outcomes in the absence of any antiretroviral treatment during the first 10 years of infection. Our studies have demonstrated a narrow source clade B' HIV-1 infection in this cohort, and there are strong associations between HLA type and mutations found in the virus, without confounding by founder effect (17).

We selected 22 HLA-B51$^+$ individuals from the cohort, and the immunodominance hierarchy of B51-restricted CTL responses was mapped using 17 known HLA-B51–restricted epitopes from the Los Alamos Molecular Immunology database. Three epitopes —Gag 327-345(NI9) NANPDCKTI, Pol 743-751(LI9) LPPVVAKEI, and Pol 283-289 (TI8) TAFTIPSI—were found to be the most dominant epitopes among HLA-B51$^+$ individuals. Disease progression was found to be associated with the sequential selection of mutations within the epitope and the T cell responses generated in each patient. We postulate that HLA-B51$^+$ HIV-infected patients might benefit from having several effective immunodominant epitopes, which enable them to form multiple layers of defense against virus escape, starting with LI9 responses, followed by TV8 and NI9, making it much harder for the virus to escape immune control by HLA-B51–restricted T cell responses.

Materials and Methods

Patient cohort

A total of 282 HIV-infected individuals infected with HIV-1 through exposure to contaminated blood in the early 1990s were recruited. None of the patients had received antiretroviral drug treatment before 2003. Personal history of infection, treatment history and general clinical data were collected (including viral load and CD4$^+$ counts). Informed consent has been obtained and ethical approval was obtained from Beijing You An Hospital, Ditan Hospital, and the University of Oxford Tropical Ethics Committee.

HLA typing, peptide synthesis, and HIV-1 Nef, Gag, and Pol sequencing

Molecular HLA typing was performed using the Amplification Refractory Mutation system with sequence-specific primers as described previously (18). HIV-1 *Gag*, *Pol*, and *Nef* genes were sequenced using modified primers and nested PCR as described previously (18). Peptides were synthesized by Sigma-Aldrich.

Human IFN-γ ELISPOT assay

A total of 200,000 PBMCs with 10 μg/ml peptide or 400 T cell clones with 20,000 peptide-pulsed B cell lines were used in a standard Human IFN-γ ELISPOT assays as described elsewhere (19). In brief, assays were carried out in 96-well MultiScreen filter plates (Millipore) coated with 15 μg/ml anti–IFN-γ mAb (1-DIK; Mabtech). A total of 5 μg/ml PHA (final concentration, 1 μg/ml) were used as positive control. Plates were incubated for 16 h at 37°C, 5% CO_2. Spot enumeration was performed with an AID ELISPOT reader system (Autoimmun Diagnostika). To quantify Ag-specific responses, mean spots of the control wells were subtracted from the positive wells, and results were expressed as spot-forming units (SFUs) per 10^6 PBMCs. Responses were regarded as positive if results were at least three times the mean of the quadruplicate negative control wells and >50 SFUs/10^6 PBMCs. If background wells were >30 SFUs/10^6 PBMCs or positive control wells (PHA or Flu, EBV, and CMV epitopes pool stimulation) were negative, the assay was excluded from further analysis.

CTL clones and HIV-1 permissive target cells

CTL clones specific for HLA-B51 epitopes were generated by limiting dilution from the PBMCs of HIV-infected patients responding to the NI9, LI9, and TV9 epitopes and maintained as described by Dong et al. (20). The human T cell leukemia MT2 line stably expressing CD4$^+$ and HLA-B51 was maintained in R10 media (RPMI 1640 with 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine, and 10% heat-inactivated FCS from Sigma-Aldrich).

Live virus ELISPOT

Differing HIV-1 strains (IIB and MN) were used to infect MT2 cells. Infected cells were washed twice and then resuspended in 2 ml R10 at a concentration of 1×10^6 cells/ml and cultured in a 25-ml flask for a period of 72 h postinfection at 37°C/5% CO_2. Cells were then washed, counted, and cocultured with the panel of HLA-matched CTL clones in triplicate at one E:T ratio of 400:20,000 on the precoated IFN-γ ELISPOT plates at a final volume of 100 μl/well. Negative controls included the individual CTL clones cocultured with uninfected target cell line in triplicate, and positive control included each CTL clone cocultured with uninfected target cells pulsed with 2 μM specific peptide. ELISPOT plates were incubated for 6 h at 37°C/5% CO_2 and subsequently washed and developed as described previously. SFUs were counted using the ELISPOT reader system AID ELISPOT 4.0.

Generating HIV mutant virus

Molecular clones of NL432 Pol TI8 and pNL432 Pol LI9 mutant virus were generated by PCR-based point mutagenesis of pNL432 by using PrimeSTAR HS DNA Polymerase (Takara, Japan) as described previously (7). To obtain pNL432 wild-type (WT) and mutant viruses, 293T cells were transfected with WT and mutant plasmids using Lipofectamine 2000 (Invitrogen). Supernatants from transfected 293T cell cultures were stored at −80°C.

Viral suppression assay

High-titer HIV-1 strains (1000 tissue culture-infective dose 50) were used to infect MT2 cell as target cells for T cell clones, and cell pellets were resuspended in a total volume of 200 μl R10 and then incubated for 90 min at 37°C. Infected cells were subsequently washed (twice) to remove free virus. A total of 5×10^4 infected cells were cocultured with HLA-matched HIV-1–specific CTL clones at differing E:T ratios of 1:1, 1:2, 1:4, 1:8, and 1:16 in H10-IL-2 (RMPI 1640 + 10% human serum + 200 μl/ml IL-2) on a flat-bottom 96-well plate, in a final volume of 200 μl/well, at 37°C for 4 d. Each condition was performed in triplicate, including one HLA-mismatched clone as a negative control and virus-infected cells in the

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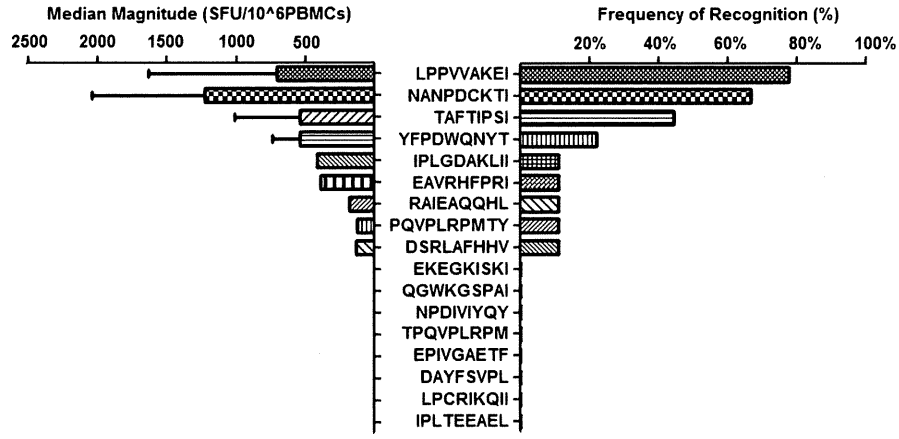

FIGURE 1. The average frequency of recognition and magnitude of response to the individual epitopes restricted by HLA-B*51. The responses to 17 known HLA-B51 epitope peptides was measured ex vivo using ELISPOT assay among 22 HLA-B51$^+$ individuals. The median magnitudes of response are represented with SFUs/10^6 PBMCs on the left side, and the mean frequencies of recognition are displayed on the right side.

absence of CTL as a positive control. The extracellular p24 content in the supernatant was assayed on day 4 by quantitative p24 Ag ELISA (Immunodiagnostics) in accordance with the manufacturer's protocol (21, 22).

Statistical analysis

Correlation with HIV viral load was analyzed using nonparametric Mann–Whitney *U* tests, and correlation with CD4$^+$ T cell counts was analyzed using the unpaired Student *t* test. Mutations associated with HLA-B51 were analyzed using χ^2 test. Statistical test differences were considered significant if $p < 0.05$.

Results

Three dominant HLA-B51–restricted HIV-1–specific T cell epitopes in a slow-progressor plasma donor cohort

A total of 282 HIV-1–infected individuals from rural China who were exposed to HIV-1 through contaminated blood in the early 1990s were recruited from 2005 to 2007. None of the patients had received antiretroviral drug treatment before 2003. Bulk proviral DNA sequences were generated for *Gag*, *Nef*, and *Pol*, from which consensus protein sequences were derived, and HLA typing was completed for all individuals. All members of this cohort are believed to have been infected with HIV-1 at a similar time between 1993 and 1995 by the same infection route and have subsequently progressed to diverse disease outcomes without antiretroviral treatment during the first 10 years of infection (17).

Twenty-two HLA-B51$^+$ individuals selected from this cohort were studied in detail. The clinical details and HLA typing of those individuals are shown in Supplemental Table I. Seventeen known HLA-B51 epitope peptides from the Los Alamos database, based on consensus sequences from the cohort, were synthesized, and ELISPOT assays were performed using patients' PBMCs. Epitopes TV8: TAFTIPSV (Pol283-289), NI9: NANPDCKTI (Gag327-15), and LI9: LPPVVAKEI (Pol743-750) were the most immunodominant epitopes in this cohort at frequencies of 42, 64, and 72% (Fig. 1).

Selection hierarchy of B51 escape mutations

We examined *Gag* and *Pol* viral sequences containing the three dominant epitopes (TV8, NI9, and LI9) in genomic DNA extracted from 22 HLA-B51$^+$ individuals and 126 HLA-B51$^-$ individuals and found that mutations in each of these three dominant epitopes were significantly associated with HLA-B51 (Table I). Seventeen of the 22 HLA-B51$^+$ individuals showed a mutation of L243I at position 1 of the LI9 epitope, 17 of 22 showed a mutation of V289X at position 8 of the TV8 epitope, and 6 of 22 showed a mutation of A328S at position 2 of the NI9 epitope. Interestingly, we found a pattern of mutation suggesting that there may have been a sequential selection hierarchy of mutations in these three epitopes (Table II): individuals with the A328S mutation in the NI9 epitope also showed mutations in the other two Pol epitopes (LI9 and TV8) and individuals with the V8L mutation in the TV8 epitope also showed the L1I mutation in the LI9 epitope. These results strongly suggest that L743I in position 1 of the LI9 epitope seems to mutate the most readily and is likely to be the first to mutate, followed by V289L in position 8 of the TV8 epitope, and then A328S in position two of the NI9 epitope.

HLA-B51–restricted NI9 and TV8 T cell responses are associated with viral control and a better clinical outcome when epitopes are not mutated

We next examined the association among mutated epitopes, viral loads, and CD4$^+$ counts (Fig. 2). A significantly higher viral load was observed when more than one epitope had mutated, whereas a significant decrease of CD4$^+$ counts was observed when all three epitopes had mutated. Once the data obtained on the selection

Table I. Epitope mutations associated with HLA-B51 in the Chinese cohort

Epitope	Mutant	1	2	3	4	5	6	7	8	9	B51$^+$ Donor (n = 22)	B51$^-$ Donor (n = 126)	p Value (Mutation Associated with HLA-B51)
LI9	WT	L	P	P	V	V	A	K	E	I	5	114	<0.001
	L1***I***	***I***	P	P	V	V	A	K	E	I	17*	12	
TV8	WT	T	A	F	T	I	P	S	V		8	97	
	V 8***I***	T	A	F	T	I	P	S	***I***		0	23	0.025
	V8***L***	T	A	F	T	I	P	S	***L***		10*	5	<0.001
	V8***T***	T	A	F	T	I	P	S	***T***		2*	0	0.021
	V8***R***	T	A	F	T	I	P	S	***R***		2*	0	0.021
NI9	WT	N	A	N	P	D	C	K	T	I	15	123	<0.001
	A2***S***	N	***S***	N	P	D	C	K	T	I	7*	3	

Bold and italic formatting indicates the variant amino acid within the epitope. Asterisks indicate these mutations have significant associations with HLA-B51.

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Table II. Epitope mutation selection hierarchy in HLA-B51–restricted dominant epitopes in SM cohort

SM ID	NANPDCKTI	TAFTIPSV	LPPVVAKEI
200	0	0	0
379	0	0	0
40	0	0	0
166	0	0	0
192	0	0	0
209	0	0	1
446	0	0	1
349	0	0	1
176	0	1	1
64	0	1	1
8	0	1	1
55	0	1	1
181	0	1	1
215	0	1	1
381	0	1	1
350	1	1	1
455	1	1	1
47	1	1	1
14	1	1	1
342	1	1	1
63	1	1	1
353	1	1	1

0, no mutation in epitope; 1, mutation developed within epitope (Table I).

Table III. Patient groups

	NI9		TV8		LI9			
	M*	T*	M*	T*	M*	T*	VL-07	$CD4^+$-07
Group 1								
40	0	—	0	—	0	—	42,000	248
215	0	—	+	+	+	+	200,000	514
381	0	—	+	+	+	+	100,000	106
350	+	—	+	—	+	+	200,000	8
47	+	—	+	—	+	+	88,000	143
14	+	—	+	—	+	+	100,000	113
342	+	+	+	—	+	+	200,000	222
353	+	—	+	—	+	+	100,000	169
181	0	—	+	—	+	—	300,000	252
Group 2								
166	0	—	0	+	0	+	11,000	590
176	0	+	+	—	+	—	1,200	621
446	0	+	0	+	+	+	14,000	421
209	0	+	0	—	+	—	35	508
349	0	+	0	+	+	—	820	378
8	0	+	+	—	+	—	23,000	370

Patients were grouped according to T cell responses detected with no epitope mutation for at least one of the epitopes (group 2) or no T cell response detected in any nonmutated epitopes (group 1).
M*, mutation detected in epitope.

hierarchy of epitopes are combined (Table II), it is evident that if there is only one epitope mutation in the patient, the mutation is more likely to be that of LI9, and if two epitope mutations are detected, they are likely to be LI9 and TV8. Therefore, the results suggest that, when mutations developed in TV8 and NI9, this is associated with loss of viral control in HLA-B51$^+$ patients; moreover, an S328A mutation developing in the NI9 epitope is significantly associated with low $CD4^+$ counts.

There are two main reasons why the virus might not develop epitope escape mutations under CTL pressure: the first being that the individual did not generate the T cell responses against the epitope and that therefore there is no immune pressure on the epitope region of the virus; the second being that when T cells target an important region of the virus, any change within that region will affect the function of the virus and mutations will therefore incur a fitness cost. We hypothesized that in the case of HLA-B51, individuals who are able to generate a response toward at least one of the three dominant epitopes in which the targeted epitope remains unmutated should have a better clinical outcome and, more specifically, a lower viral load and higher $CD4^+$ counts. In contrast, individuals that have three mutated epitopes, or no detectable T cell responses to unmutated epitopes, would have higher viral loads and lower $CD4^+$ counts. Therefore, we divided patients into two groups (Table III): group 1 consisted of individuals with mutations in all three epitopes or with no T cell responses against unmutated epitopes, and group 2 consisted of individuals with at least one detectable T cell response against epitopes that had not mutated. We found that $CD4^+$ counts were significantly higher ($p < 0.001$) and viral loads significantly lower ($p < 0.001$) in patients with no epitope mutation (group 1) and with at least one detectable T cell response against the TV8 and NI9 epitopes (group 2) (Fig. 3). Because most of the patients developed an I243L mutation in the LI9 epitope, the T cell responses against unmutated epitopes in group 2 were all NI9- or TV8-specific responses. These data strongly imply that the eventual loss of viral control in HLA-B51$^+$ HIV-infected individuals was due to the development of escape mutations in all three epitopes and that single T cell responses against unmutated epitopes were sufficient for viral control.

T cell escape mutations developed in three epitopes (NI9, LI9, and TV8)

TV8-, NI9-, and LI9-specific CTL lines and clones were generated from HLA-B51$^+$ HIV-infected individuals, and recognition of epitope variants was tested in ELISPOT assays. The A2S NI9 epitope variant peptide was tested using gag-specific T cell clones and could not be recognized by NI9 specific clones (Fig. 4*A*), which indicates that this represents an escape mutation. High cross-reactivity was observed when variant epitope peptides and

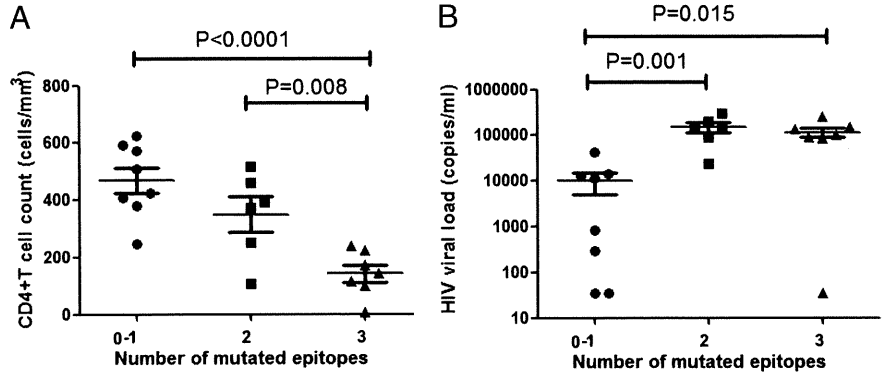

FIGURE 2. Increased numbers of mutated epitopes are significantly associated with lower CD4 counts and higher viral load in this cohort. The HIV viral load (*A*) and $CD4^+$ T cell counts (*B*) were compared among people with mutations developed in none or one (LI9 only), two (LI9 + TV8), or three epitopes (LI9 + TV8 + NI9).

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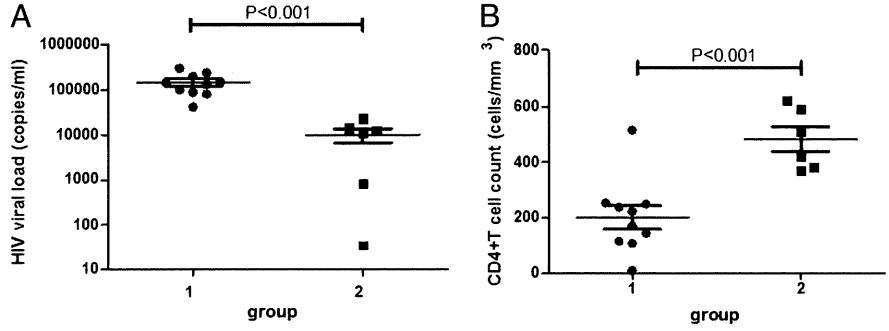

FIGURE 3. CTL detected in patients with no mutation in at least one HLA-B51–restricted epitopes are significantly associated with better clinical outcome. The HIV viral load (*A*) and $CD4^+$ T cell counts (*B*) were compared between group 2 (patients responded to at least one of the epitopes without mutation) and group 1 (patients did not respond to any nonmutated epitopes).

T cell clones specific for the TV8 and LI9 epitopes were used (Fig. 4*B*, 4*C*); those results were further confirmed by ex vivo ELISPOT assay using HLA-$B51^+$ patient's PBMCs stimulating with both WT and variant peptides (Supplemental Fig. 1). Interestingly, when we tested the recognition of virus-infected target cells, using HIV-1 containing the 289L variant, which is the major variant occurring in $B51^+$ individuals (59%), TV8-specific clones failed to recognize the 8L mutant virus (Fig. 5*A*), strongly suggesting that this mutation had affected Ag processing and presentation of the epitope. Although we didn't observe escape recognition of the LI9 epitope through the 743I mutation, we found that 82% (14 of 17) of $B51^+$ patients with the 743I mutation in the LI9 epitope also developed mutations in the downstream flanking region (I or V to L mutation in position 760) and 41% developed a mutation in position 754 (C to S) (Supplemental Table II), whereas none of the $B51^-$ individuals with WT LI9 sequences showed these mutations. Mutant virus was made with the single mutation L743I as well the combinations L743I/L760I and L743I/L760I/S754C. We tested the ability of LI9-specific CTL clones to inhibit viral replication and found significantly reduced antiviral efficacy against the L743I virus (2-fold increment of p24 level compared with the WT), which was further reduced while using virus-containing L743I/L760I and L743/L760I/S754C mutations (4- and 8-fold increment in p24 level when compared with WT) (Fig. 5*B*), suggesting that these mutations have an impact on Ag processing and presentation, therefore affecting the antiviral efficacy of LI9-specific T cell responses against HIV-1 expressing those mutations. Comparable levels of P24 production were observed between WT and mutant virus-infected MT2 cells in the absence of clones.

NI9, TV8, and LI9 T cell clones could effectively recognize and control virus-infected target cells in live virus ELISPOT assays and in in vitro virus suppression assays

We tested the ability of NI9-, LI9-, and TV8-specific T cells to recognize IIIB and MN virus-infected target cells in live virus ELISPOT assays (Fig. 6*A*). NI9 CTL showed the most effective recognition of virus-infected cells, followed by TV8 CTL. LI9 CTL showed very poor recognition of virus-infected target cells, which could be due to a mutation detected at position 4 (V4I) of the MN virus (Supplemental Fig. 1). *Pol* and *Gag* proviral DNA sequences were examined by extracting genomic DNA from HIV-1 IIIB- and MN-infected MT2 cells. NI9, TI8, and LI9 WT sequences were confirmed in IIIB virus-infected cells, whereas an

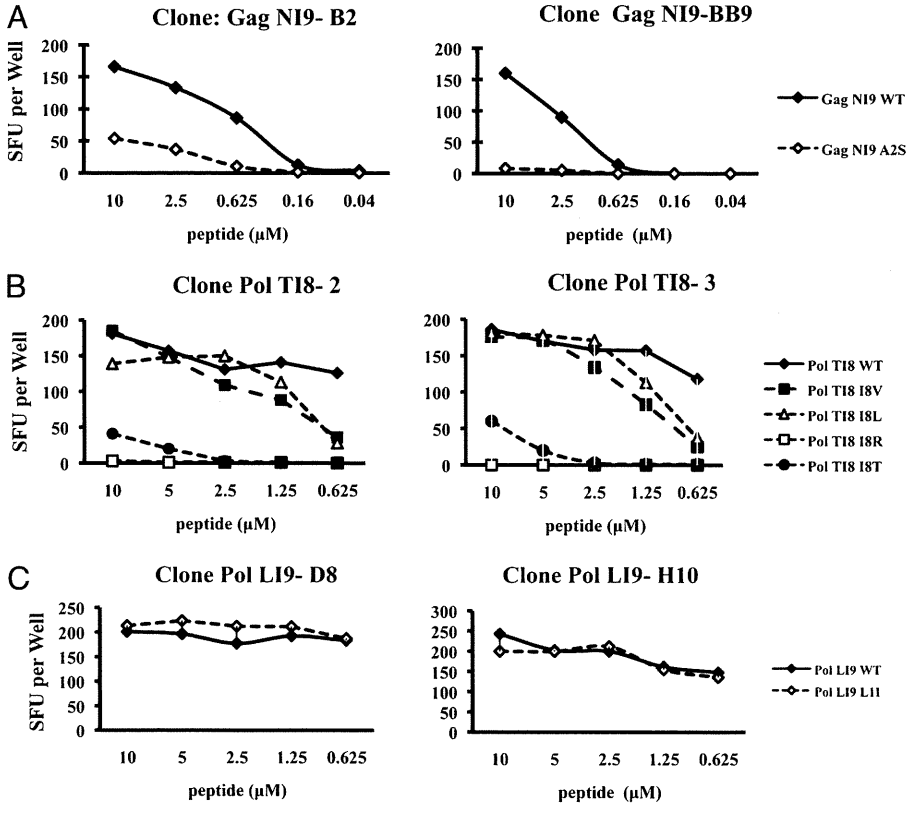

FIGURE 4. Recognition of major epitope variants by Ag-specific CTL clones. Ag-specific T cell clones were cocultured with target cells. *A–C* show the recognition of epitope variants by Gag NI9-, pol TI8-, and LI9-specific CTL clones, respectively; $n = 3$.

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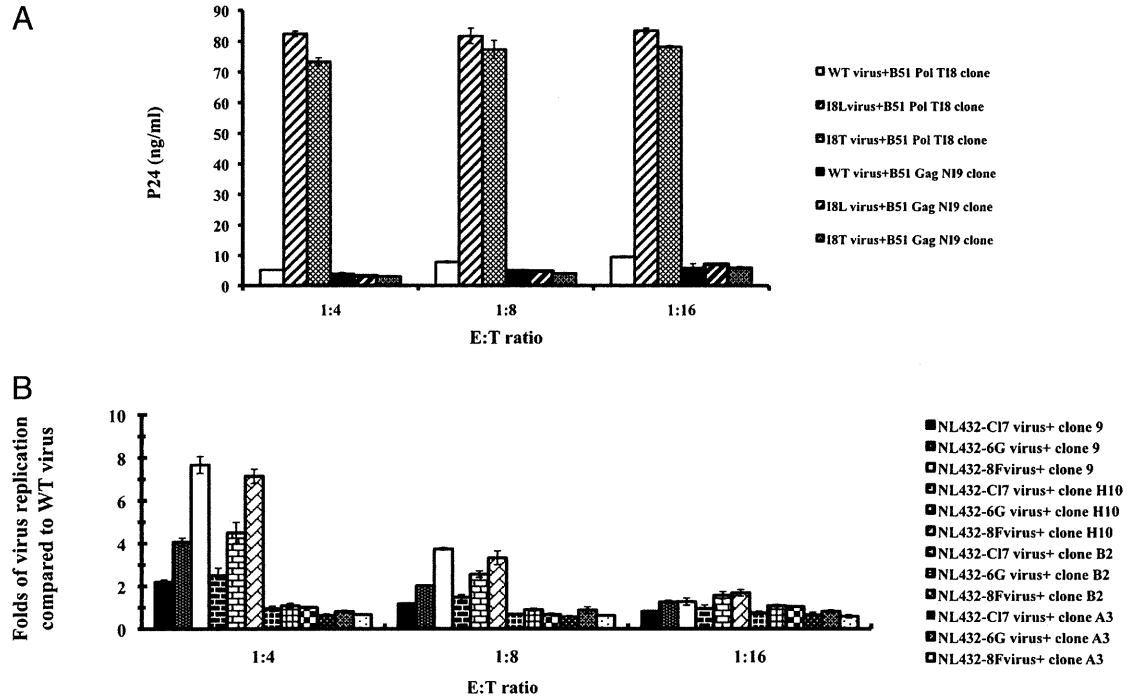

FIGURE 5. In vitro inhibition of WT and mutant viruses in presence of Ag-specific CTL clones at different E:T ratios. *A*, Targets were infected with WT (NL432 pol TI8) or mutant (I8L/T) viruses and incubated with PolTI8-specific CTL clones. Gag NI9-specific CTL clone was used as control; $n = 3$. *B*, CTL clones were cocultured with target cells infected with mutant viruses NL432-cl7 (pol L743I), 6G (pol L743I/L760I), and 8F (pol L743I/S754C/L760I) infected. Data are expressed as fold increase versus WT virus NL432. Clones 9 and 10 are pol LI9-specific, whereas B2 is gag NI9-specific. HLA-mismatched CTL clone (A3) is also included; $n = 3$.

intraepitope mutation, I746V, at position 4 of LI9 epitope was detected in MN virus-infected cells (data not shown), which explains the weak recognition by LI9-specific T cell clones.

To confirm the antiviral efficacy of all three epitope-specific T cells, we also performed in vitro virus suppression assays by coculture of NI9, TV8, and LI9 T cell clones with HIV-1 IIIB or MN virus-infected MT2 cells, harvesting the culture supernatant on day 4 to measure HIV-p24 levels by ELISA. All three epitope-specific T cell clones could control both viruses effectively when WT epitopes are presented (Fig. 6*B*, 6*C*).

Discussion

In most of the existing cohort studies, HIV infection route, length of infection, and infecting viral strain almost always differ among individuals, leading to complexity of data analysis, in particular, applied to HIV Ag-specific T cell responses, T cell escape, and their association with clinical outcome. In this paper, we have been able to study a unique cohort of patients who have been infected with a narrow source virus with a similar length of infection time (17). We found that HLA-B51–restricted immunodominant HIV-specific T cell responses play an important role in controlling disease progression in this cohort. A marked selection hierarchy of epitope mutations among the three most dominant HLA-B51–restricted epitopes was observed. Good control of viral load and higher $CD4^+$ counts were found in patients with T cell responses against unmutated epitopes, whereas lower $CD4^+$ counts and higher viral loads were detected in patients with mutations in all three epitopes or with no detected T cell responses against epitopes that did not mutate. We conclude that the patients with HLA-B51 have benefited from having a hierarchy of protective epitopes, which provide multiple layers of defense against the development of immune escape mutations, particularly when CTL target the epitope (NI9), which is relatively conserved and appears to escape slowly.

Interestingly, we observed that there was a significant difference in $CD4^+$ T cell count but not viral load when two versus three escape mutations are present, suggesting CD4 cell counts might be a better indicator for disease progression than viral load in this cohort.

Unlike the Japanese cohort studied by Kawashima et al. (7), the Chinese cohort showed less dependence on the TI8 epitope response, presumably because this epitope already expresses a mutation in the consensus sequence (valine, instead of isoleucine, was dominant, found in 77% of non-B51 individuals), indicating that 289V was likely to be present in the infecting virus (17). The pattern of escape mutation that developed was also very different between the two cohorts: for example, we observed very few V289T mutations in the TV8 epitope. However, this mutation was frequently observed in the Japanese and other cohorts, and this led to the direct escape of recognition by CTL. Instead, most of our patients developed a V8L mutation in this epitope, which did not lead to direct escape from CTL recognition but probably impaired efficient Ag processing of this epitope. Overall, we have shown clear evidence that the development of epitope mutations and the sequence of the infecting virus are very different in the Chinese compared with the Japanese cohort, which might suggest the existence of different mechanisms of immune protection through HLA-B51.

Strong linkage of mutations in the flanking region of the LI9 epitope (L760X and S754C) to the intraepitope mutation (L743I) was observed in HLA-B51 individuals (these mutations are also significantly associated with HLA-B51). We demonstrated that CTL show reduced antiviral efficacy against virus containing these mutations, strongly suggesting that the virus with these mutations in $B51^+$ individuals in our cohort (82%) will evade LI9-specific T cell responses because of impaired presentation of this epitope.

Live virus ELISPOT assays and suppression assays showed that NI9, LI9, and TV8 T cells could recognize MN or IIIB virus-

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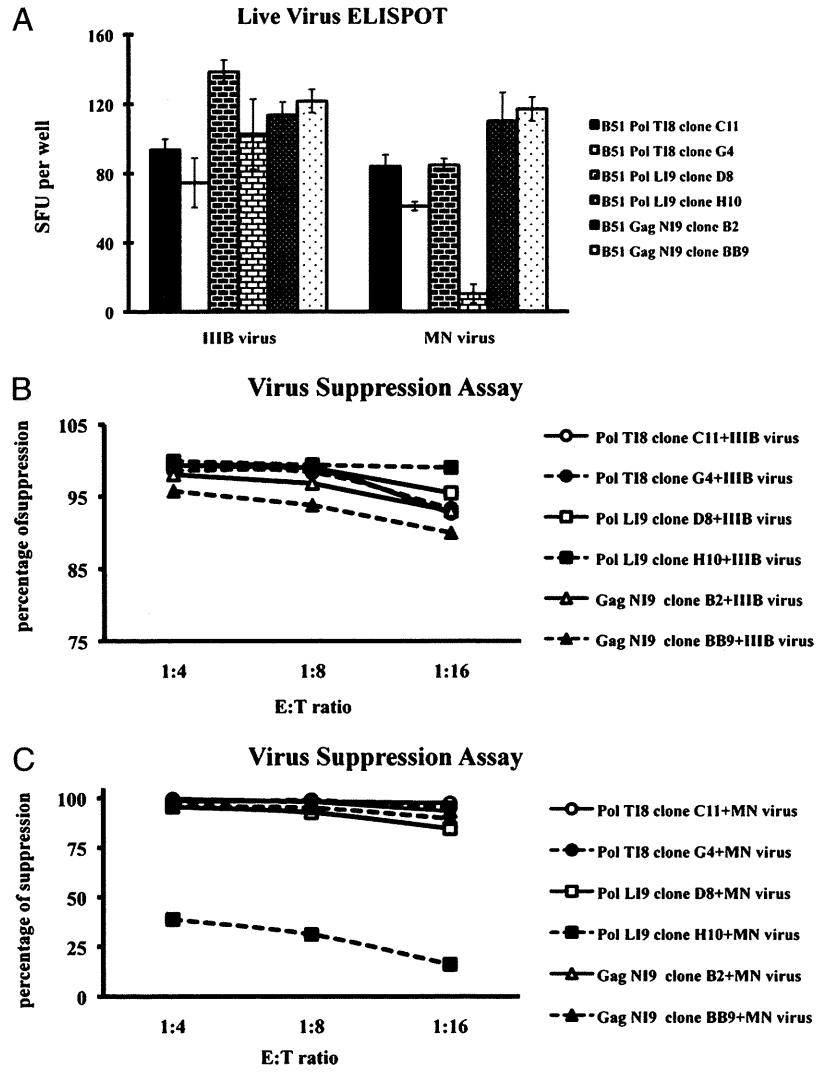

FIGURE 6. NI9-, TV8-, and LI9-specific T cell clones can effectively recognize and control virus-infected target cells. *A*, Antiviral activity of CTL clones measured by live virus IFN-γ ELISPOT. CTL clones were cocultured with MN and IIIB virus-infected targets; $n = 3$. *B* and *C*, Ability of CTL clones to suppress HIV-1 replication in IIIB- and MN-infected MT2 cell lines at variable E:T ratios; $n = 3$.

infected cells efficiently when the WT epitope was presented, with no clear hierarchical ranking in terms of antiviral efficacy observed among these three epitope-specific clones, suggesting that these T cells could function efficiently if the infecting virus were to be processed and presented properly in target cells.

A hierarchy of rates of escape in epitopes in acute infection was well described by Brumme et al. (23) using a large seroconverter cohort. One of the HLA-B51–restricted epitopes, TI8, was mentioned in their study, with the rate of escape being 15% in the first year of infection. The LI9 and NI9 epitopes were not discussed, although LI9 responses were reported as the most immunodominant during acute infection (24), followed by TI8 responses (no data on NI9 responses in acute infection). In this study, we have shown that in an acutely HIV-1–infected individual who progressed rapidly, T cell responses to LI9 and TI8 epitopes appeared at a very early stage of infection (before day 60 postinfection), and T cell responses to the NI9 epitope were not detected in the first year postinfection, which is in agreement with previous findings. This case study further highlights the importance of multilayered defense in HLA-B51–associated viral control.

In the complex and long-lasting battle between the virus and the host T cell response, there are factors that either favor the host (efficient T cell function and multiple T cell responses targeting conserved proteins where escape mutations have a significant fitness cost) or the virus (fast development of escape mutations with no fitness cost or ineffective host immune control of viral replication). This complexity makes it difficult to determine the appropriate immunogen for an HIV-1 vaccine. Our data suggest that a multilayered defense system could help the host combat the virus effectively, particularly by targeting epitopes, which mutate slowly, such as the gag NI9 epitope. Unlike B57-associated protection or what was observed in T cell control of SIV replication in preclinical vaccine trials (25, 26), the fitness cost of virus containing escaped mutations seemed not to be a major factor in B51-associated protection, because high viral loads were detected in patients with mutant LI9 or TI8 epitopes and in B51-negative individuals (Table III). We propose that when considering a future HIV vaccine that would elicit effective T cell responses, it should be possible to design a more precise construct, with the aim of eliciting the most beneficial CTL-specific responses by targeting epitopes, that mutate with difficulty and that are associated with viral control for common HLA alleles in a target population.

Our data are consistent with other studies suggesting that the emergence of escape mutations coincides with increased viral replication (9); moreover, these mutations do not occur at random but develop in the same position in different patients, consistent with T cell driven selection pressure. However, without detailed longitudinal studies, we could not exclude the possibility that increased viral replication may lead to more mutations, rather than escape mutations preceding the rise in viral load. De novo CTL responses may also develop in response to new viral variants (27), which merits future investigation.

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In conclusion, our results provide an example of the constant battle between the human immune system and HIV. In this case, the presence of HLA-B51 and T cells targeting these three immunodominant epitopes could be advantageous, and more importantly, having an immunodominant response to the slow mutating epitope NI9 is beneficial for long-term control of viral replication.

Disclosures

The authors have no financial conflicts of interest.

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Short communication

Selection of HLA-B57-associated Gag A146P mutant by HLA-B*48:01-restricted Gag140-147-specific CTLs in chronically HIV-1-infected Japanese

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Abstract

We previously showed the possibility that Gag A146P, which is an escape mutant from HLA-B*57-restricted CTLs, was selected by HLA-B*48:01-restricted Gag138–147(LI10)-specific CTLs in a Japanese cohort in which HLA-B*57 individuals were not detected. We herein demonstrated Gag140–147(GI8) to be the optimal epitope rather than LI10 and that GI8-specific T cells failed to recognize the A146P mutant virus-infected cells. The sequence analysis of Gag146 in 261 chronically HIV-1-infected Japanese showed the accumulation of the A146P mutation in HLA-B*48:01$^+$ individuals. These findings together indicate that the A146P mutant is accumulating in Japanese by selection by GI8-specific CTLs.
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Keywords: HIV-1; CTL; HLA-B*57; HLA-B*48:01; Escape

1. Introduction

HLA-B*57 is well known to be associated with the control of HIV-1 [1,2]. The presence of HLA-B*57-restricted CTLs specific for the Gag147–155 (ISPRTLNAW: ISW9) epitope is associated with a slow progression to AIDS [3,4], suggesting that these epitope-specific CTLs play an important role in the control of HIV-1. The substitution from Ala to Pro at residue 146 of Gag (A146P) in the N-terminal flanking region of ISW9 is associated with the HLA-B*57 genotype [5,6]. These findings suggest that HLA-B*57-restricted ISW9-specific CTLs selected this mutant. Since A146P is located at the N-terminal flanking region, it is assumed that this mutation would affect the processing of this epitope. Indeed, this A146P mutation prevents N-terminal trimming by the ER aminopeptidase I [7].

Our previous analysis of 52 chronically HIV-1-infected Japanese showed that 38% of these subjects have the HLA-B*57-associated A146P variant, although the HLA-B*57 genotype is not detected in them and that there is a strong association between this substitution and the Asian allele HLA-B*48:01 [8]. In addition, we showed that HLA-B*48:01-restricted Gag138–147(LQGQMVHQAI: LI10)-specific T cells, which can be induced by LI10 peptides from PBMC of chronically HIV-1-infected HLA-B*48:01$^+$ individuals, fail to recognize the A146P mutant epitope peptide and A146P mutant virus-infected cells [8]. These results together suggest that HLA-B*48:01-restricted LI10-specific CTLs selected the A146P mutant in HIV-1-infected Japanese. However, since Gag138–147 (LI10) contains glutamine (*Q*) at positions 2 and 4, which is known as HLA-B*48:01 anchor residue [9], it is assumed that Gag140–147 (GQMVHQAI: GI8) would also be

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an HLA-B*4801-restricted epitope and that the A146P mutant would be selected by GI8-specific CTLs.

In the present study, we sought to clarify whether both the 10-mer and 8-mer peptides are HLA-B*48:01-restricted CTL epitopes and whether GI8-specific CTLs fail to recognize the A146P mutant virus-infected cells if GI8 was the CTL epitope. Furthermore, we analyzed the association between A146P variant and HLA-B*48:01 using a larger number of chronically HIV-1-infected Japanese individuals in order to confirm the accumulation of this mutation in Japanese.

2. Materials and methods

2.1. CTL clones

CTL clones were generated from an established peptide-specific bulk CTL culture from a chronically HIV-1-infected HLA-B*48:01$^+$ patient KI-092 by seeding 50 cell/well into U-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 μl cloning mixture (RPMI 1640 medium supplemented with 10% fetal calf serum [FCS] and 200 U/ml recombinant human IL-2, 1×10^5 irradiated allogeneic PBMC from a healthy donor, and 5×10^4 irradiated C1R-B*4801 cells pre-pulsed with 1 μM of the corresponding peptide, GI8 or LI10. The clones were examined for CTL activity by the standard ^{51}Cr release assay.

2.2. Intracellular cytokine staining (ICC) assay

PBMCs from chronically HIV-1-infected HLA-B*48:01$^+$ individuals were stimulated with GI8 or LI10 peptides for 2 weeks. These cultured cells were stimulated with C1R-B*4801 cells pre-pulsed with GI8 or LI10 peptides at 100 nM at an effector-to-stimulator (E: S) ratio of 1:4. The CTL clones were also stimulated with C1R-B*4801 cells pre-pulsed with the various concentrations of the 4 different peptides (GI8, GI8-7P, LI10 or LI10-9P) at the same E: S ratio. .221-CD4-B*4801 cells were generated by transfecting an HLA class I-defecting .221 cell line with HLA-B*48:01 and human CD4 genes. They were infected with NL432 or NL432-A146P mutant virus and then these HIV-1-infected cells (>30% p24 Ag-positive cells) were used as stimulator cells at an effector-to-stimulator ratio of 1:1. Stimulator cells were washed in RPMI 1640-10% FCS before use. The cells were incubated for 6 h at 37 °C in 5% CO_2. Brefeldin A (Sigma–Aldrich) at a concentration of 10 μg/ml was added 2 h after stimulation. After 6 h incubation, the cells were washed in PBS supplemented with 5% FCS. Cell surface staining was performed for 30 min at 4 °C with PacificBlue-conjugated anti-human CD8 MAb. Then cells were washed twice in PBS supplemented with 5% FCS. After a wash, the cells were fixed with 4% paraformaldehyde for 20 min at 4 °C and subsequently made permeable by incubation for 10 min at 4 °C in PBS supplemented with 0.1% saponin containing 10% FCS. The cells were resuspended in permeabilizing buffer and then were stained with a FITC-conjugated anti-human IFN-γ MAb at room temperature for 30 min. Finally, the cells were washed twice with permeabilizing buffer and were resuspended in 2% paraformaldehyde. The percentages of intracellular IFN-γ positive cells among CD8$^+$ cells were analyzed using FACS Aria (Becton Dickinson, San Jose, CA).

2.3. Sequencing of plasma RNA

Two hundred and sixty-one chronically HIV-1-infected Japanese individuals were recruited for the present study, which was approved by the ethic committees of Kumamoto University and International Medical Center of Japan. Viral RNA was extracted from the plasma of these HIV-1-infected individuals by using a QIAamp MinElute Virus Spin Kit (QIAGEN). cDNA was synthesized from the RNA with Superscript II and random primer (Invitrogen). We amplified sequence of Gag region by nested PCR. Sequencing was done with a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed by use of an ABI PRISM 3500 genetic analyzer.

2.4. Statistical analysis

Statistical analysis was performed with SPSS 17 software (SPSS Inc, Chicago, IL). Standard deviation and means were calculated. A Mann–Whitney U test was used to assess the significance of between groups. A p-value <0.05 interpreted as a significant difference.

3. Results

3.1. Recognition of GI8 and LI10 peptides by HLA-B*48:01-resticted CTLs established by stimulation with GI8 or LI10 peptide

To clarify whether both the 10-mer and 8-mer peptides are HLA-B*48:01-restricted CTL epitopes, we investigated HLA-B*48:01-restricted CD8$^+$ T cell responses to GI8 or LI10 peptide. We stimulated PBMC from a chronically HIV-1-infected Japanese individual, KI-092, with GI8 or LI10 peptide and then cultured them for 2 weeks. The ability of bulk-cultured cells to respond to these peptides was investigated by performing the ICC assay. Although both CD8$^+$ bulk-cultured cells recognized not only target cells pulsed with GI8 but also those pulsed with LI10, they recognized GI8 much more effectively than LI10 (Fig. 1A). To confirm this result, we established HLA-B*48:01-restricted CTL clones from these bulk-culture cells. Both CTL clones established by stimulation with GI8 or LI10 peptides from the bulk-cultured cells showed similar results (Fig. 1B). These results indicate that GI8 was the optimal epitope rather than LI10.

We further analyzed the CD8$^+$ T cell responses to these peptides by using PBMCs from 9 other chronically HIV-1-infected HLA-B*48:01$^+$ individuals. These were cultured for 2 weeks after stimulation with either peptide and then the responses were analyzed by ICC assay. The LI10-stimulated bulk-cultured cells from these individuals failed to respond to the LI10 peptide (data not shown). On the other hand, the GI8-stimulated ones from 5 of the 9 individuals responded to the GI8

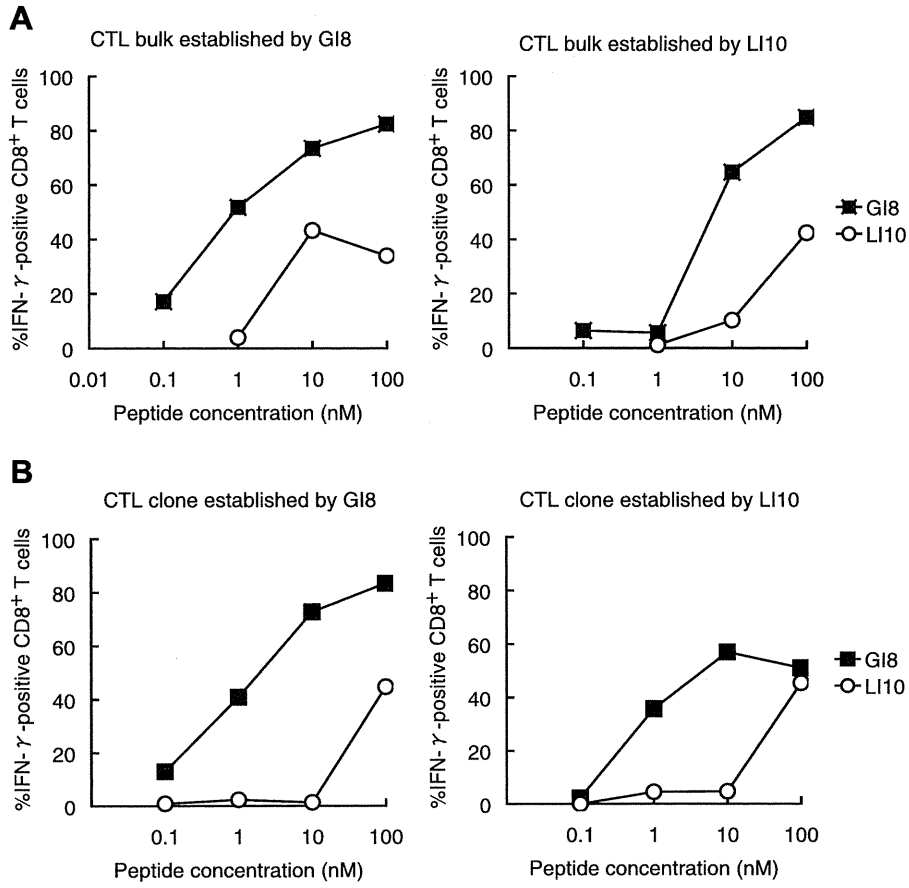

Fig. 1. Recognition of GI8 and LI10 peptides by HLA-B*48:01-resticted CTLs established by stimulation with GI8 or LI10 peptide. Antigen sensitivity of bulk PBMC culture from KI-092 stimulated with GI8 (left panel) or LI10 (right panel) peptide (A) and that of the HLA-B*48:01-restricted CTL clones established from these cultured cells by stimulation with GI8 (left panel: GI8-H2 clone) or LI10 (right panel; LI10-A7 clone) peptide (B) were investigated by using C1R-B*4801 cells pre-pulsed with GI8 or LI10. These T cells were stimulated with C1R-B*4801 cells pre-pulsed with GI8 or LI10 at the concentrations of 0.1–100 nM, and then IFN-γ production was measured by using the ICC assay. Similar results were found in 5 and 4 clones established by GI8 and LI10, respectively.

peptide but not to the LI10 peptide (Fig. 2). These results confirmed GI8 to be the optimal epitope.

3.2. Ability of GI8-specific CTLs to recognize the mutant peptides and the mutant HIV-1-infected cells

To clarify whether CTL clones established from GI8 peptide recognize A146P variant peptides, we investigated the recognition by the CTL clones of GI8-7P (GQMVHQ**PI**) and LI10-9P (LQGQMVHQ**PI**). They hardly recognized these variant peptides (Fig. 3A), suggesting that A146P was an escape mutation. To confirm this, we investigated whether GI8-specific CTLs would fail to recognize the A146 mutant virus-infected cells. .221-CD4-B*4801 cells were infected with NL432 or NL432-A146P mutant virus and then they were cultured for 4 days. The infectivity of these viruses (NL432 and NL432-A146P) was 39.2% and 47.2%, respectively. These cells were used as stimulator cells for the GI8-specific CTL clones. The CTL clones failed to recognize .221-CD4-B*4801-infected with NL432-A146P mutant virus but effectively recognized those infected with the NL432 wild-type one (Fig. 3B). These results together indicate that A146P was an escape mutation from GI8-specific CTLs.

3.3. Frequency of the Gag A146X variant in the Japanese cohort

A previous analysis of 52 chronically HIV-1-infected Japanese showed a positive association between the presence of HLA-B*48:01 and the A146P mutant [8]. To confirm this association, we additionally analyzed this residue in 209 chronically HIV-1-infected Japanese. Taken together with previous data on those 52 subjects, the results revealed that 79.2% of 24 HLA-B*48:01$^+$ individuals and 41.8% of 237 HLA-B*48:01$^-$ individuals had A146X (mostly A146P) variants (Fig. 3C). Thus, this result showed a strong association between A146X (A146P) and HLA-B*48:01 ($P = 0.00049$). These results confirm the previous study and strongly suggest that HLA-B*48:01-restricted GI8-specific CTLs selected the A146P mutation.

4. Discussion

A previous study demonstrated that HLA-B*57-restricted ISW9-specific CTLs select the A146P mutant that affects the processing of the ISW9 epitope by preventing N-terminal trimming by the ER aminopeptidase I [7]. On the other hand, this

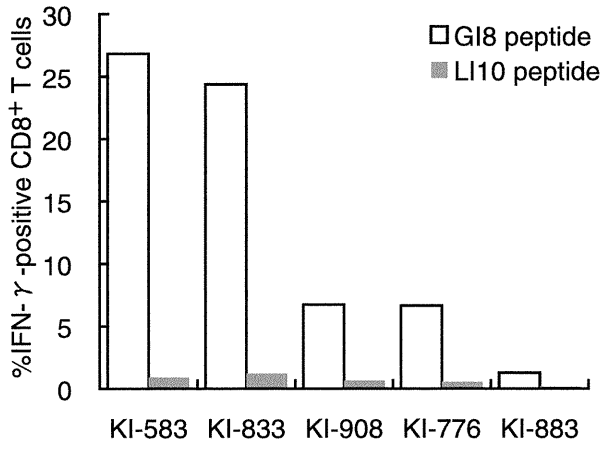

Fig. 2. GI8-specific $CD8^+$ T cell response in chronically HIV-1-infected HLA-B*48:01^+ individuals. PBMCs from 9 chronically HIV-1-infected HLA-B*48:01^+ individuals were stimulated with GI8 or LI10 peptide and cultured for 2 weeks. Cultured bulk cells were stimulated with C1R-B*4801 cells pre-pulsed with GI8 or LI10 peptide at a concentration of 100 nM, and IFN-γ production was measured by using the ICC assay. The cultured $CD8^+$ T cells from 5 of the 9 individuals produced IFN-γ after stimulation with C1R-B*4801 cells pre-pulsed with GI8 peptide, whereas those from all 9 individuals failed to produce IFN-γ after stimulation with C1R-B*4801 cells pre-pulsed with LI10 peptide. The frequency of IFN-γ-producing $CD8^+$ T cells among the total $CD8^+$ T cell population after stimulation with C1R-B*4801 cells pre-pulsed with GI8 peptide is shown.

mutation might affect the binding to the TCR of GI8-specific HLA-B*48:01-restricted CTLs or that of the mutant peptide to HLA-B*48:01 molecules. Since residue 146 is located at position 7 of the epitope peptide, which position is not anchor for HLA-B*48:01, it is speculated that the substitution from Ala to Pro at position 7 would not affect the binding of the peptide to HLA-B*48:01 molecules. Therefore this substitution may affect the binding to the TCR. Thus A146P is a case in which the same mutation is selected by different CTLs by different mechanisms.

We established CTL clones by stimulating PBMC from patient KI-092 with GI8 or LI10 peptide. The CTL clones established from PBMC stimulated with GI8 recognized GI8 peptide more than those established from PBMC stimulated with LI10 although both clones recognized LI10 peptide only at a concentration of 100 nM (Fig. 1). This finding suggests that the former CTL clones carry a different TCR from the latter ones. Thus this patient may have at least 2 GI8-specific CTLs with different TCRs.

The frequency of this variant is 14.1% in HLA-B*57 negatives in a Durban cohort where 22.3% of population are HLA-B*57-positives [5]. A strong association between the A146X variant and HLA-B*57 allele was further detected in 3 African cohorts [8]. In the present study, we showed that 41.8% of HLA-B*48:01^- individuals had this variant in a Japanese cohort where HLA-B*48:01 was detected in only 9.4% of the population. Thus, this mutant is accumulating more in the Japanese population than in the African populations. These findings suggest the following possibilities: 1) HLA-B*48:01-restricted GI8-specific CTLs can select the A146P mutant more strongly than HLA-B*57-restricted ISW9-specific CTLs. 2) This mutant is also selected by other HLA class I-restricted CTLs. The latter possibility is speculated based on the findings that this variant is associated with HLA-B*39 and HLA-B*15:10 [10,11]. Although HLA-B*39-restricted epitope is known not to be near Gag146, Gag143–152 (VHQAISPRTL) was previously reported as HLA-B*15:10 epitope [11]. If Gag143–152-specific HLA-B*15:10-restricted CTLs selected this mutation, TCR of the CTLs may not bind to the mutant epitope. Further analysis is expected to clarify the mechanism of the accumulation of A146P mutation in Japanese.

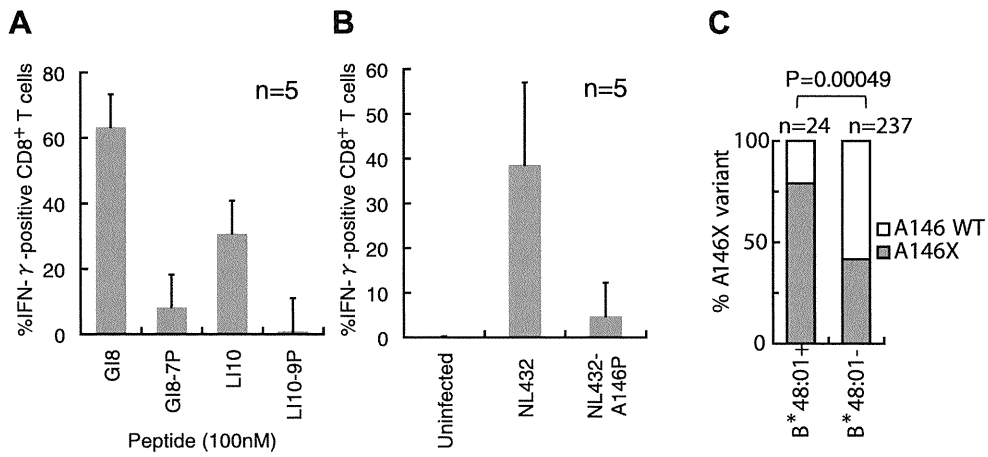

Fig. 3. Ability of GI8-specific CTL clones to recognize the mutant and frequency of the Gag A146X variant in the Japanese cohort. (A) Five CTL clones established by GI8 were stimulated with C1R-B*4801 cells pre-pulsed with GI8, GI8-7P, LI10 or LI10-9P peptides at a concentration of 100 nM, and then IFN-γ production was measured by performing the ICC assay. The frequency of IFN-γ-producing $CD8^+$ cells is shown. (B) Ability of GI8-specific CTLs to recognize A146P mutant virus-infected cells. .221-CD4-B*4801 cells were infected with wild-type (NL432) or mutant (NL432-A146P) viruses. The infectivity of these viruses was 39.2% (NL432) and 47.2% (NL432-A146P). Five GI8-specific CTL clones were stimulated with these cells, and then IFN-γ production was measured by using ICC assay. The frequency of IFN-γ-producing $CD8^+$ cells is shown. (C) The frequency of the A146X variant in 24 HLA-B*48:01^+ and in 237 HLA-B*48:01^- chronically HIV-1-infected individuals is shown. HLA-B*57^+ was not found in these 261 subjects. The frequency of the amino-acid X: $P = 100\%$ in HLA-B*48:01^+ subjects, $P = 94\%$, $S = 2\%$, $T = 2\%$, $G = 1\%$, and $N = 1\%$ in HLA-B*48:01^- ones.

In the present study, we demonstrated that A146P mutation was selected by GI8-specific T cells rather than LI10-specific CTLs. In addition, we confirmed the selection of this mutation in HLA-B*48:01^{+} individuals by analyzing a large number of chronically HIV-1-infected Japanese. The present study shows that the same mutation was selected by different HLA alleles and by different mechanisms.

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Selection of escape mutant by HLA-C-restricted HIV-1 Pol-specific cytotoxic T lymphocytes carrying strong ability to suppress HIV-1 replication

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HIV-1 mutants escaping from HLA-A- or HLA-B-restricted CTL have been well studied, but those from HLA-C-restricted CTL have not. Therefore we investigated the ability of HLA-C-restricted CTL to select HIV-1 escape mutants. In the present study, we identified two novel HLA-Cw*1202-restricted Pol-specific CTL epitopes (Pol328-9 and Pol463-10). CTL specific for these epitopes were detected in 25–40% of chronically HIV-1-infected HLA-Cw*1202$^+$ individuals and had strong abilities to kill HIV-1-infected cells and to suppress HIV-1 replication *in vitro*, suggesting that these CTL may have the ability to effectively control HIV-1 in some HLA-Cw*1202$^+$ individuals. Sequence analysis of these epitopes showed that a V-to-A substitution at the 9th position (V9A) of Pol 463-10 was significantly associated with the HLA-Cw*1202 allele and that the V9A mutant was slowly selected in the HLA-Cw*1202$^+$ individuals. Pol 463-10-specific CTL failed both to kill the V9A virus-infected cells and to suppress replication of the V9A mutant. These results indicate that the V9A mutation was selected as an escape mutant by the Pol463-10-specific CTL. The present study strongly suggests that some HLA-C-restricted CTL have a strong ability to suppress HIV-1 replication so that they can select HIV escape mutants as in the case of HLA-A-restricted or HLA-B-restricted CTL.

Key words: CTL · Escape mutation · Fitness · HLA-C · HIV infection

Supporting Information available online

Introduction

CTL are involved in the control of HIV-1 replication during acute and chronic phases of HIV-1 infections [1–8]. However, CTL cannot completely eradicate HIV-1 because HIV-1 escapes from the cell-mediated immune system of the host by various mechanisms [9–17]. One such mechanism is the appearance of a single amino acid mutation within CTL epitopes, which is crucial for preventing their binding to HLA class I molecules or for the interaction between the TCR of the HIV-1-specific CTL and the peptide-HLA class I complex. The escape mechanisms result in the loss of CTL activities against HIV-1-infected target cells and

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contribute to the selection of viruses capable of escaping from HIV-1-specific CTL [4, 9–11, 18, 19]. Many studies demonstrated that the immune pressure mediated by HIV-1-specific CTL selects escape variants during both acute and chronic HIV-1 infections and that the selection of escape mutants could result in the loss of immune control, leading to progression to AIDS [9, 20–25].

The majority of previous studies concerning HIV-1-specific CTL focused on HLA-A- or HLA-B-restricted ones. However, the role of HLA-C-restricted CTL in HIV infections has not been well documented. It is speculated that HLA-C-restricted CTL do not contribute to the control of HIV-1 replication, because the expression level of HLA-C molecules is approximately 10% of that of HLA-A or -B molecules [26, 27]. In contrast, HIV-1 Nef-mediated HLA class I down-regulation affects HLA-A- or HLA-B-restricted CTL recognition but not the HLA-C-restricted one [28], suggesting a role for the HLA-C allele in HIV-1 infections. In addition, a whole-genome association study indicated that a variant located 35 kb upstream of the HLA-C gene (rs9264942) is associated not only with HLA-C mRNA expression but also with HIV viral load (VL) and AIDS progression [29, 30]. These studies suggest that the HLA-C-restricted immune responses play an important role in the control of HIV-1.

There are very few studies on HIV-1-specific HLA-C-restricted T cells. A previous study using HIV-1-specific HLA-C-restricted CTL clones demonstrated that HLA-HLA-C*03, 07, 15-restricted HIV-1-specific CTL clones effectively suppress HIV-1 replication *in vitro* [28]. A recent study revealed that HLA-C*04-restricted CTL have functional and phenotypic characteristics similar to those of HLA-A or B-restricted CTL [31]. Previous population analyses showed the association of some HLA-C alleles with the substitutions of HIV [32, 33]. Although these studies suggest the possibility that HLA-C-restricted CTL can select HIV-1 escape mutants, they did not directly show that HLA-C-restricted CTL actually do so.

In the present study, we investigated whether HLA-C-restricted CTL could select escape mutants. We focused on HLA-Cw*1202-restricted CTL because this allele, which forms a haplotype with HLA-A*2402 and HLA-B*5201, is frequently detected in Japan. To clarify the role of HLA-Cw*1202-restricted CTL in the selection of escape mutants, we first identified HLA-Cw*1202-restricted epitopes and then measured the ability of the HLA-Cw*1202-restricted CTL to suppress HIV-1 replication. Furthermore, we analysed mutations of HIV that had escaped from the CTL.

Results

Identification of 2 HLA-Cw*1202-restricted HIV-1 Pol-specific CTL epitopes

To identify HLA-Cw*1202-restricted CTL epitopes, we stimulated PBMC from chronically HIV-1-infected donor KI-069 (HLA-A*2402/-, B*5201/4006, Cw*1202/0304) with peptide cocktails including eight 17-mer overlapping peptides from Gag and Pol regions of HIV-1 and cultured the cells for 12–14 days. After stimulation with autologous B-lymphoblastoid cell lines (B-LCL) prepulsed with the corresponding peptide cocktail, each bulk culture was assessed by performing the intracellular cytokine assay. Bulk cultures from KI-069 responded specifically to 1 Gag cocktail, 3 Pol cocktails, and 3 Nef cocktails (data not shown). Further analysis using a single peptide demonstrated that 2 Gag (Gag 17–13 and Gag 17–14) and 3 Pol (Pol 17–40, Pol 17–48, and Pol 17–78) induced specific $CD8^+$ T cells responses (data not shown). HLA restriction of these T-cell responses was subsequently determined using a panel of B-LCL sharing 1 HLA class allele with KI-069. The results showed that $CD8^+$ T-cell responses against Gag 17-13, Pol 17–48, and Pol 17–78 peptides were restricted by a haplotype of HLA-A*2402, HLA-B*5201, and HLA-Cw*1202 (data not shown). Further analysis using C1R transfectant cells expressing each HLA molecule showed that only responses of $CD8^+$ T cells specific for the Pol 17–78 peptide were restricted by HLA-Cw*1202 (Fig. 1A top). Next, we generated a panel of 11-mer peptides covering the 17-mer amino acid sequences of the Pol 17–78 peptide and then tested IFN-γ production of each bulk culture in response to C1R-HLA-Cw*1202 cells prepulsed with these 11-mer peptides. Only the Pol 11–232 peptide induced the specific responses (Fig. 1A middle). To determine minimum length of the epitope, we generated four truncated peptides, Pol 11-232(IV9), Pol 11-232(C9), Pol 11-232(N10), and Pol 11-232(C10). Pol11-232-induced $CD8^+$ T cells recognised Pol 11-232(C10) but neither the IV9 nor the C9 (Fig. 1A bottom), indicating Pol 11-232(C10) to be the optimal epitope.

On the other hand, the ELISPOT assay using 11-mer overlapping Nef, Gag, and Pol peptides for KI-108 carrying HLA-A*2402/A*2402, B*5201/B*5201, and Cw*1202/Cw*1202 showed that 3 Pol peptide cocktails (Pol11-G17, Pol11-G27, and Pol11-G47) induced specific CD8 T-cell responses from this patient (data not shown). Subsequent analysis using single 11-mer peptides demonstrated that Pol11-164, Pol11-263, and Pol11-463 peptide-specific $CD8^+$ T cells were included among the PBMC cultured with Pol11-G17, Pol11-G27, and Pol11-G47, respectively (data not shown). In order to determine HLA class I restriction molecules of these peptide-specific T-cell responses, we employed C1R transfectants expressing each HLA molecule as stimulator cells and found that only Pol11-164-specific T-cell response was restricted by HLA-Cw*1202 (Fig. 1B top). To identify the optimal epitope recognised by $CD8^+$ T cells specific for Pol11-164, we synthesised a set of truncated peptides, Pol 11-164(RY10), Pol 11-164(RI9), Pol 11-164(KY9), Pol 11-164(QY8), and Pol11-164(KQ10) and tested which peptide the bulk cultured cells recognise. Pol 11-164(RY10), Pol 11-164(KY9), and Pol11-164(KQ10) peptides induced high IFN-γ responses of $CD8^+$ T cells in the culture (Fig. 1B middle). In addition, the analysis of peptide titration showed that the Pol11-164(KY9) peptide induced stronger IFN-γ responses of the $CD8^+$

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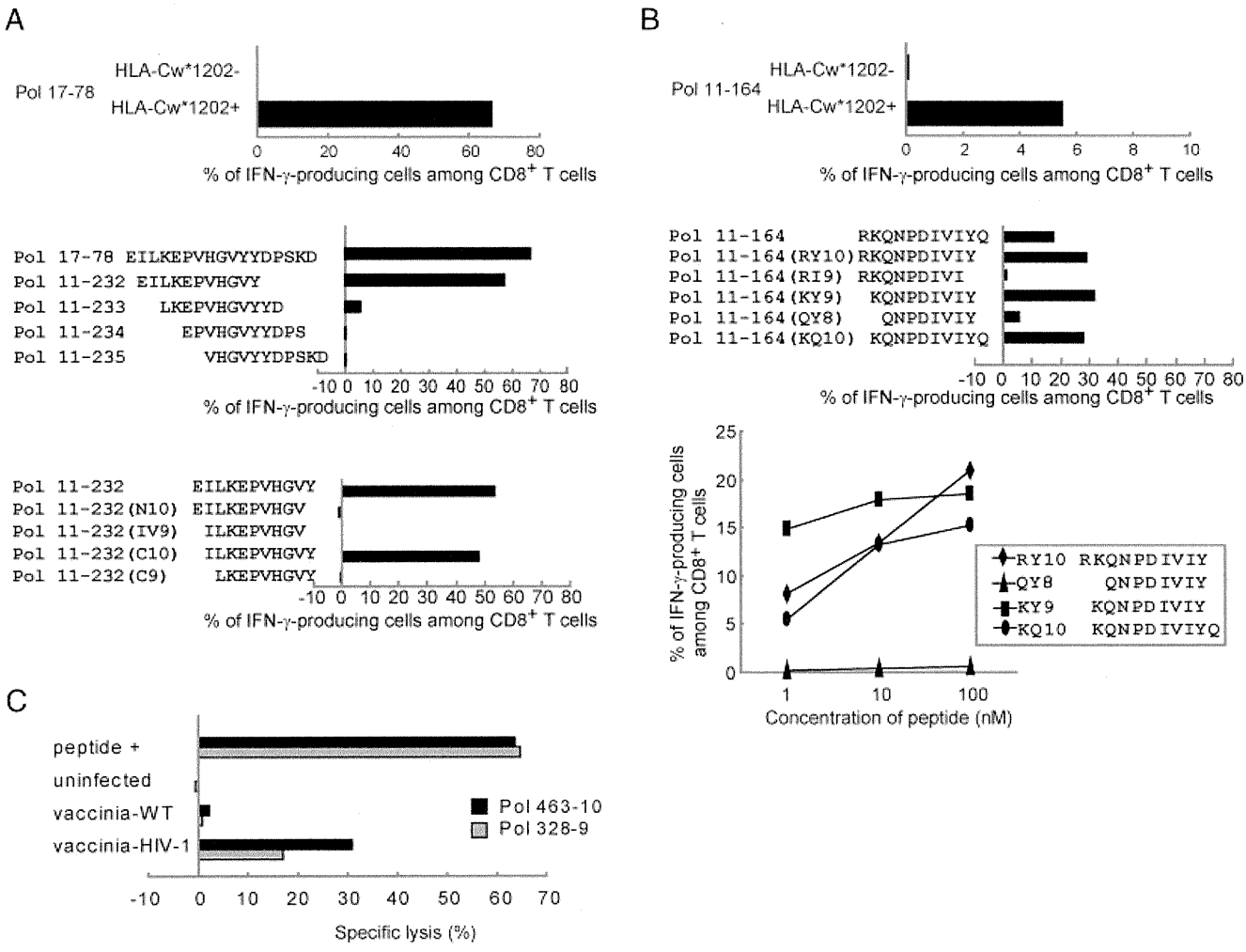

Figure 1. Identification of two HIV-1 Pol-specific epitopes using overlapping peptides. Candidates of HLA-Cw*1202-restricted HIV-1 CTL epitopes were identified using overlapping 17-mer or 11-mer HIV-1 peptides. PBMC from HLA-Cw*1202$^+$ HIV-1-seropositive individuals (KI-069 and KI-108) were stimulated with the 17-mer peptide cocktails and the 11-mer peptide cocktails, respectively, and then cultured for 12–14 days. (A) Top: A candidates of 17-mer Pol epitope peptide. The cultured PBMC cells form KI-069 were stimulated with the corresponding peptide-pulsed C1R cells expressing HLA-Cw*1202 (Cw*1202$^+$) or C1R cells (Cw*1202$^-$). Middle: Identification of 11-mer HIV-1 Pol peptides including HLA-Cw*1202-restricted epitope. The 17-mer cocktail peptide-specific bulk CD8$^+$ T cells were stimulated with C1R-Cw*1202 cells prepulsed with each of four overlapping 11-mer peptides. Bottom: Recognition of the 9-mer and 10-mer truncated peptides by the 11-mer-specific CD8$^+$ T cells. The 11-mer cocktail peptide-specific bulk CD8$^+$ T cells were stimulated with C1R-Cw*1202 prepulsed with each 8- to 10-mer truncated peptide. Peptide-specific CD8$^+$ T cells were detected using the intracellular IFN-γ staining assay. The percentages of IFN-γ-producing cells among CD8$^+$ T cells are shown at each figure. Each bar presents the data from one bulk T cells in a single experiment. (B) Top: A candidates of 11-mer Pol epitope peptides. The cultured PBMC cells from KI-108 were stimulated with the corresponding peptide-pulsed C1R cells expressing HLA-Cw*1202 (Cw*1202$^+$) or C1R cells (Cw*1202$^-$). Middle and Bottom: Pol11-164-specific bulk CTL were co-cultured with C1R-Cw*1202 prepulsed with each truncated peptide at concentrations of 100 nM (middle) or from 1 to 100 nM (bottom). The responsiveness of the bulk CD8$^+$ T cells toward each truncated peptide was measured by conducting the intracellular IFN-γ staining assay. The percentages of IFN-γ-producing cells among CD8$^+$ T cells are shown at each figure. Each bar or graph presents the data from one bulk T cells in a single experiment. (C) Presentation of two Pol epitopes by HLA-Cw*1202 on C1R-Cw*1202 cells infected with HIV-1 recombinant-HIV-1 vaccinia virus. The CTL activity of Pol 328-9-specific and Pol 463-10-specific bulk T cells against C1R-Cw*1202 cells prepulsed with a 1 μM concentration of the epitope peptide (peptide+) or infected with recombinant vaccinia virus expressing the corresponding HIV-1 Gag/Pol proteins (vaccinia-HIV-1) or WT vaccinia virus (vaccinia WT) was tested at an E:T ratio of 2:1. Each bar presents the data from one bulk T cells in a single experiment.

T cells than Pol the 11-164(RY10) or Pol 11-164(KQ10) one (Fig. 1B bottom). These results indicate that Pol 11-164(KY9) is the optimal epitope.

To clarify whether these two peptides were endogenously processed and presented by HLA-Cw*1202, we generated CTL clones specific for Pol 11-164(KY9) [9-mer peptide starting from position 328: Pol 328-9] or Pol 11-232(C10) [10-mer peptide starting from position 463: Pol 463-10], and then investigated whether these CTL clones could kill C1R-HLA-Cw*1202 cells infected with recombinant vaccinia virus expressing the HIV-1 Gag/Pol protein. These CTL clones effectively killed C1R-Cw*1202 infected with the recombinant vaccinia virus, but not those cells infected with WT vaccinia or uninfected cells (Fig. 1C), indicating that Pol 328-9 and Pol 463-10 are naturally processed CTL epitopes.

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Frequency of HLA-Cw*1202-restricted HIV-1-specific CD8$^+$ T cells in chronically HIV-1-infected individuals

Next we investigated the frequency of the two Pol-specific HLA-Cw*1202-restricted CTL in chronically HIV-1-infected individuals carrying HLA-Cw*1202 in order to clarify whether they were immunodominant epitopes. We detected Pol 328-9-specific and Pol 463-10-specific T cells *ex vivo* among CD8$^+$ T cells from the HLA-Cw*1202$^+$ individuals by performing the ELISPOT assay (Fig. 2). Ten of 25 individuals tested showed positive responses for the Pol328-9 epitope. Since Pol 463-9 (ILKEPVHGV) is reported to be an HLA-A*02 epitope [34], we selected HLA-Cw*1202$^+$ individuals who did not have HLA-A*02 for Pol 463-10-specific T cells. Four of 15 individuals carrying HLA-Cw*1202 but not HLA-A*02 showed positive responses for Pol463-10. These indicate that the specific T cells were frequently elicited in chronically HIV-1-infected individuals carrying HLA-Cw*1202.

Strong abilities of HLA-Cw*1202-restricted CTL clones to suppress HIV-1 replication

To investigate the ability of HLA-C*1202-restricted HIV-1-specific CTL to suppress HIV-1 replication, we measured the ability of CTL clones specific for Pol 328-9 or Pol 463-10 to suppress HIV-1 replication in primary CD4$^+$ T cells infected with HIV-1 NL432. Three Pol 328-9-specific HLA-Cw*1202-restricted and three Pol 463-10-specific HLA-Cw*1202-restricted CTL clones completely suppressed HIV-1 replication at an E:T ratio of 1:1 (Fig. 3). Our previous studies showed that approximately 70% of HLA-A-restricted or HLA-B-restricted CTL clones weakly suppress HIV-1 replication (less than 50% suppression at E:T ratio of 1:1), whereas others such as HLA-B*5101-restricted Pol 283-specific and HLA-A*2402-restricted Nef 138-specific ones strongly suppress it (Supporting Information Table 1) [12, 35–37]. These results show that the two HLA-Cw*1202-restricted CTL had a strong ability to suppress HIV-1 replication *in vitro*.

Pol 463-10-9A is a mutant that escaped from Pol 463-10-specific CTL

To clarify whether Pol 328-9-specific or Pol 463-10-specific CTL select escape mutants at the population level, we analysed the sequences of these epitopes and their flanking regions in viruses from HLA-Cw*1202$^+$ and HLA-Cw*1202 HIV-1 infected-donors. Analysis of 16 HLA-Cw*1202$^+$ and 66 HLA-Cw*1202$^-$ individuals showed that several mutations were found in the Pol 328-9 epitope region (data not shown), but these mutations were not significantly associated with HLA- Cw*1202 ($p > 0.05$). We also analysed the sequence of Pol 463-10 from 33 HLA-Cw*1202$^+$ and 108 HLA-Cw*1202$^-$ HIV-1 infected-donors. Several mutations were found at positions 3 and 9 (Fig. 4A). The frequency of the 9A mutation was significantly higher in the HLA-Cw*1202$^+$ donors than in the HLA-Cw*1202$^-$ ones ($p = 0.001$, Fig. 4A), suggesting that the 9A was a mutant that escaped from the Pol 463-10-specific CTL. Since Pol 463-9 (ILKEPVHGV) is known to be an HLA-A*02 epitope [34], the 9A may be selected by Pol 463-9-specific HLA-A*02-restricted CTL. To clarify this possibility, we analysed the sequences at this position from 55 HLA-A*02$^+$ and 88 HLA-A*02$^-$ HIV-1-infected donors. Frequencies of HLA-A*02$^+$ and HLA-A*02$^-$ individuals having the 9A are 10.9 and 19.8%, respectively, indicating that HLA-A*02 is not significantly associated with the 9A mutation. Indeed, the 9A mutation has not been reported as escape mutant from Pol 463-9-specific HLA-A*02-restricted CTL. Further analysis of 26 HLA-Cw*1202$^+$ HLA-A*02$^-$ and 60 HLA-Cw*1202$^-$ HLA-A*02$^-$ HIV-1-infected-donors showed that the frequency of the 9A mutation was significantly higher in the HLA-Cw*1202$^+$ HLA-A*02$^-$ donors

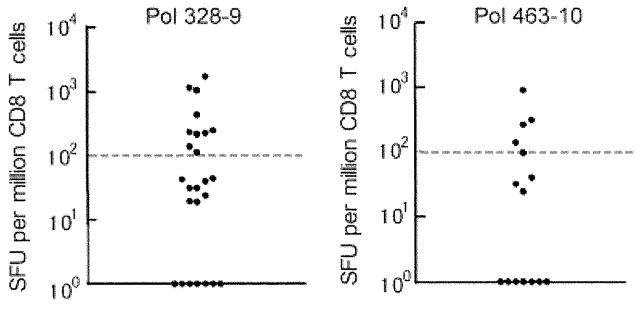

Figure 2. Frequency of HLA-Cw*1202-restricted Pol epitopes-specific CD8$^+$ T cells in chronically HIV-1-infected individuals. The frequencies of Pol 328-9-specific and Pol 463-10-specific CD8$^+$ T lymphocytes in chronically HIV-1-infected HLA-Cw*1202$^+$ individuals were measured using IFN-γ ELISPOT. Ten of 25 individuals tested showed positive responses for Pol328-9 epitope (mean SFU = 234), whereas four of 15 individuals who did not have HLA-A2 tested showed positive responses for Pol463-10 (mean SFU = 121). The subjects revealing a response of less than 100 SFU were evaluated as non-responders. SFU: spot-forming unit. Each dot represents one individual.

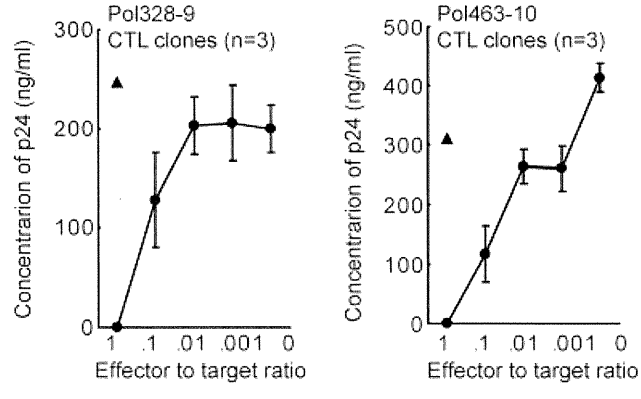

Figure 3. Strong abilities of two HIV-1 Pol-specific CTL to suppress HIV-1 replication. CD4$^+$ T cells from an HLA-Cw*1202$^+$ donor were infected with NL-432, and then co-cultured with the Pol-specific CTL clones ($n = 3$) at E:T ratios of 1:1, 0.1:1, 0.01:1, and 0.001:1 (circles). As a negative control, HLA-A*1101-restricted Pol675-specific CTL clone ($n = 1$) was used at an E:T ratio of 1:1 (triangle). HIV-1 p24 Ag in the supernatant were measured on day 7 after infection by an enzyme immunoassay. The data shown are the means and SD of assays for three HIV-1-specific CTL clones. They were from one out of two independent experiments.

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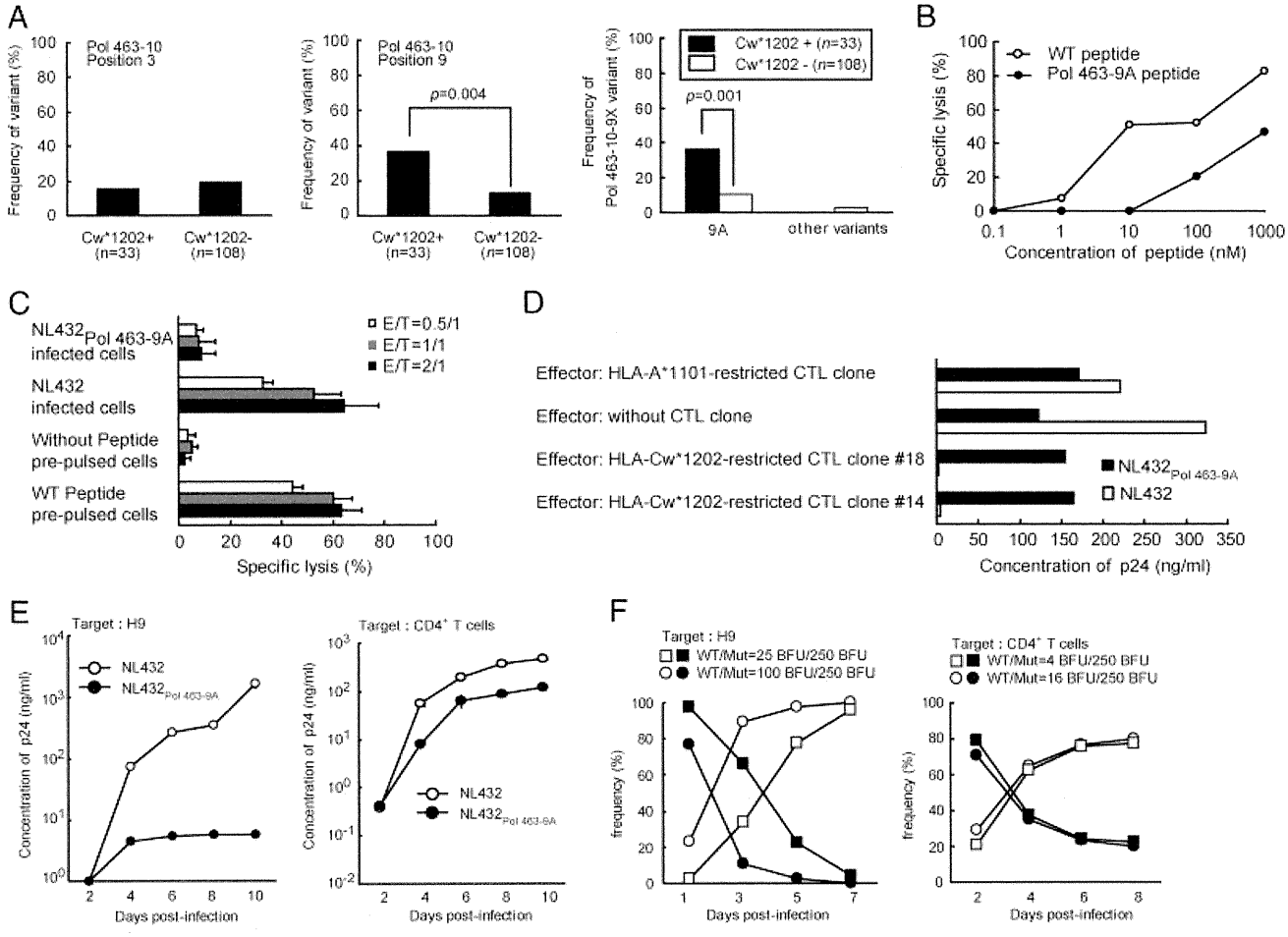

Figure 4. Characterization of escape Pol 463-9A escape mutation. (A) Frequency of mutations in Pol 463-10 epitope among chronically HIV-1-infected HLA-Cw*1202$^+$ and HLA-Cw*1202$^-$ HIV-1-infected individuals. The sequence of Pol 463-10 epitope was analysed in a single sample from both HLA-Cw*1202-positive or HLA-Cw*1202-negative individuals chronically infected with HIV-1. Thirty-three HLA-Cw*1202-positive or 108 HLA-Cw*1202-negative individuals were analysed. The consensus sequence of the Pol 463-10 epitope in clade B is ILKEPVHGVY. The frequency of mutations at positions 3 and 9 of the epitope are shown for both HLA-Cw*1202-positive and HLA-Cw*1202-negative donors. Frequency of Ala mutation at position 9 was significantly higher in HLA-Cw*1202-positive donors than in HLA-Cw*1202-negative donors. The p values were determined by Fisher's exact test. (B) Cytotoxic activities of Pol463-10-specific CTL clone toward C1R-HLA-Cw*1202 cells pulsed with Pol 463-10 or Pol 463-10-9A peptide. C1R-Cw*1202 cells were prepulsed with various concentrations of Pol 463-10 or Pol 463-10-9A peptide. Cytotoxic activity of a Pol463-10-specific CTL clone was measured at an E:T ratio of 2:1. The results were from a single T cell clone in one of two independent experiments. (C) Cytotoxic activity of Pol 463-10-specific CTL clones against 721.221-CD4-HLA-Cw*1202 cells infected with the 9A mutant virus; 721.221-CD4- HLA-Cw*1202 cells were infected with NL-432 or NL-432 $_{\text{Pol 463-10-9A}}$ mutant virus. NL-432-infected or NL-432 $_{\text{Pol 463-10-9A}}$-infected 721.221-CD4-HLA-Cw*1202 were used as target cells at an E:T ratio of 2:1, 1:1, or 0.5:1. The data shown are the means and SD of assays for three HIV-1-specific CTL clones. They were from one of two independent experiments. (D) Ability of HIV-1-specific CTL clones to suppress HIV-1 replication in the 9A mutant virus-infected CD4$^+$ T cells. CD4$^+$ T cells from an HLA-Cw*1202$^+$ HLA-A*1101$^-$ healthy donor were infected with NL-432 or NL-432 $_{\text{Pol 463-10-9A}}$, and then co-cultured with the Pol 463-10-specific CTL clone (clone #14 or clone #18) or HLA-A*1101-restricted CTL clone at an E:T ratio of 2:1 or without the CTL clone. HIV-1 p24 Ag in the supernatant were measured on day 7 after infection by performing an enzyme immunoassay. The results were from one T-cell clone in one of two independent experiments. (E) Fitness of the 9A mutant virus. Production of p24 Ag in culture supernatant was determined by an enzyme immunoassay. Profiles of replication kinetics (p24 production) of NL-432 (closed circles), NL-432 $_{\text{Pol 463-10-9A}}$ (open circles) were determined with H9 cells and CD4$^+$ T cells. The data shown are the means and SD of triplicates in one of two independent experiments. (F) A competitive HIV-1 replication assay using the 9A mutant and WT virus. To compare the replication kinetics of NL-432 (open symbols) and NL-432 $_{\text{Pol 463-10-9A}}$ (closed symbols), H9 cells (left), and CD4$^+$ T cells (right) were infected with both viruses at different viral titers. The frequency of each virus at day 1, 3, 5, and 7 (H9 cells) or at day 2, 4, 6, and 8 (CD4$^+$ T cells) was determined from the relative peak height on sequencing electrograms. The data were from one sample in a single experiment.

than in the HLA-Cw*1202$^-$HLA-A*02$^-$ ones (34.6 *versus* 14.4%, $p = 0.037$). These results together suggest that HLA-Cw*1202-restricted Pol 463-10-specific CTL selected the 9A mutant.

To clarify whether the 9A was indeed an escape mutant of Pol 463-10-specific CTL, we investigated the ability of Pol 463-10-specific CTL to recognise the Pol 463-10-9A mutant epitope. We first tested the activity of Pol 463-10-specific CTL clones to kill target cells prepulsed with the Pol 463-10-9A mutant peptide. Three Pol 463-10-specific CTL clones effectively killed target cells prepulsed with the Pol 463-10 WT peptide but showed reduced ability to kill those prepulsed with the Pol 463-10-9A mutant peptide (Fig. 4B), suggesting that the 9A mutant had escaped

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Table 1. Longitudinal analysis of Pol 463-10 epitope sequence in HIV-1-infected individuals

ID	HLA-Cw*1202	Sample date Month/day/year	Sequence ILKEPVHGVY
KI-037	Positive	01/29/2002	----------
		06/17/2004	--------A-
KI-163	Positive	08/30/2002	----------
		06/28/2004	----------
		08/29/2005	--------A-
		02/27/2006	--------A-
KI-428	Positive	03/12/2003	----------
		06/28/2006	----------
		09/27/2006	----------
		07/12/2007	--------A-
KI-452	Positive	12/30/1999	----------
		01/30/2007	--------A-
KI-097	Negative	04/25/2003	--------A-
		02/18/2005	--R-------
KI-091	Negative	09/10/2001	--------A-
		07/09/2003	--R-------
KI-161	Negative	07/25/2002	--E-----A-
		05/07/2004	----------
		09/29/2004	----------

from Pol 463-10-specific CTL. Therefore we generated the 9A mutant virus from NL432 (NL-432-$_{\text{Pol 463-10-9A}}$) to further analyse the ability of Pol 463-10-specific CTL to kill target cells infected with the 9A mutant virus. The Pol 463-10-specific CTL clones effectively killed the target cells infected with NL-432 whereas they failed to kill those infected with NL-432-$_{\text{Pol 463-10-9A}}$ (Fig. 4C). In addition, by performing a replication suppression assay we analysed whether these CTL could suppress the replication of mutant virus and WT virus *in vitro*. Pol 463-10-specific CTL clones (clone #14 and #18) effectively suppressed the replication of the WT virus, whereas they failed to suppress that of the 9A mutant virus (Fig. 4D). These results indicate that the 9A is indeed escape mutant of Pol 463-10-specific CTL.

We performed longitudinal analysis of the Pol 463-10 epitope in 14 HLA-Cw*1202$^+$ individuals. Four HLA-Cw*1202$^+$ individuals showed the WT sequence of Pol 463-10 in the early phase and the Pol 463-10-9A mutant appeared more than 3 years later (Table 1), supporting that the 9A is escape mutant from Pol 463-10-specific CTL. KI-037 is a haemophiliac patient who had been infected with HIV-1 before 1985, indicating that the 9A mutant appeared more than 17 years after HIV-1 infection. Thus, this mutant may be slowly selected in HLA-Cw*1202$^+$ individuals.

Reversion of the 9A mutant

To examine the effect of the 9A mutation on viral fitness, we compared the replication ability of NL432 (WT) and the 9A mutant using the p24 production assay. The results using H9 cells and primary CD4$^+$ T cells as target cells showed that fitness cost of the 9A was much higher than that of WT (Fig. 4E). In addition, we performed a competitive HIV-1 replication assay for further comparison of replication kinetics in H9 cells and primary CD4$^+$ T cells. During 7 days culture, we observed that the 9A had higher fitness cost than WT in both cells (Fig. 4F). These results suggest that this mutant is able to revert to WT in HLA-Cw*1202$^-$ HIV-1-infected individuals. To clarify the reversion, we performed a longitudinal analysis of this epitope sequence on five HLA-Cw*1202$^-$ individuals who could be followed from early stage of the infection and had the 9A mutation at the early stage. Three of these five HLA-Cw*1202$^-$ individuals showed the reversion within approximately 2 years after the 9A had been found (Table 1). These results support the finding that the 9A mutant did not remarkably accumulate in the HLA-Cw*1202$^-$ individuals.

Discussion

HLA-C molecules are believed to play a less important role in the presentation of various Ag than HLA-A and -B ones, because the former molecules are expressed on the cell surface at a level that is approximately 10% of that of the latter molecules [26, 27]. On the other hand, HLA-A and -B molecules are down-regulated in HIV-1-infected cell mostly due to the effect of Nef whereas HLA-C molecules are not, implying that HLA-C-restricted HIV-specific CTL can be elicited and have some role in the control of HIV-1. A previous study demonstrated that HLA-C-restricted responses are elicited in an African cohort infected with HIV-1 clade C, although it showed that HLA-B-restricted T-cell responses are much stronger than those of HLA-C-restricted or HLA-A-restricted ones [38]. A study using HLA-C-restricted HIV-1-specific CTL clones previously demonstrated that the ability of HLA-C-restricted CTL to suppress HIV-1 replication *in vitro* is similar

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