学位論文

Doctoral Thesis

Role of $CD8^+$ T cells specific for escape mutant in suppression of HIV-1 replication and co-evolution with HIV-1

（HIV-1 増殖抑制と HIV-1 との共進化に及ぼす逃避変異特異的 $CD8^+$ T 細胞の役割）

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1. ABSTRACT

[Background] The accumulation of HIV-1 escape mutations affects HIV-1 control by HIV-1-specific T cells. Some of these mutations can elicit escape mutant-specific T-cells, but it still remains unclear whether they can suppress the replication of HIV-1 mutant viruses. HLA-B*52:01-restricted RI8 (Gag 275-282) is a protective T cell epitope in HIV-1 subtype B-infected Japanese individuals, but 3 Gag280A/S/V mutations were found in 26% of them. We investigated the co-evolution of HIV-1 with RI8-specific T-cells and suppression of HIV-1 replication by its escape mutant-specific T-cells in vitro and in vivo.

[Methods] We analyzed the elicitation and function of T-cells specific for Gag280 mutant epitopes by performing ELISPOT assays, ICS assays, and viral suppression assays. In addition, the ability of these T cells to suppress HIV-1 replication in vivo was evaluated by comparing clinical parameters between HLA-B*52:01+ responders and non-responders to RI8 or RI8-6V epitopes.

[Results] The HLA-B*52:01+ individuals infected with Gag280A/S mutant viruses failed to elicit these mutant epitope-specific T-cells, whereas those with the Gag280V mutant one effectively elicited RI8-6V mutant-specific T-cells. These RI8-6V-specific T cells suppressed the replication of Gag280V virus and selected wild-type virus, suggesting a mechanism affording no accumulation of the Gag280V mutation in the HLA-B*52:01+ individuals. The responders to wild-type (RI8-6T) and RI8-6V mutant peptides had significantly higher CD4 counts than non-responders, indicating that the existence of not only RI8-6T-specific T cells but also RI8-6V-specific ones was associated with a good clinical outcome.

[Conclusion] Gag280V virus, which were selected by HLA-B*52:01-restricted CD8+ T-cells specific for GagRI8 protective epitope, frequently elicited GagRI8-6V mutant-specific CD8+ T-cells having a strong ability to suppress the replication of the Gag280V mutant virus both in vitro

and in vivo. We thus demonstrated the role of escape mutant-specific T cells in the control of HIV-1 and co-evolution with HIV-1.

2. PUBLICATION LIST

I Reference Publications

1. Yu Zhang, Nozomi Kuse, Tomohiro Akahoshi, Takayuki Chikata, Hiroyuki Gatanaga, Shinichi Oka, Hayato Murakoshi, and Masafumi Takiguchi. (2020). Role of escape mutant-specific T cells in suppression of HIV-1 replication and co-evolution with HIV-1 (J Virol, in press)
2. Hayato Murakoshi, Nozomi Kuse, Tomohiro Akahoshi, Yu Zhang, Takayuki Chikata, Mohamed Ali Borghan, Hiroyuki Gatanaga, Shinichi Oka, Keiko Sakai, and Masafumi Takiguchi. Broad recognition of circulating HIV-1 by HIV-1-specific CTLs with strong ability to suppress HIV-1 replication. J Virol. 93: e01480-18, 2019.
3. Yosuke Maeda, Taichiro Takemura, Takayuki Chikata, Takeo Kuwata, Hiromi Terasawa, Riito Fujimoto,Nozomi Kuse, Tomohiro Akahoshi, Hayato Murakoshi, Giang Van Tran, Yu Zhang, Chau Ha Pham, Anh Hong Quynh Pham, Kazuaki Monde, Tomohiro Sawa, Shuzo Matsushita, Trung Vu Nguyen, Kinh Van Nguyen, Futoshi Hasebe, Tetsu Yamashiro, Masafumi Takiguchi. Existence of Replication-Competent Minor Variants with Different Coreceptor Usage in Plasma from HIV-1-Infected Individuals. J Virol. 94:e00193-20, 2020.

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4. ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
CTL	Cytotoxic T lymphocyte
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	IFN-γ Enzyme-Linked ImmunoSpot
ICS	Intracellular cytokine staining
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
APC	Allophycocyanin
IFN-γ	Anti-interferon γ
PBMC	Peripheral blood mononuclear cell
PE	Phycoerythrin
pVL	Plasma viral load
FITC	Fluorescein isothiocyanate
APC	Antigen presenting cell
DC	Dendritic cell
NK	Natural killer cell
TCR	T-cell receptor
nAbs	Neutralizing antibodies
WT	Wild-type

MT	Mutant-type

5. BACKGROUND

5.1 The natural course of HIV infection

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus that infects primarily $CD4^+$ T cells as well as dendritic cells and macrophages. Infection with HIV-1 results in progressive loss of $CD4^+$ T-lymphocytes, leading to Acquired Immune Deficiency Syndrome (AIDS) characterized by a susceptibility to infection with opportunistic pathogens or by the occurrence of an aggressive form of Kaposi's sarcoma or B-cell lymphoma (1-2). During the natural course of untreated infection with HIV, an abundance of virus in the peripheral blood and a marked decrease on the numbers of circulation CD4 T cells were the characteristics in the acute phase (3).

The acute viremia is associated in virtually all patients with the activation of $CD8^+$ T cells and the elicitation of HIV-specific $CD8^+$ cytotoxic T lymphocyte (CTL), which is important in reducing the plasma viral load (pVL) and increasing the $CD4^+$ T cells. In the chronic phase, profound $CD4^+$ T cell depletion, escape mutant from HIV-specific CTL and immune exhaustion leading to disease progression and AIDS (Figure A) (4).

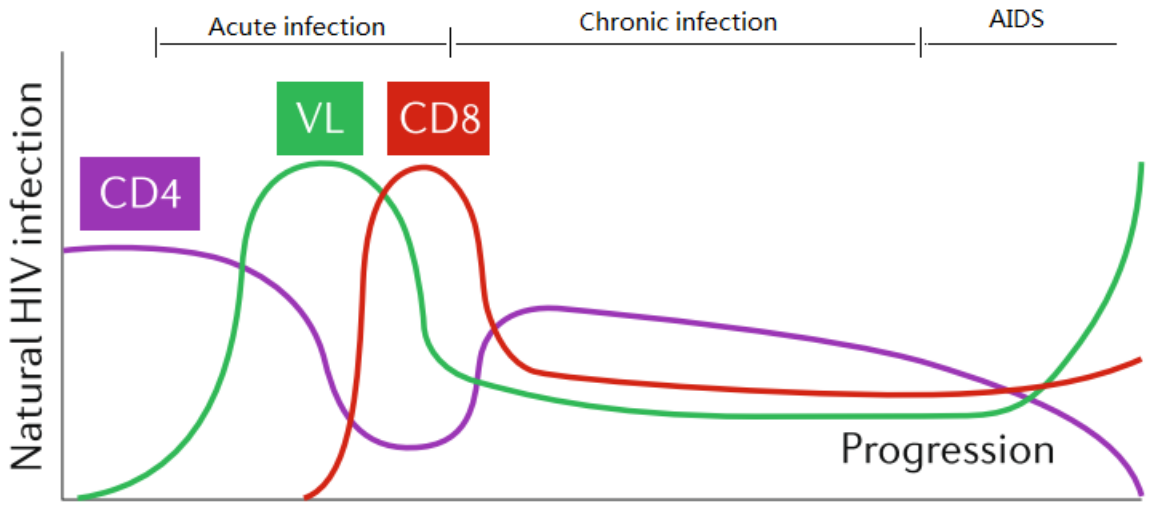


Figure A. A typical T cell immune response to untreated HIV infection. After initial exposure, viral load increase rapidly in the early acute phase. $CD4^+$ T cells decline with the increase in viral load. HIV-specific $CD8^+$ cytotoxic T cell responses reduce the viral load and an increase in $CD4^+$ T cells. $CD4^+$ T cells decline slowly and viral load remains stable during chronic infection. Incomplete $CD8^+$ T cell-mediated control of pVL eventually leading to opportunistic infection and AIDS (4).

5.2 Immune activation and T cell immune response to HIV infection

During HIV infection, HIV replication leads to activation of the innate and adaptive immune systems (5). Human leukocyte antigen (HLA) Class II- restricted T cell activation leads to the release of interleukins, the activation of B cells, and antibody production (6-7). On the other hand, the antigen-presenting cells (APC) present viral epitopes together with class I HLA molecules, thereby activation the cellular immune response (6-7). Beside the destruction of $CD4^+$ T cells, persistent immune activation is another hallmark of HIV infection and plays an important role in the pathogenesis of HIV disease (8).

Macrophages, monocytes and dendritic cells are the main professional APCs of the immune system, while dendritic cells (DC) are the most potent inducer of specific immune responses. T cells express different T cell receptors (TCR) that may bind to the peptide-HLA class I complexes on the surface of dendritic cells to allow activation of $CD8^+$ T cells, or to the HLA Class II molecules, to activate CD4 T cells. The ability of DC to activate T cells also depends on the secretion of stimulatory cytokines such as Type 1 IFNs, which is a key cytokine for the activation of natural killer (NK) cells. In acute HIV infection, peripheral blood NK cells become activated and are capable of mediating potent effector and immunoregulatory functions (9-10). The killer immunoglobulin-like receptors (KIRs) influence NK cell activation which is governed by the

integration of activating and inhibitory signals. Population−level genetic studies in HIV-1 infected individuals have demonstrated that HLA/KIR combinations are associated with HIV-1 clinical outcomes and disease progression, implying that NK cell might contribute markedly to the control of HIV-1 infections (11-13). Later studies showed that the presence of KIR3DL1 and its HLA-Bw4-80I ligand confers stronger killing of HIV-1-infected cells, suggesting that NK cells contribute to containment of viral replication in HIV-infected individuals (14).

In the case of adaptive immunity, $CD4^+$ T cells are the major target for HIV and massive depletion of $CD4^+$ T cells occurs during acute infection in adults (15-16). It is known that activated $CD4^+$ T cells could provide help to primary and long-lived memory CD8 T-cell responses (10, 17). In addition, HIV-specific $CD4^+$ T cells have intrinsic direct cytotoxic activity against infected cells which has previously been associated with viral control in chronically infected patients and predict disease outcome in acute HIV infection (18-20). HIV-specific $CD8^+$ T cells play a key role in control of viral replication based on studies in humans (21-22) and SIV infected monkey models (23-24). The stimulation of $CD8^+$ T lymphocytes and the formation of HIV-specific cytotoxic T cells (CTL), which is thought to be important in controlling virus levels. $CD8^+$ T cells recognize structurally constrained HIV-specific peptide presented via HLA class I molecule on the surface

of infected cells via TCR, inducing proliferation and perforin/granzyme-mediated cytolysis of infected cells (Figure B).

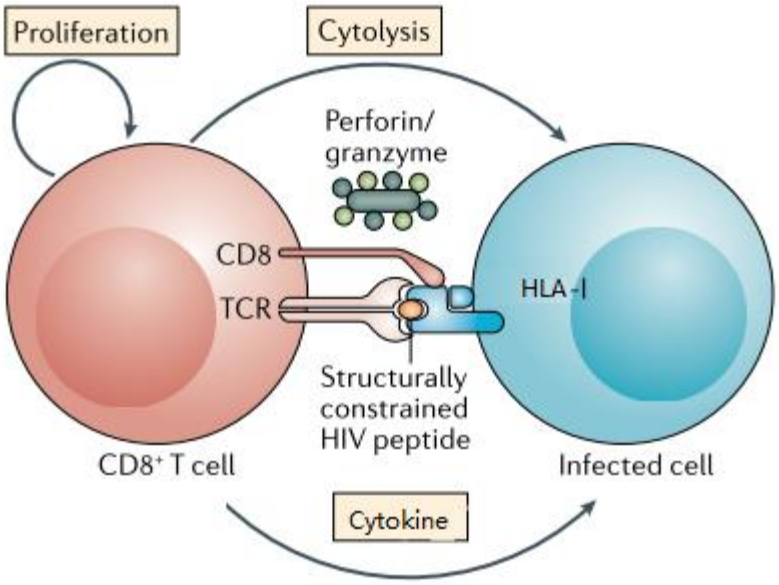


Figure B. $CD8^+$ T cell interactions with infected cells. Following $CD8^+$ T cell recognition of the infected cell, the $CD8^+$ T cell effector mechanisms are triggered. $CD8^+$ effector mechanisms can be killing of the infected cell and/or secretion of cytokines such as IFN-γ and TNF-α which reduce the probability of cell infection and viral production (4).

5.3 $CD8^+$ T cells in HIV control and cure

Numerous studies have emphasized that HIV-1-specific $CD8^+$ T-cells play an important role in the control of HIV-1 during the acute and chronic phases of an HIV-1 infection (4, 21-22, 25-30). A strong expression of T-bet and effector molecules such as perforin and granzyme B were shown to correlate with antiviral efficacy (31). A recent study demonstrated that the induction of $CD8^+$ T cells displaying high levels of T-bet and perforin during the early days following an HIV infection showed a direct benefit on HIV reservoir seeding in vivo (32), suggesting that HIV-1-specific CTLs with high function can be expected to prevent HIV-1 infection and to eradicate the HIV-1 reservoir. Although the majority of current vaccine strategies focus on the induction of neutralizing antibodies, there is substantial evidence that cellular immunity mediated by $CD8^+$ T cells can

sustain long- term disease- free and transmission- free HIV control and may be harnessed to induce both therapeutic and preventive antiviral effects (4). A recent study in HSIV infected animal models showed that, the immunization with HVVs, that inducing high levels of $CD8^+$ and $CD4^+$ T cells, with nAbs-inducing ones could confer enhanced protection against infection with the homologous SHIV virus, suggesting that combining T-cell reducing and nAbs will be of interest to vaccinology (33).

The so-called "kick-and-kill" treatment, which combines latency-reversing agents with CTLs or NK cells, is proposed to eradicate latent HIV-1 reservoirs from ART-treated individuals (34-35). A previous study in a non-human primate model of simian immunodeficiency virus showed that mosaic vaccines in combination with an immune modulator TLR7 agonist improved virologic control and delayed viral rebound following discontinuation of antiretroviral therapy and that the breadth of cellular immune responses correlated inversely with set point viral loads and correlated directly with time to viral rebound (36), suggesting that effective cellular immunity is required in "kick-and-kill" treatment. A recent clinical trial study of a therapeutic vaccine in 26 ART-suppressed HIV-infected individuals who had started with ART during an acute infection demonstrated that the mosaic vaccine induced high levels of polyfunctional $CD4^+$ T cells and $CD8^+$ T cells, as well as Env-specific antibodies, but the effect of this vaccine to delay viral rebound following discontinuation of antiretroviral therapy was small as compared to that of placebo controls (37).

5.4 Co-evolution of HIV with $CD8^+$ T cells

However, HIV-1 can escape from the host immune system through Nef-mediated HLA class I down regulation, selection of escape mutations and skewed maturation of memory HIV-specific $CD8^+$ T lymphocytes, resulting in impaired activities of HIV-1-specific CTLs to kill target cells

infected with escape mutant virus as well as in the selection and accumulation of escape mutant (38-42). Amino acid mutations within the CTL epitope can reduce epitope-HLA binding, impair TCR recognition, and affect antigen presentation (38-39). Mutations in epitope flanking regions, which impair intracellular peptide processing and presentation, have also been described (43). In the past decade, quite a bit of studies have revealed that some of these mutations are HLA-adapted ones, indicating that HLA-restricted immune responses could driving and shaping HIV-1 evolution in vivo within an infected host an at the population level (44-46).

A well-documented case for the dynamic interaction between CTL response and HIV-1 evolution is investigated in HLA-B*27-restricted, HIV-1 Gag-derived KK10-specific CTLs. Highly effective wild-type (WT)-specific CTLs control HIV-1 replication at a relatively early phase of the infection (47), whereas an escape mutation at position 6 (L286M) in KK10 can evade this CTL recognition (48). Subsequently, cross-reactive CTLs that recognized both WT and L286M mutant can be induced and control the mutant virus. However, in some individuals, such cross-reactive CTL drive a selection of another mutation at position 2 (R264K) in KK10, leading to the abrogation of the binding of the KK10 epitope to the HLA-B27 molecule, loss of control of the mutant virus (49).

The existence of escape mutations in reservoir viruses and circulating viruses is a critical barrier for the eradication of latent HIV-1 reservoirs and prevention of HIV-1 infections. Previous studies showed that escape mutant viruses can elicit mutant-specific T cells in some cases (50-53). A recent study showed that the transmission of HLA-adapted mutations affects the clinical outcome in the acute phase of an HIV-1 infection (54). T cell responses to epitopes including HLA-adapted mutations are frequently detected in HIV-1 chronic infections (55), whereas they are rarely found in the acute infection (54). Although some HLA-adapted mutations are known to be escape ones,

it remains unknown whether T cells specific for epitopes having HLA-adapted or escape mutations can effectively suppress HIV-1 replication in chronic infections. Previous studies demonstrated that escape mutant-specific T cells fail to suppress replication of the mutant virus *in vitro* (51, 56-57).

5.5 HLA-B*52:01 and HLA-B*52:01-restricted CD8$^+$ T cells in Japanese cohort

The presence of HLA class I alleles or haplotypes have consistently been associated with the rate of progression to AIDS and/or with clinical markers of disease progression, such as pVL and CD4 T cell count (58-66). HLA-B*57 and HLA-B*27 are well-known to associate with successful control of HIV-1 or slow progression to disease in Caucasians and Africans (58, 59, 63, 65-68), whereas HLA-B*35, HLA-B*58:02, and HLA-A*29:01-B*07:05-C*15:05 are associated with a rapid progression (60, 64, 66, 69-72). However, HLA-B*27 and HLA-B*57 are very rare alleles in Japan and other Asian countries. Whole-genome association analyses showed that HLA-B*52:01 are the second strongest protective alleles in Caucasian and/or African individuals (65, 73). A previous study demonstrated that HLA-B*52:01-C*12:02 is a protective haplotype in Japan (63,74), which is found in more than 20% of Japanese and is an allele with relatively high frequency in East Asian countries, whereas it is detected in only 2-3 % of Caucasians and is very rare in Africa (75, 76). Therefore, HLA-B*52:01-restricted immune responses to HIV-1 play an important role in HIV-1 control in Japanese and East Asian individuals more than in other ethnic groups (28, 77).

Recent studies on HIV-1 subtype B-infected Japanese individuals demonstrated that HLA-B*52:01-restricted HIV-1-specific CD8$^+$ T-cells for 4 epitopes (GagMI8: Gag 198-205, GagWV8: Gag 316-323, GagRI8: Gag 275-282, and PolSI8: Pol 654-661) have the ability to suppress HIV-1 replication both *in vivo* and *in vitro* (28, 77).

5.6 HLA-B*52:01 RI8-specifc T cells and Gag280 mutations

Of four HLA-B*52:01-restricted epitopes, GagMI8, GagWV8, and PolSI8 are conserved ones among the subtype B viruses, whereas GagRI8 (Gag 275-282, RMYSPTSI) has 3 substitutions at Gag280 (Gag280S, Gag280A and Gag280V) in 26% of HIV-1 subtype B-infected Japanese individuals (Figure C) (28, 78). A previous study on HLA-associated HIV-1 polymorphisms in HIV-1 subtype B-infected Japanese individuals showed that Gag280S and Gag280A accumulate in HLA-B*52:01^{+} individuals whereas Gag280V do not (79), suggesting that Gag280S and Gag280A are escape mutations selected by HLA-B*52:01-restricted RI8-speciifc T-cells. However, it is unknown whether Gag280V is escape mutant or not and why RI8 is a protective epitope even though 26% of circulating viruses have these mutations.

HLA	Epitope	Sequence	Frequency in our cohort (Percentage)
B*52:01	Gag MI8	MQMLKETI	328 / 346 (94.8)
	Gag WV8	WMTETLLV	356 / 367 (97.0)
	Pol SI8	SQYALGII	306 / 330 (92.7)
	Gag RI8	RMYSPTSI	236 / 346 (68.2)
		-----V--	44 / 346 (12.7)
		-----S--	30 / 346 (8.7)
		-----A--	16 / 346 (4.6)
		-----I--	5 / 346 (1.4)

Figure C. Frequencies of amino acid sequences for the 4 epitope regions (78).

In the present study, we investigated the mechanisms for the selection and accumulation of escape mutations at Gag280 in HIV-1 subtype B-infected Japanese and for elicitation of escape mutant-specific T cells. Furthermore, we investigated the role of HLA-B*52:01-restricted T-cells specific for the RI8 epitope or its mutants in the clinical outcome of Japanese individuals.

6. Materials and Methods

6.1 Ethics statement

This study was approved by the ethics committees of Kumamoto University (RINRI-1340 and GENOME-342) and the National Center for Global Health and Medicine (NCGM-A-000172-01). Informed consent was obtained from all individuals according to the Declaration of Helsinki.

6.2 Study subjects

All treatment-naive Japanese individuals chronically infected with HIV-1 subtype B were recruited from the National Center for Global Health and Medicine, Japan. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood. HLA types of HIV-infected individuals were determined by standard sequence-based genotyping. The pVLs of the individuals at their first visit were measured by using the Cobas TaqMan HIV-1 real-time PCR version 2.0 assay (Roche Diagnostics, NJ, USA).

6.3 Cell Lines

C1R cells expressing HLA-B*52:01 (C1R-B*52:01), 721.221 cells expressing CD4 molecules and HLA-B*52:01 (721.221-B*52:01), and RMA-S cells expressing HLA-B*52:01 (RMA-S-B*52:01) were previously generated (80, 81). These cells were maintained in RPMI 1640 medium (Invitrogen) containing 5% fetal calf serum (FCS, R5) and 0.15 mg/ml of hygromycin B or 0.2 mg/ml neomycin.

6.4 HIV-1 mutant clones

NL4-3 mutants (NL4-3-Gag280V, -Gag280-6S, and -Gag280-6A) were previously generated (28).

6.5 Sequence of autologous virus

Determination of the epitope sequence for RI8 was performed as previously described (79). The RI8 sequence data from 390 chronically HIV-1 subtype B-infected treatment-naïve Japanese individuals were analyzed after excluding individuals having a mixture of amino acid sequence at Gag280 from previously analyzed ones (79) and adding new data from 16 individuals.

6.6 Intracellular cytokine staining (ICS) assay.

C1R and 721.221 cells prepulsed with peptide or 721.221 cells infected with the HIV-1, strain NL4-3, were added to the effector STCLs in a 96-well plate and incubated for 2 h at 37 °C. Brefeldin A (10 μg/ml) was then added and the cells were incubated further for 4 h. Cells were fixed with 4% paraformaldehyde and incubated in permeabilization buffer [0.1% saponin–10% FBS–phosphate-buffered saline (PBS)] after staining with allophycocyanin (APC)-labeled anti-CD8 monoclonal antibody (mAb) (Dako, Glostrup, Denmark). Thereafter, the cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-interferon γ (IFN-γ) mAb (BD Bioscience, CA). The percentage of IFN-γ-producing cells among the $CD8^+$ T-cell population was determined by flow cytometry.

6.7 IFN-γ Enzyme-Linked ImmunoSpot (ELISPOT) assay

1×10^5 PBMCs from HIV-1-positive individuals and peptides at a concentration of 100 nM or 1 μM were added to 96-well polyvinylidene plates (Millipore, Bedford, MA) that had been precoated with 5 μg/ml anti-IFN-γ mAb; 1-D1K (Mabtech, Stockholm, Sweden). The plates were then incubated for 16 h at 37 °C before the addition of biotinylated anti-IFN-γ mAb (Mabtech) at 1 μg/ml at room temperature for 90 min, streptavidin-conjugated alkaline phosphatase (Mabtech) for at room temperature 60 min. Individual cytokine-producing cells were visualized as dark spots

after a 20-min reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium in the presence of an alkaline phosphatase-conjugated substrate (Bio-Rad, Richmond, CA, USA). The spots were counted with an Eliphoto-Counter (Minerva Teck, Tokyo, Japan). The frequencies of the responding cells were represented as spot-forming units (SFU)/10^6 $CD8^+$ T cells by measuring frequency of $CD8^+$ T cells using a flow cytometry. A mean+5 SD of the SFUs of samples (N=3) from 12 HIV-1-naïve individuals for the peptide pool was 115 SFU/106 CD8+ T cells. Therefore, we defined a positive ELISPOT response as larger than 200 SFU/106 CD8+ T cells to exclude false positive (82).

6.8 Tetramer staining

HLA-B*52:01-RI8-6T/6V tetrameric complexes (tetramers) were generated as previously described (83, 84). PBMCs or HIV-1-specific T-cell clones/lines were stained with a combination of PE-conjugated RI8-6T and APC-conjugated RI8-6V-HLA-B*52:01 tetramers at 100 nM at 37 °C for 30 min. The cells were subsequently stained with FITC-conjugated anti-CD3 (Dako, Glostrup, Denmark), Pacific blue-conjugated anti-CD8 mAb (BD Biosciences), and 7-AAD (BD Pharmingen) at 4 °C for 30 min and analyzed with a FACS Canto II (BD Bioscience, CA). The frequency of HLA-$tetramer^+$ cells was measured after gating the $CD3^+CD8^+$ population.

6.9 Generation of epitope-specific T-cell clones or lines

PBMCs were stained with PE or APC-conjugated tetramers, FITC-conjugated anti-CD3 (Dako, Glostrup, Denmark), Pacific blue-conjugated anti-CD8 mAb (BD Biosciences), and 7-AAD (BD Pharmingen), after which $CD3^+CD8^+7AAD^-$ $tetramer^+$ T cells were sorted in U-bottomed 96-well microtiter plates (1 cell/well for T-cell clones and 100-500 cells/well for T-cell lines) by using a FACS Aria (BD Biosciences). The sorted cells were stimulated with the corresponding epitope peptide and cultured as previously described (84). After 2-3 weeks in culture, epitope-specific

$CD8^+$ T-cells were used in functional assays after their purity had been confirmed by flow cytometry analysis using tetramers.

6.10 HLA stabilization assay

The affinity of peptide binding to HLA-B*52:01 was examined using by RMA-S-B*52:01 cells as previously described (85, 86). Briefly, these RMA-S transfectant cells were cultured at 26 °C for 16 hr and then pulsed with peptides at 26 °C for 1 hr and subsequently incubated at 37 °C for 3 hr. Staining of cell-surface HLA molecules was performed by using anti-HLA class I α3 domain mAb TP25.99(87) and FITC-conjugated sheep anti-mouse IgG (Jackson ImmunoResearch). The fluorescence intensity was measured with the FACS-Canto II.

6.11 In vitro virus inhibition assay

The ability of epitope-specific $CD8^+$ T cells to suppress HIV-1 replication was measured as described previously (65, 88). $CD4^+$ T cells isolated from HLA-matched healthy donor PBMCs were infected with HIV-1 virus and then co-cultured with epitope-specific T-cells at E:T ratios of 1:1, 0.1:1, and 0:1. When RI8-6V-specific bulk T cells were used as effector T cells (Figure 4C), the number of effector T cells was calculated as a total number of T cells × % RI8-6V $tetramer^+$ T cells. On day 5 post infection, the concentration of p24 antigen in the culture supernatant was measured by using an enzyme-linked immunosorbent assay kit (HIV-1 p24 Ag ELISA kit, ZeptoMetrix). The percentage of suppression was calculated as follows: (concentration of p24 without CTLs – concentration of p24 with CTLs) / concentration of p24 without CTLs × 100.

6.12 Statistical analysis

The frequency of the mutation between HLA^+ and HLA^- individuals was statistically analyzed by using Fisher's exact test. Groups were compared by performing the unpaired t-test or two-tailed Mann-Whitney U tests. P values <0.05 were considered significant

7. RESULTS

7.1 Selection and accumulation of Gag280S/A mutant viruses in HIV-1-infected HLA-B*52:01+ individuals

To investigate HLA-B*52:01-associated mutations at Gag280 in HIV-1 subtype B infections, we analyzed the sequences around this position from 390 treatment-naïve Japanese individuals chronically infected with HIV-1 subtype B (99 HLA-B*52:01^{+} and 291 HLA-B*52:01^{-} ones). The frequencies of Gag280S and Gag280A mutants were significantly higher in the HLA-B*52:01^{+} individuals than in the HLA-B*52:01^{-} ones ($P=6.74E^{-11}$, $q=3.37E^{-10}$ *and* $P=0.00837$, $q=0.0140$, respectively), whereas no significant difference was observed in the frequency of Gag280V mutants between HLA-B*52:01^{+} and HLA-B*52:01^{-} individuals (**Figure 1**). These results indicate that Gag280S and Gag280A mutants had accumulated in the HLA-B*52:01^{+} individuals but that Gag280V ones had not.

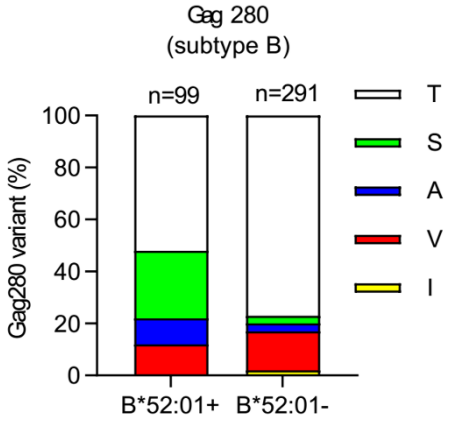
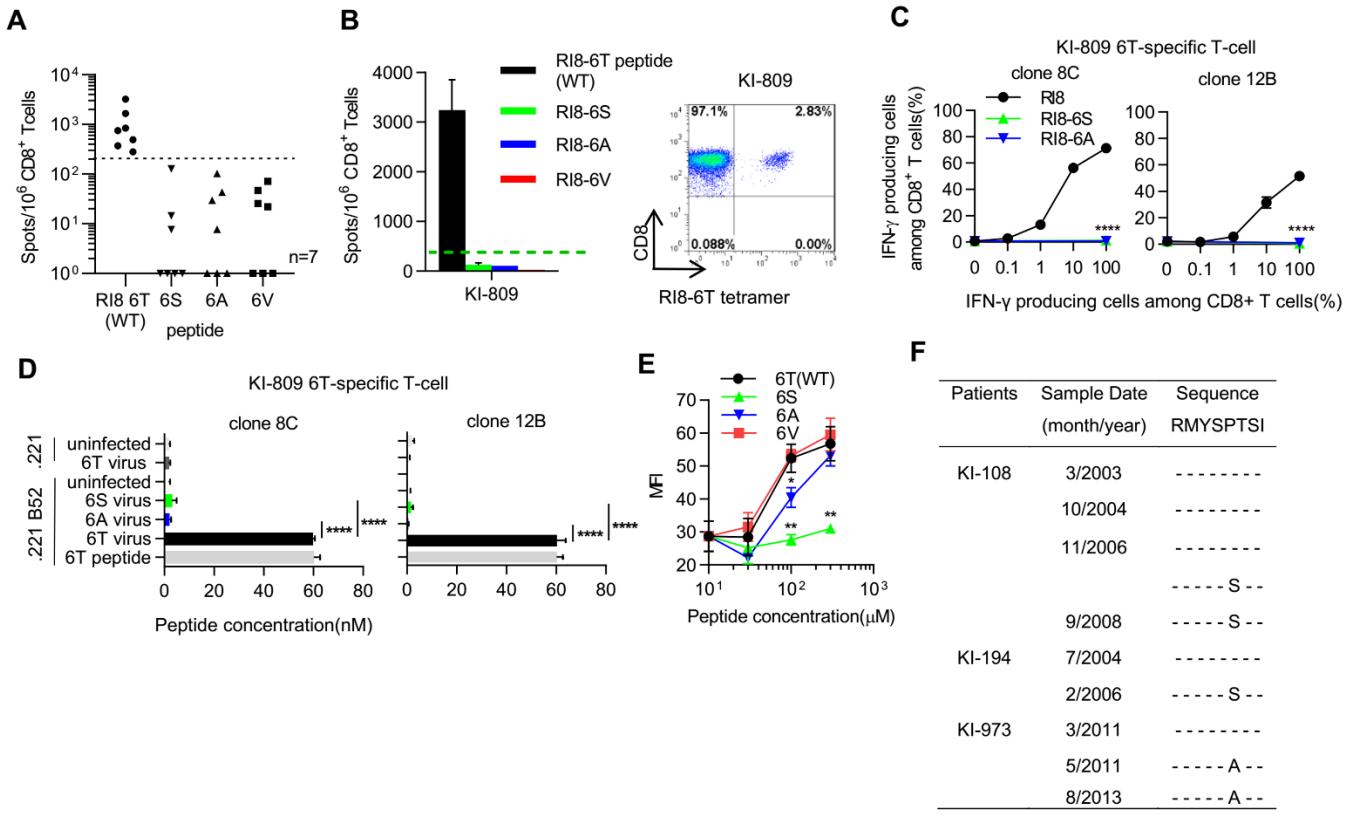


Figure 1. Association of HLA-B*52:01 with mutations at Gag280 in 390 HIV-1 subtype B-infected Japanese individuals. Gag280S, Gag280A, Gag280V, and Gag280T were found in 26, 10, 12, and 51 HLA-B*52:01^{+} individuals, respectively; and Gag280S, Gag280A, Gag280V, Gag280I, and Gag280 T in 8, 8, 45, 6, and 224 HLA-B*52:01^{-} ones, respectively.

A previous study revealed that T cell responses to 3 mutant epitopes, RI8-6S, -6A, and -6V, were not detectable in 3 HLA-B*52:01^{+} individuals having T-cells specific for the RI8-6T(RMYSPTSI) wild-type epitope (28). To confirm this result, we analyzed an additional 7 HLA-B*52:01^{+} individuals infected with Gag280T wild-type virus who had RI8-6T-specific T-cells. T cell responses to RI8-6S, -6A or -6V mutant peptides were not found in these individuals (**Figure 2A**), indicating that these mutant epitopes could not be recognized by RI8-6T-specific T-cells. To investigate in detail the recognition of RI8-6T-specific T-cells for these mutant epitopes, we established RI8-6T-specific T-cell clones from wild-type virus-infected individual KI-809, who had a strong T cell response to the RI8-6T peptide (**Figure 2B, left**) and a high number of RI8-6T-HLA-B*52:01 tetramer-binding T-cells (**Figure 2B, right**). Two RI8-6T-specific T-cell clones failed to recognize not only 721.221 cells expressing HLA-B*52:01 (.221-B*52:01) prepulsed with RI8-6S or RI8-6A mutant peptide (**Figure 2C**) but also those infected with these mutant viruses (**Figure 2D**). An HLA class I stabilization assay using RMA-S-B*52:01 cells revealed that the binding affinity of RI8-6S and RI8-6A peptides for HLA-B*52:01 molecules was weaker than that of the RI8-6T peptide, though that of the RI8-6S peptide was much weaker than that of the RI8-6A one (**Figure 2E**). These findings together suggest that the Gag280S mutation critically affected the epitope presentation in the cells infected with the Gag280S mutant virus and that the Gag280A mutation may have affected TCR recognition rather than the presentation of the epitope.

We performed a longitudinal sequence analysis at Gag280 in 13 HLA-B*52:01^{+} individuals infected with the wild-type virus at the first sampling. The results demonstrated the T to S substitution (KI-108 and KI-194), the T to A substitution (KI-973), and the T to V substitution (KI-906) in 4 of them after the second sampling (**Figure 2F and Figure 4A**). These results support the idea that these mutations were selected by RI8-6T-specific T-cells and accumulated in the

HLA-B*52:01^{+} individuals.



Patients	Sample Date (month/year)	Sequence RMYSPTSI
KI-108	3/2003	- - - - - - - -
	10/2004	- - - - - - - -
	11/2006	- - - - - - - -
		- - - - - S - -
	9/2008	- - - - - S - -
KI-194	7/2004	- - - - - - - -
	2/2006	- - - - - S - -
KI-973	3/2011	- - - - - - - -
	5/2011	- - - - - A - -
	8/2013	- - - - - A - -

Figure 2. Recognition of RI8-6S and RI8-6A mutant epitopes by HLA-B*52:01-restricted RI8-6T-specific T cells. **(A)** T cell responses to RI8-6T peptide or its mutant ones. The dotted line indicates the threshold for a positive response. **(B)** Response (left) and identification (right) of RI8-specific T-cells in PBMCs from KI-809 were analyzed by using the ELISPOT assay and RI8-6T tetramer, respectively. **(C and D)** Recognition of RI8-6S or -6A mutant epitopes by RI8-6T-specific T cell clones. T cell responses to 721.221-B*52:01 cells prepulsed with RI8-6T, RI8-6S or RI8-6A peptide at various concentrations (C) and to those infected with NL43-Gag280T (wild-type), -Gag280S, or -Gag280A were analyzed by performing ICS assays (D). The frequencies of p24 antigen-positive cells among 721.221-B*52:01 cells infected with NL43-Gag280T, -Gag280S, and -Gag280A, and 721.221 cells infected NL43-Gag280T were 59.6%, 54.6%, 59%, and 59%, respectively (D). **(E)** Binding affinity of RI8 and its mutant peptides to HLA-B*52:01. **(G)** Longitudinal sequence analysis at Gag280 in 3 HLA-B*5201^{+} Japanese individuals. Results are given as mean with SD (n=3). Statistical analysis was performed by using the unpaired t test (d-f). *P < 0.05, **P < 0.01, ****P < 0.0001.

We further investigated whether RI8-6A- or RI8-6S-specific T cells were elicited in HLA-B*52:01^{+} individuals infected with Gag280A or Gag280S virus by performing the ELISPOT assay. The result showed that these mutant epitope-specific T cells were not elicited in them (**Figure 3**). Thus, these mutations critically affected the elicitation of these mutant epitope-specific T cells *in vivo*.

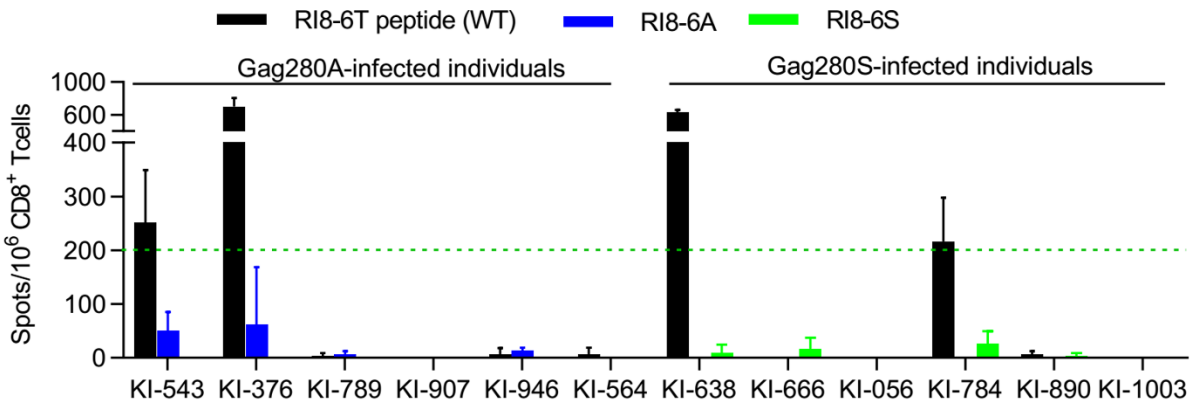
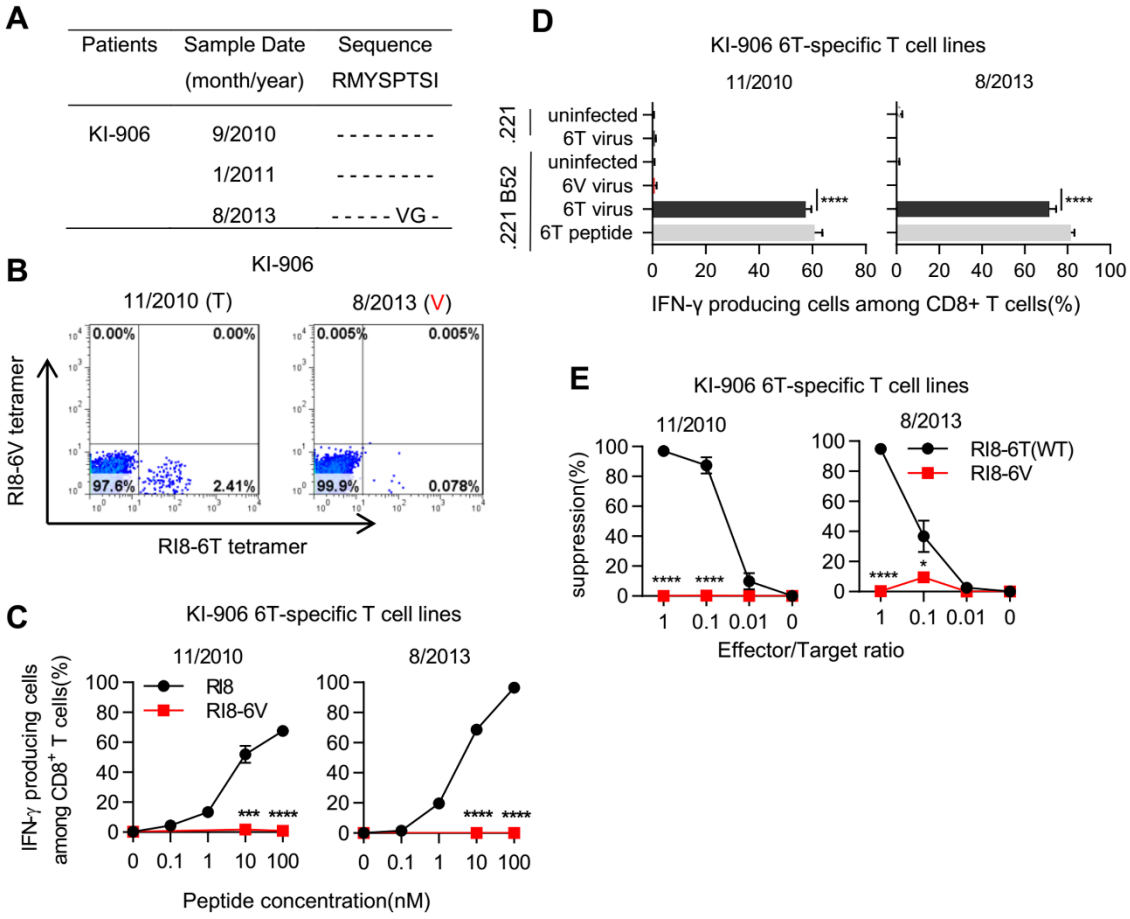


Figure 3. T cell responses to RI8 and RI8-6A peptides. T cell responses to RI8 and RI8-6A peptides in 6 Gag280A-infected and those to RI8 and RI8-6S in 6 Gag280S-infected individuals. Results are given as mean with SD (n=3).

7.2 Selection of Gag280V mutant virus by RI8-6T-specific CD8+ T-cells

T cell responses to the 6V mutant were not found in 7 HLA-B*52:01^{+} individuals who were infected with the wild-type virus and had RI8-6T-specific T-cells **(Figure 2A),** suggesting that RI8-6T-specific T-cells could not recognize the RI8-6V mutant. To clarify the ability of RI8-6T-specific T-cells to recognize the mutant, we investigated RI8-6T-specific T-cells in individual KI-906, who was infected with the Gag280T virus before January 2011 followed by the emergence of the Gag280V mutant one in August 2013 (**Figure 4A**). Flow cytometric analysis using RI8-6T- and RI8-6V-HLA-B*52:01 tetramers revealed that KI-906 had RI8-6T-specific T-cells as 2.41% of total CD8^{+} T-cells in November 2010 but only as 0.08 % of them in August 2013 (**Figure 4B**).

We established RI8-6T-specific T-cell lines from PBMCs at these time points by sorting for RI8-6T-specific T-cells and analyzed the ability of these T-cell lines to recognize target cells prepulsed with RI8-6V peptide and those infected with Gag280-6V virus. Two RI8-6T-specific T-cell lines recognized .221-B*52:01 cells prepulsed with RI8-6T peptide and those infected with Gag280-6T virus, whereas they failed to recognize those prepulsed with RI8-6V peptide and those infected with Gag280-6V virus (**Figure 4C** and **4D).** In addition, a viral suppression assay showed that these T cell lines strongly suppressed the replication of the Gag280T virus but not that of the Gag280-6V one (**Figure 4E**). Taken together, these results support the idea that the Gag280V mutation could be selected by RI8-6T-specific T-cells. However, it remains unclear as to why the



Gag280V mutant did not accumulate in the subtype B-infected HLA-B*52:01^{+} individuals.

Figure 4. Recognition of RI8-6V mutant epitope by HLA-B*52:01-restricted RI8-6T-specific T cells. (A) Longitudinal sequence analysis at Gag280 in an HLA-B*5201^{+} Japanese individual. **(B)** Identification of RI8-specific T-cells among PBMCs from KI-906 before and after the emergence of the Gag280V mutant virus. **(C and D)** Recognition of RI8-6V mutant epitope by RI8-6T-specific T-cell lines. T cell responses to 721.221-B*52:01 cells prepulsed with RI8-6T or -6V peptide (C) and to those infected with NL43-Gag280T or -Gag280V were analyzed (D). Frequencies of p24-positive cells among 721.221-B*52:01 cells infected with NL43-Gag280T and -Gag280V and 721.221 cells infected with -Gag280T were 60.8%, 58.0%, and 66.0% respectively (D). **(E)** Ability of RI8-6T-specific T cell to suppress the replication of Gag280T and Gag280V virus. Results are given as mean with SD (n=3). Statistical analysis was performed by using the unpaired t test (b-d). ***P < 0.001, ****P < 0.0001.

7.3 Selection of Gag280T wild-type virus by RI8-6V-specific and cross-reactive T-cells

We performed a longitudinal analysis at Gag280 in 5 HLA-B*52:01^{+} individuals infected with the Gag280V virus at the first sampling and found that HLA-B*52:01^{+} individual KI-855 had been infected with the Gag280V virus in June 2010 and then showed the presence of both Gag280V and Gag280T viruses in December 2010, and finally that of only the Gag280T virus in September 2013 (**Figure 5A**). Analysis using RI8-6T- or RI8-6V-HLA-B*52:01 tetramers demonstrated the existence of both RI8-6T-specific and RI8-6V-specific HLA-B*52:01-restricted T-cells in PBMCs collected from KI-855 in December 2010 (**Figure 5B**). To investigate these T cells, we established RI8-6T-specific and RI8-6V-specific T-cell clones from this individual's PBMCs collected in Dec 2010. Two RI8-6V-specific T-cell clones, 6C and 11B, had strong ability to recognize .221-B*52:01 cells prepulsed with RI8-6V peptide (**Figure 5C)** and those infected with the Gag280V virus **(Figure 5D**). On the other hand, 2 RI8-6T-specific T-cell clones, 2F and 8F, effectively recognized .221-B*52:01 cells prepulsed with RI8-6T peptide, though the latter one had a weak

ability to cross-recognize those prepulsed with RI8-6V peptide at a high concentration (**Figure 5C)**. Both clones effectively recognized .221-B*52:01 cells infected with the Gag280T virus, whereas clone 2F and clone 8F failed to recognize and weakly recognized, respectively, those infected with the Gag280V virus **(Figure 5D**). Analysis using the B*52:01-tetramers demonstrated that clone 2F and clones 6C and 11B were RI8-6T-specific and RI8-6V-specific T-cells, respectively, and that clone 8F was cross-reactive T-cells that strongly bound to the RI8-6T tetramer but weakly to the RI8-6V one (**Figure 5E**). Thus, 3 types of RI8-specific T-cells (RI8-6V-specific, RI8-6T-specific, and cross-reactive T-cells) were elicited in KI-855. Further analyses using viral suppression assays demonstrated that the RI8-6V-specific T-cell clone effectively suppressed the replication of the Gag280V virus but not that of the Gag280T one and that the RI8-6T-specific T-cell clone suppressed the replication of the Gag280-6T virus but not that of the Gag280-6V one (**Figure 5F**). The cross-reactive T-cell clone revealed a strong ability to suppress the replication of both viruses, though the viral suppression ability for the Gag280-6V virus was weaker than that for the Gag280-6T one (**Figure 5F**). These results indicate that T-cells having a strong ability to suppress the replication of the Gag280V virus were elicited in HLA-B*5201^{+} individuals infected with the Gag280V virus. KI-855 revealed a reversion of Gag280V to Gag280T after the elicitation of RI8-6V-specific and cross-reactive T-cells. This finding supports the idea that RI8-6V-specific T-cells and/or cross-reactive T-cells selected the wild-type virus.

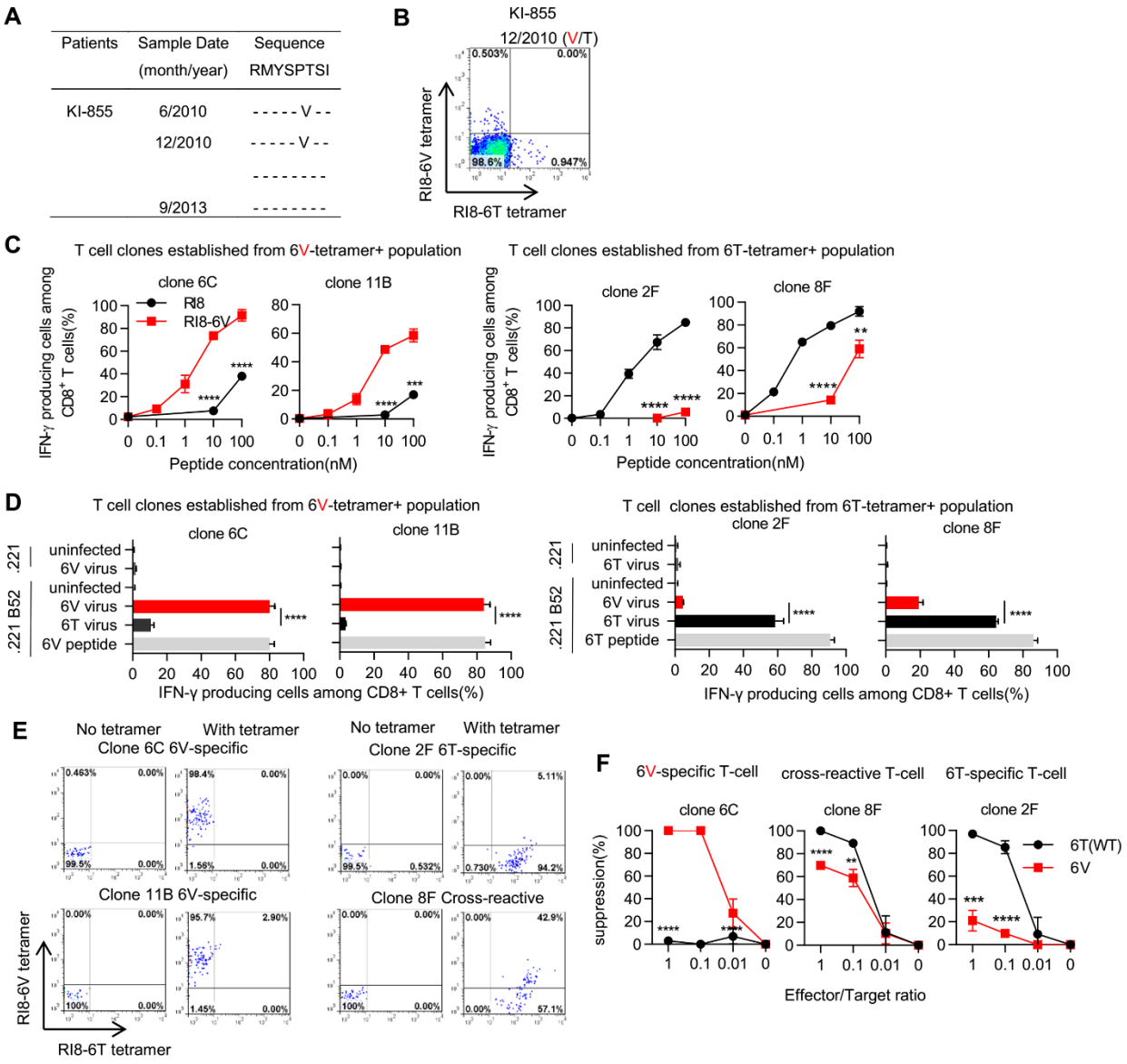


Figure 5. Ability of RI8-6V-specific T-cells to recognize RI8-6V-infected cells and to suppress RI8-6V replication. (A) Longitudinal sequence analysis at Gag280 in an HLA-B*5201^{+} Japanese individual infected with HIV-1 subtype B virus. **(B)** Identification by tetramer staining of RI8-specific T-cells in PBMCs from KI-855 infected with a mixture of Gag280T and Gag280V viruses in December 2010. **(C and D)** Recognition of RI8-6T or -6V epitope by T cell clones established from the RI8-6V tetramer^{+} or RI8-6T tetramer^{+} T cell population. Responses of these clones to 721.221-B*52:01 cells prepulsed with RI8-6T or -6V peptide (C) and to those infected with NL43-Gag280T or -Gag280V were analyzed by using the ICS assay (D). The frequencies of p24 antigen-positive cells among 721.221-B*52:01 cells infected with NL43-Gag280T and Gag280V were

35.3% and 28.9%, respectively; whereas those of 721.221 infected with NL43-Gag280T and -Gag280V were 30.3% and 34.8%, respectively (D). **(E)** Staining of RI8-6V-specific, RI8-6T-specific, and cross-reactive T cell clones with both HLA-B*52:01-RI8-6T and HLA-B*52:01-RI8-6V tetramers. **(F)** Ability of RI8-6V-specific, cross-reactive, and RI8-6T-specific T cell clones to suppress the replication of Gag280-6T and -6V viruses. Results are given as mean with SD (n=3). % suppression of HIV-1 replication is presented. Statistical analysis was performed by using the unpaired t test. **P < 0.01, ***P < 0.001, ****P < 0.0001 (c, d, and f).

We next analyzed RI8-6V-specific and/or cross-reactive T-cells in all 12 HLA-B*5201^{+} individuals infected with the Gag280V virus. RI8-6V-specific T-cells were detected in 10 of these individuals, though RI8-6T-specific T-cells were also found in 4 of them (**Figure 6A**). The analysis of PBMCs from 5 individuals, performed by using specific tetramers, confirmed the existence of RI8-6V-specific T-cells in these 5 individuals (**Figure 6B**). RI8-6V-specific T cell lines established from 4 individuals demonstrated a strong ability to suppress replication of Gag280V mutant virus, though those from KI-917 exhibited strong ability to suppress the replication of both viruses (**Figure 6C**). These results demonstrated that RI8-6V-specific T-cells and/or cross-reactive T-cells were frequently elicited in HLA-B*5201^{+} individuals infected with Gag280V virus and that these T-cells could suppress the replication of the Gag280V mutant virus.

A

KI#	Date(M/Y)	T cell response to epitope (spots/10^6 $CD8^+$ T cells) Res to 6V	Res to 6T
KI504	8/2010	648	408
KI621	2/2009	63	5721
KI628	3/2009	1771	0
KI698	8/2009	677	0
KI705	3/2010	2614	0
KI728	10/2009	40	NA
KI797	2/2010	360	NA
KI852	6/2010	234	0
KI855	6/2010	633	696
KI903	10/2010	3065	82
KI904	9/2010	555	7925
KI917	11/2010	305	408

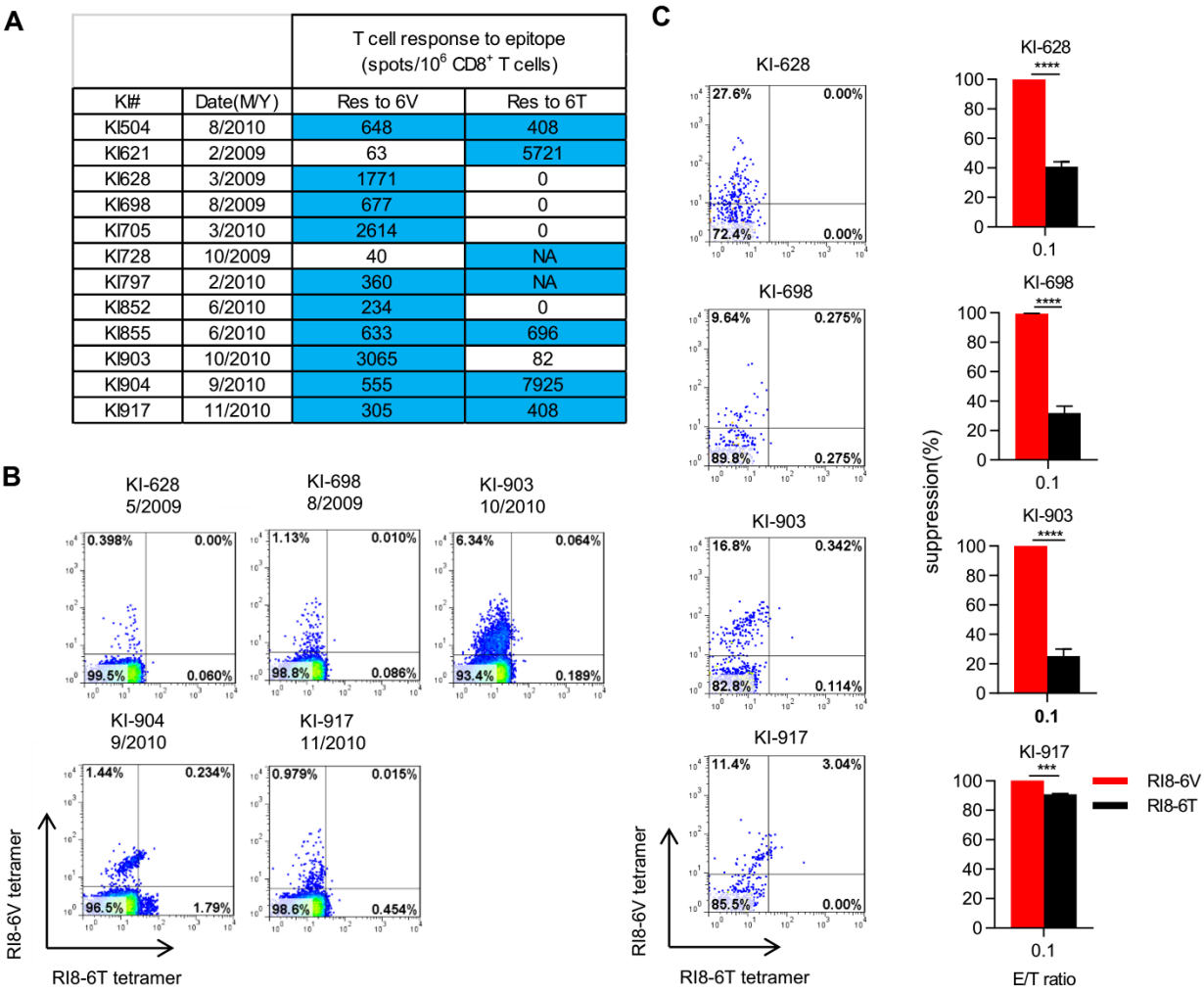


Figure 6. Detection of RI8-6V-specific T-cells and their ability to suppress RI8-6V replication in Gag280V-infected HLA-B*5201$^+$ Japanese individuals. (A) T-cell responses to RI8-6V (6V) or RI8-6T (6T) epitopes were analyzed by performing the IFN-γ ELISPOT assay. The blue shading indicates a positive response in the ELISPOT assay (>200 spots/10^6 $CD8^+$ T cells). NA, not analyzed. **(B)** Identification by tetramer staining of RI8-specific T-cells among PBMCs from 5 Gag280V-infected individuals. PBMCs were stained with HLA-B*52:01-RI8-6T and HLA-B*52:01-RI8-6V tetramers. **(C)** Ability of RI8-6V-specific T-cells to suppress the replication of Gag280-6T and -6V viruses. RI8-6V-specific T cell lines were induced from PBMCs of 4 individuals by stimulating the PBMCs with RI8-6V peptide and culturing them for 14 days. The frequency of RI8-6V-specific and RI8-6T-specific T-cells were measured by staining with both HLA-B*52:01-RI8-6T and HLA-B*52:01-RI8-6V tetramers (left). Activated $CD4^+$ T cells from an HLA-B*52:01$^+$ individual infected with NL43-Gag280T or -Gag280V were co-cultured with

RI8-6V-specific T-cells at an E:T ratio of 0.1:1 (right). Results are given as mean with SD (n=3). % suppression of HIV-1 replication is presented. Statistical analysis was performed by using the unpaired t test. ***P < 0.001, ****P < 0.0001.

7.4 Contribution of RI8-6T and RI8-6V-specific CD8+ T-cells to control of HIV-1 in subtype B infection

Next, we analyzed the effect of Gag280 mutations on the clinical outcome in the subtype B-infected HLA-B*52:01^{+} Japanese individuals. The individuals infected with the Gag280T virus had significantly higher CD4 counts than those with Gag280S/A virus, whereas the Gag280V-infected individuals showed a trend for a higher CD4 count than the Gag280S/A-infected ones (**Figure 7A**). These results suggest that RI8-6T/6V-specific T-cells may have suppressed the replication of HIV-1 in these individuals. We therefore investigated the association of T cell responses to RI8-6T/6V with the clinical outcome. Responders to RI8-6T or 6V peptide showed significantly higher CD4 counts and trends toward a lower pVL than non-responders (**Figure 7B**), indicating that both RI8-6T-specific and RI8-6V-specific T-cells contributed to the suppression of HIV-1 replication in subtype B-infected HLA-B*52:01^{+} Japanese individuals.

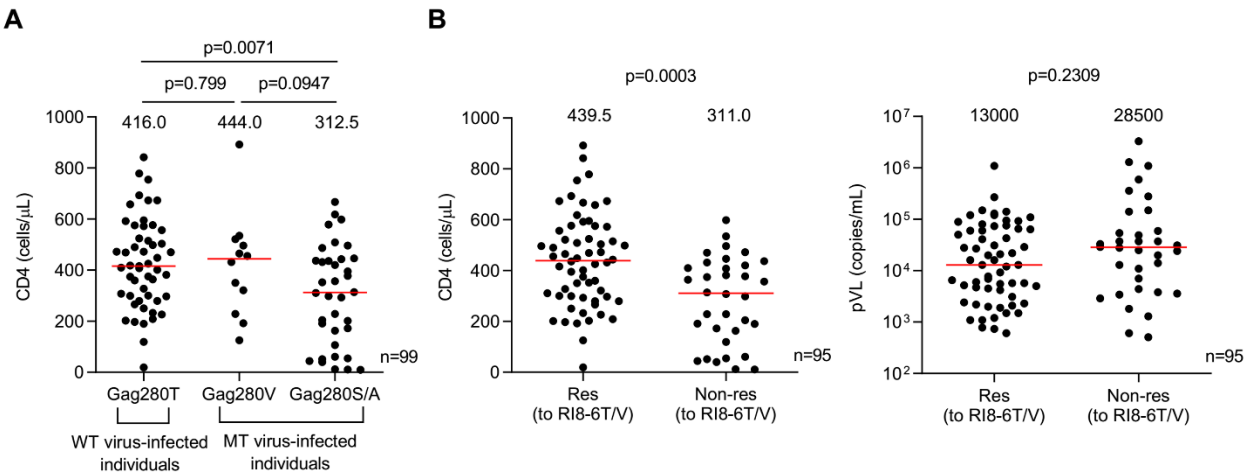


Figure 7. Comparison of clinical outcome among individuals infected with HIV-1 having different Gag 280 mutations and between T cell responders to RI8 and non-responders. (A) Comparison of CD4 count for individuals infected with Gag280T (wild-type), Gag280V or Gag280S/Gag280A virus among 99 subtype B-infected HLA-B*52:01^{+} Japanese individuals. **(B)** Association of T cell responses to RI8-6T/6V with clinical outcome. Comparison of CD4 count and pVL between T cell responders and non-responders to RI8-6T/6V among 95 subtype B-infected HLA-B*52:01^{+} Japanese individuals. Statistical analysis was performed by using the Mann-Whitney test. The value indicated by the red line in each figure represents the median of the CD4 count.

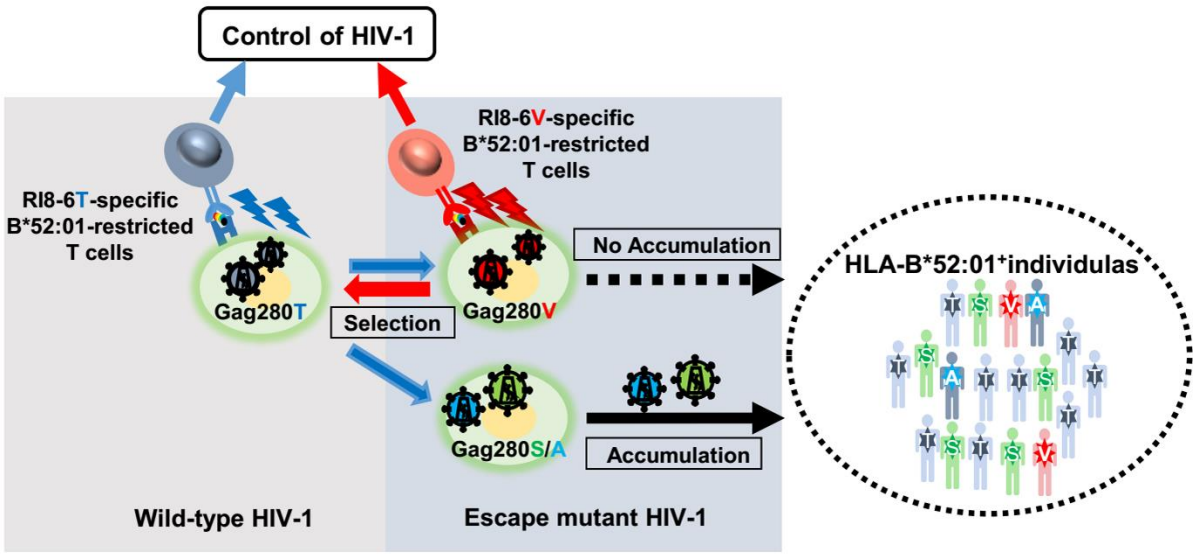


Figure 8. Summary of this study.

8. DISCUSSION

A previous study on HLA-associated HIV-1 polymorphisms in HIV-1 subtype B-infected Japanese individuals showed that Gag280S and Gag280A are HLA-B*52:01-associated mutations whereas Gag280V is not (79). This finding suggested that Gag280S and Gag280A, but not Gag280V, are escape mutations selected by HLA-B*52:01-restricted RI8-specific T-cells. However, the present study clearly demonstrated that HLA-B*52:01-restricted RI8-specific T-cells failed to recognize cells infected with Gag280V, Gag280A, or Gag280S mutant virus, indicating that these mutations were escape ones. Gag280V had not accumulated in the HLA-B*52:01^{+} individuals, whereas this mutation was found more frequently than the Gag280A or Gag280S mutation in Japanese individuals. These findings together suggested the presence of a mechanism responsible for no accumulation of the Gag280V mutation in the HLA-B*52:01^{+} individuals. Our hypothesis is that RI8-6V-specific T cells were elicited in HLA-B*52:01^{+} individuals infected with the Gag280V mutant virus and that these T cells selected the wild-type virus. Indeed, we demonstrated that RI8-6V-specific T cells were detected in most of the HLA-B*52:01^{+} individuals infected with Gag280V mutant virus and that these T cells had a strong ability to suppress replication of the Gag280V mutant virus.

The results of an HLA class I stabilization assay showed that the binding affinity of RI8-6S peptide for HLA-B*52:01 molecules was much weaker than that of the RI8-6T peptide but that the affinity of the RI8-6V peptide was identical to that of the RI8-6T. These findings together suggest that position 6 is a critical site for both the peptide binding to HLA-B*52:01 and TCR recognition, though this position is not an anchor residue (80). The affinity of the RI8-6S peptide was much weaker than that of the RI8-6T or the RI8-6V one, suggesting that the RI8-6S epitope peptide could not be presented in the cells infected with the Gag280S mutant virus. On the other

hand, the Gag280A mutation weakly affected the peptide binding affinity, suggesting this mutation may have affected TCR recognition. RI8-6T-specific T cells failed to recognize the RI8-6V epitope, whereas RI8-6V-specific T cells were elicited in the individuals infected with Gag280V mutant virus. These findings suggest that there were 2 T cell repertoires for RI8 in HLA-B*52:01^{+} individuals, one having high-affinity TCRs for RI8-6T and the other one for RI8-6V.

HLA-B*52:01 is protective allele in the subtype B and C infections (62, 65, 73) whereas Gag RI8 is one of protective T cell epitopes restricted by HLA-B*52:01 (28). RI8-6T-specific T-cells failed to recognize the cells infected with Gag280S/A mutant viruses, and T-cells specific for RI8-6A/6S mutant epitopes were not elicited in the individuals infected with these viruses (**Figure 3**). These findings suggest that the accumulation of Gag280S/A mutations would critically affect suppression of HIV-1 replication by these specific T cells *in vivo*. Indeed, HLA-B*52:01^{+} Japanese individuals infected with Gag280S/A mutant viruses had significantly lower CD4 counts than those infected with the wild-type virus. In contrast, RI8-6V-specific T-cells, which were frequently elicited in Gag280V virus-infected HLA-B*52:01^{+} individuals, had a strong ability to suppress replication of Gag280V mutant viruses *in vitro*. Indeed, our analysis showed that no significant difference in CD4 count was found between individuals infected with Gag280T virus and those with the Gag280V one, suggesting that the Gag280V mutation did not affect the control of HIV-1. Since the accumulation of Gag280S/A mutations was found in only 20% of the HLA-B*52:01^{+} individuals, GagRI8 is still a protective T cell epitope in them.

Three of 4 HLA-B*52:01-restricted epitopes are conserved among circulating HIV-1 subtype B viruses (28), and T-cells specific for these epitopes have a strong ability to suppress HIV-1 replication *in vivo* (28, 77). These epitopes may be targets for prophylactic T cell vaccines and a cure for HIV-1. The wild-type sequence of RI8 is found in only 60% of Japanese individuals

infected with the subtype B virus, suggesting that this epitope may not be useful for a T cell vaccine and AIDS cure. However, the Gag280V mutant virus could elicit RI8-6V mutant virus-specific T-cells in individuals infected with this mutant virus, and these T cells could suppress replication of the mutant virus. Since approximately 80 % of circulating viruses have Gag280T/V, chimeric antigens containing both RI8-6T and RI8-6V epitopes could be useful for a vaccine and cure of AIDS. Thus, the present study showed that a T cell epitope including an escape mutation could be target for a T cell vaccine and AIDS cure. However, since it is still unknown whether other escape mutant epitopes also could elicit specific T cells that could effectively suppress HIV-1 mutant viruses, further studies on T cell recognition for escape HIV-1 mutants are required for generation of chimeric vaccine antigens that should contribute to the development of a prophylactic T cell vaccine and AIDS cure.

9. CONCLUSION

In the present study, we demonstrated a mechanism for the accumulation of different Gag280 mutations in subtype B-infected Japanese and for co-evolution of HIV-1 with HIV-1-specific T-cells as well as the important role of mutant-specific T-cells in the suppression of HIV-1 replication *in vivo* (**Figure 8**). The results of the present study strongly impact our understanding of the role of mutant epitope-specific T-cells in the control of HIV-1 and imply their use for a prophylactic AIDS vaccine and AIDS cure.

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