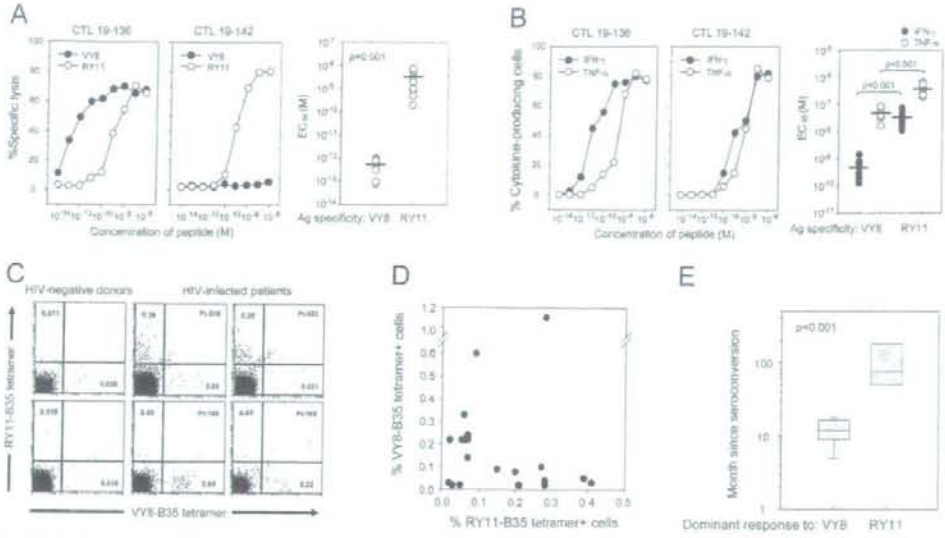

FIGURE 2. HLA-B35-restricted CTL responses toward PxxP region of Nef. *A* and *B*, Cytotoxic activity (*A*) and cytokine-producing activity (*B*) of VY8 or RY11-specific CTL clones generated from multiple donors (patients 01, 03, 19, and 136) were analyzed by using C1R-B3501 cells pulsed with various concentrations of the indicated peptides (Effector to target cell ratio = 2). Representative peptide-titration data obtained by CTL clones 19-136 and 19-142 (generated from PBMC of patient 19) specific for VY8 and RY11, respectively, are shown (*left* and *middle panels*). EC_{50} values thus obtained from an additional nine clones (total 10 clones each) generated from multiple donors are shown (*right panels*). Horizontal bars indicate means, and statistic analysis were performed by using the two-tailed *t* test. *n.s.*, not significant. Cytotoxic activity and cytokine-producing activity in the absence of the cognate peptide were always below 3 and 0.1%, respectively. *C* and *D*, PBMC samples isolated from 5 HIV-negative and 19 HIV-positive donors were analyzed by flow cytometry by using HLA-B*3501 tetramers in complex with VY8 or RY11 peptides. Cells that were $CD3^+CD8^+$ were gated and then analyzed for their frequency of HLA-tetramer$^+$ cells. Some representative dot plots of 2 HIV-negative and 4 HIV-positive donors are shown with frequencies of HLA-tetramer$^+$ cells in each dot plot (*C*). The frequencies of HLA-tetramer$^+$ cells for VY8 and RY11 epitopes in each individual subject are shown (*D*). It should be noted that reversing the fluorochromes of the tetramers gave identical results and that the background level of HLA-tetramer staining was 0.022%, as determined by the data from 5 HIV-negative donors (mean + 3 SD). *E*, Differences in months since seroconversion between the subject groups who showed dominant CD8 T cell responses to VY8 or RY11 epitope. Boxes indicate values between 25th and 75th percentiles. Horizontal lines across boxes indicate the median value ± SD. Lines extend from the box to the highest and lowest values. Statistical analysis was performed by using the Mann-Whitney *U* test.

the type of Nef variants changed from RF to TY by two amino acid substitutions, the Nef variant with two mutations, i.e., TPQVPLRPMTF (referred to as TF), was not apparently selected. Rather, the T75 mutation appeared to arise from a different lineage of viral quasispecies in this host (Fig. 1*C*). In addition, the TF double mutation was barely found in Los Alamos HIV database (1 of 443 entries), suggesting that the combination of these two mutations causes a significant fitness cost in viral replication in vivo.

Fine epitope mapping of HLA-B35-restricted CD8 T cells to PxxP region of Nef

We next examined HLA-B35-restricted CD8 T cell responses toward the PxxP region of Nef. Although HLA-B*35 prefers proline at position 2 in its binding peptide and this region can provide various candidate peptides for CTL epitopes, only two peptides, VY8 (Nef_{78-85}: VPLRPMTY) and RY11 (Nef_{75-85}: RPQVPLRPMTY), showed substantial CTL responses in the HLA-B35$^+$ subjects (data not shown), confirming previous observations (29, 35, 36). However, it is possible that VY8 is the minimum epitope for CTL, because VY8 is entirely contained within RY11. To clarify this issue, we generated CTL clones by stimulating PBMC of HLA-B35$^+$ HIV-infected patients with either VY8 or RY11 peptide and then analyzed their Ag specificity by cytotoxic assays. CTL clone 136 generated from subject patient 19 (designated CTL 19-136) with VY8 stimulation showed cytolytic activities toward target cells pulsed with either peptide, although VY8 was a ~1000-fold more sensitive ligand than RY11 (Fig. 2*A*). In contrast, another CTL clone, CTL 19-142, which had been stimulated with RY11, showed cytolytic activity toward C1R-B3501 cells pulsed with RY11 but not toward those pulsed with VY8 (Fig. 2*A*). Furthermore, when staining CTL clones with HLA-B*3501 tetramers in complex with VY8 and RY11, CTL 19-136 and 19-142 exclusively bound the VY8- and RY11-B35 tetramers, respectively (data not shown). These data indicate that VY8 and RY11 were different optimal epitopes presented by HLA-B3501 and are recognized by a different set of CTLs.

During the peptide-titration analysis, we noticed that CTL 19-136 had much higher functional avidity for its cognate peptide than CTL 19-142, with the EC_{50} values toward the cognate Ags of CTL 19-136 and 19-142 being 2.81×10^{-13} and 7.50×10^{-10} M, respectively (Fig. 2*A*). We further generated CTL clones from PBMC of three additional subjects, patients 001, 003, and 033, and determined their functional avidity toward each cognate Ag. Although the functional avidity of these CTL clones were different even within the same specificity (~30-fold), VY8-specific CTL clones had more potent functional avidity than RY11-specific ones (~5000-fold), as the mean EC_{50} values of VY8- and RY11-specific CTL clones were $5.29 \pm 1.13 \times 10^{-13}$ and $3.14 \pm 0.82 \times 10^{-9}$ M, respectively (Fig. 2*B*).

Furthermore, evaluating the CTL sensitivity by Ag-specific IFN-γ and TNF-α production revealed that VY8-specific CTLs also showed more potent functional avidity than RY11-specific ones, as mean EC_{50} values for IFN-γ secretion were $5.30 \pm 1.21 \times 10^{-10}$ and $3.50 \pm 0.61 \times 10^{-8}$ M, and those for TNF-α

secretion, $5.02 \pm 0.69 \times 10^{-8}$ M and $3.75 \pm 0.48 \times 10^{-7}$ M, for VY8- and RY11-specific clones, respectively (Fig. 2*B*). However, it is interesting to note that the difference in avidity for cytokine production between VY8- and RY11-specific CTLs was smaller than that observed in cytotoxic activity (Fig. 2, *A* and *B*).

Analysis of HLA-B35-restricted CD8 T cell responses to the PxxP region of Nef ex vivo

We next examined the frequency of VY8- and RY11-specific $CD8^+$ cells in patients' PBMC ex vivo by using HLA-B35 tetramers in complex with VY8 and RY11 as shown in the representative data in Fig. 2*C*. The background level of the HLA-tetramer analysis was considered to be 0.022% (mean + 3 SD) as the overall frequency of HLA-tetramer$^+$ cells in HIV-negative donors ($n = 6$) was 0.0153 ± 0.0022%. The frequency of HLA-tetramer$^+$ cells in HIV-infected subjects ($n = 19$) was 0.198 ± 0.060 and 0.160 ± 0.029 for VY8 and RY11 epitopes, respectively, and both responses were not statistically different overall ($p = 0.58$, paired t test).

Interestingly, looking at the frequencies of HLA-tetramer$^+$ cells in each individual subject, every subject showed a response to either the VY8 or RY11 epitope but not to both epitopes simultaneously (Fig. 2*D*). The median ($\pm$SD) number of months since seroconversion in subjects who had dominant response to VY8 or RY11 was 13.0 ± 1.4 or 76.0 ± 19, respectively (Fig. 2*E*), suggesting an immunological shift from VY8 to RY11 in HLA-B35-restricted CD8 T cell responses during the course of their HIV infection.

Effects of antigenic variations on VY8- and RY11-specific CTLs

We next asked whether Nef mutations affected the binding between epitope peptides and HLA-B*3501. The HLA-I stabilization assay using RMA-S cells expressing HLA-B*3501 showed that the VY8 and RY11 peptides bound HLA-B*3501 comparably, as the EC_{50} values for their binding activities were $20.4 \pm 7.55 \times 10^{-5}$ and $4.65 \pm 1.63 \times 10^{-5}$ M, respectively. Although the Phe substitution at the C terminus of either peptide (VY8-8F and RY11-11F) did not change their binding activities, the Thr substitution at the N terminus of RY11 (RY11-1T) resulted in ~10-fold increased binding activity. These data indicate that the binding activity of all peptides tested were within the range of HLA-B3501-restricted CTL epitopes (29, 31, 36, 37).

We then tested the cytotoxic activity of CTL clones toward C1R-B3501 cells pulsed with the variant peptides. A VY8-specific CTL clone, CTL 19-136, showed ~1000-fold decreased sensitivity toward VY8-8F (Fig. 3*A*). A similar trend was also observed in a panel of nine additional VY8-specific CTL clones as used in Fig. 2*A*, with mean EC_{50} of $4.43 \pm 0.63 \times 10^{-13}$ and $8.23 \pm 3.08 \times 10^{-9}$ M for VY8 and VY8-8F, respectively (Fig. 3*A*). In contrast, a RY11-specific CTL clone, CTL 19-142, showed preserved sensitivity toward RY11-11F, whereas it showed >100-fold decreased sensitivity toward RY11-1T (Fig. 3*B*). Again, a panel of nine additional RY11-specific clones showed similar results, with mean EC_{50} of $2.75 \pm 0.46 \times 10^{-9}$, $4.32 \pm 0.81 \times 10^{-9}$, and $8.47 \pm 3.28 \times 10^{-7}$ M for RY11, RY11-11F, and RY11-1T, respectively (Fig. 3*B*). These data indicate that VY8- and RY11-specific CTLs had different patterns of Ag fine specificity toward naturally arising variants, suggesting a direct association between the epitope evolution in autologous Nef proteins (Fig. 1*B*) and the kinetic change of CTL immunodominance in vivo (Fig. 2*E*).

Cytotoxic activity of VY8- and RY11-specific CTLs toward HIV-infected primary CD4 T cells

As HIV-infected CD4 T cells are the predominant target of HIV-specific CTLs in vivo, we next examined the cytotoxic activity of CTL clones toward primary CD4 T cells infected with wt or variant HIV-1. CD4 T cells prepared from HIV-negative donors (HLA-B3501$^+$) were first stimulated with PHA and then infected with wt or various variant viruses. Four days later, ~30% of the cells appeared to be infected with all viruses, as revealed by intracellular flow cytometry for p24 Ag (data not shown), suggesting that all viruses had comparable replicative capacity when primary CD4 T cells were preactivated before infection (see below). Both CTLs specific for VY8 (CTL 19-136 and 33-1) and RY11 (CTL 19-142 and 03-8) were cytotoxic toward CD4 T cells infected with wt HIV-1 (Fig. 3*C*). However, the cytolytic activity of VY8-specific CTLs was more potent than that of RY11-specific ones, suggesting a link between potent functional avidity (Fig. 2*A*) and antiviral activity (Fig. 3*C*) of VY8-specific CTLs. VY8 and RY11-specific CTLs failed to kill primary CD4 T cells infected with F85 and T75 virus variants, respectively (Fig. 3*C*), consistent with the data obtained from the peptide-pulse experiments (Fig. 3, *A* and *B*). In contrast, although VY8-specific CTLs were cytotoxic toward primary CD4 T cells infected with the T75 variant virus, the activity toward the T75 variant was less than that toward the wt virus (Fig. 3*C*), suggesting that the T75 mutation, located in the region flanking the N terminus of VY8, could modulate the Ag-processing pathway for the generation of the VY8 epitope in these cells. Moreover, these data clearly show that the double mutant virus could escape from both types of CTLs (Fig. 3*C*). The fact that the mutations in autologous viruses is very rare in combination (Fig. 1*A*) suggests that the combination of both mutations imposes functional constraints on Nef.

Effects of Nef mutations on down-regulation of surface receptors

We next sought to identify such functional constraints of variants carrying these CTL escape mutations. Because Nef helps HIV-infected cells to evade CTL lysis by down-modulating cell surface HLA-I and the PxxP motif is critical for this activity (12, 17, 26, 38), we first examined whether the mutations affected the HLA-I down-regulation activity by Nef. Down-regulation of cell surface CD4, that is mediated by a different cellular pathway and does not involve the PxxP motif (27), was analyzed in parallel.

We isolated primary CD4 T cells from an HIV-negative donor, activated them with PHA, and infected them with wt or various variant viruses. In flow cytometric analysis, the surface levels of HLA-I were reduced to 40.1% of normal (uninfected cells) in cells infected with wt HIV-1, and no HLA-I down-regulation was observed in ΔNef virus-infected cells (Fig. 4*A*). In contrast, the TF double variant showed diminished down-regulation activity, as the TF variant-infected cells retained 73.1% of the normal level of HLA-I, whereas F85 and T75 variants showed HLA-I down-regulation activity comparable to that of the wt, with their surface levels being 36.3 and 46.5%, respectively (Fig. 4*A*). The same experiments using CD4 T cells isolated from three different HIV-negative donors reproducibly showed the TF variant to have a diminished activity in terms of HLA-I down-regulation (Fig. 4*B*). In stark contrast, all cells infected with variant viruses except for ΔNef showed down-regulation activity for CD4 comparable to that of the wt (Fig. 4, *A* and *B*). In addition, Western blot analysis of virus-producing cells for Nef proteins showed that all variant viruses except for ΔNef had expression levels of Nef comparable to that of the wt (data not shown). These data demonstrate that the

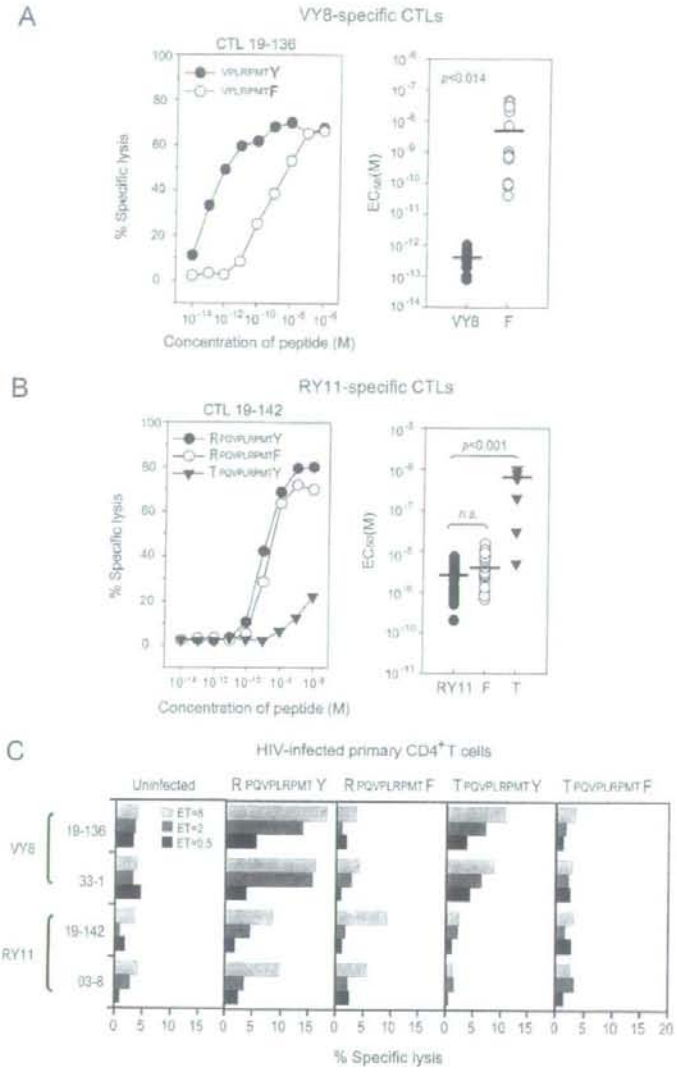

FIGURE 3. CTL responses to variant Ags. *A* and *B*, VY8 and RY11-specific CTL clones (same clones as in Fig. 2, *A* and *B*) were tested for their ability to respond to variant peptides by using C1R-B3501 cells pulsed with various concentrations of the wt or variant peptides (ET = 2). Representative peptide-titration data obtained for CTL 19-136 and 19-142 are shown (each *left panel*). EC_{50} values thus obtained for an additional 9 clones (total 10 clones) are also shown (each *right panel*). Horizontal bars indicate means, and statistic analysis was performed by using the paired *t* test. Cytotoxic activity in the absence of the peptide was always <3%. *C*, The VY8- and RY11-specific CTL clones were analyzed for their cytolytic activity toward target cells at ET = 0.5, 2, and 8 as indicated. The target cells were primary $CD4^+$ T cells that had been isolated from an HIV-negative donor ($HLA\text{-}B3501^+$), activated by PHA, and infected with wt or various variant viruses. The frequency of HIV-infected cells among target cells as determined by intracellular p24 Ag expression was 31.5, 33.2, 34.5, and 29.8% for wt, RF, TY, and TF variants, respectively. An additional experiment conducted by using a different blood donor ($HLA\text{-}B3501^+$) showed similar results.

combination of both mutations selectively diminishes the HLA-I down-regulation activity by Nef.

Effects of Nef mutations on cytolytic activity of CTL clones with other specificity

To test whether the observed differences in HLA-I down-regulation affect the susceptibility of HIV-infected cells to recognition by CTLs, we assessed the cytolytic activity of CTL clones with specificity to HIV-1 gene products other than Nef and other restriction toward primary CD4 T cells infected with wt and Nef variant viruses.

Freshly isolated CD4 T cells from an HIV-negative donor ($HLA\text{-}B35^+$ and $HLA\text{-}A24^+$) were infected with various HIV-1 as above and mixed with CTL clones specific for Pol and Env epitopes presented by HLA-B*3501 as well as with a clone specific for another Nef epitope presented by HLA-A*2402 (designate as B35-Pol, B35-Env, and A24-Nef, respectively). Although the amino acid sequences in the epitope regions of B35-Pol, B35-Env, and A24-Nef were the same among the wt and variant viruses tested, CTL-mediated killing activity appeared to be different among target cells infected with these viruses (Fig. 4*C*). Both B35-Pol and B35-Env CTLs showed most potent cytotoxic activity toward target cells infected with the ΔNef variant, whereas the same CTLs showed weak cytolytic activity toward wt virus-infected cells (Fig. 4*C*). Interestingly, CTLs markedly killed cells infected with the TF double mutant virus, whereas they weakly killed cells infected with either T75 or F85 single mutant virus (Fig. 4*C*). Moreover, in A24-Nef CTL-mediated cytotoxic activity, we also observed that the TF double mutant virus-infected cells were more potently killed than cells infected with wt or single mutant viruses (Fig. 4*C*). These data suggest that the diminished HLA-I down-regulation (i.e., increased level of cell surface HLA-I) in CD4 T cells infected with the TF double mutant virus resulted in increased susceptibility to killing by CTLs, leading to a possible selective disadvantage for the variant virus in vivo.

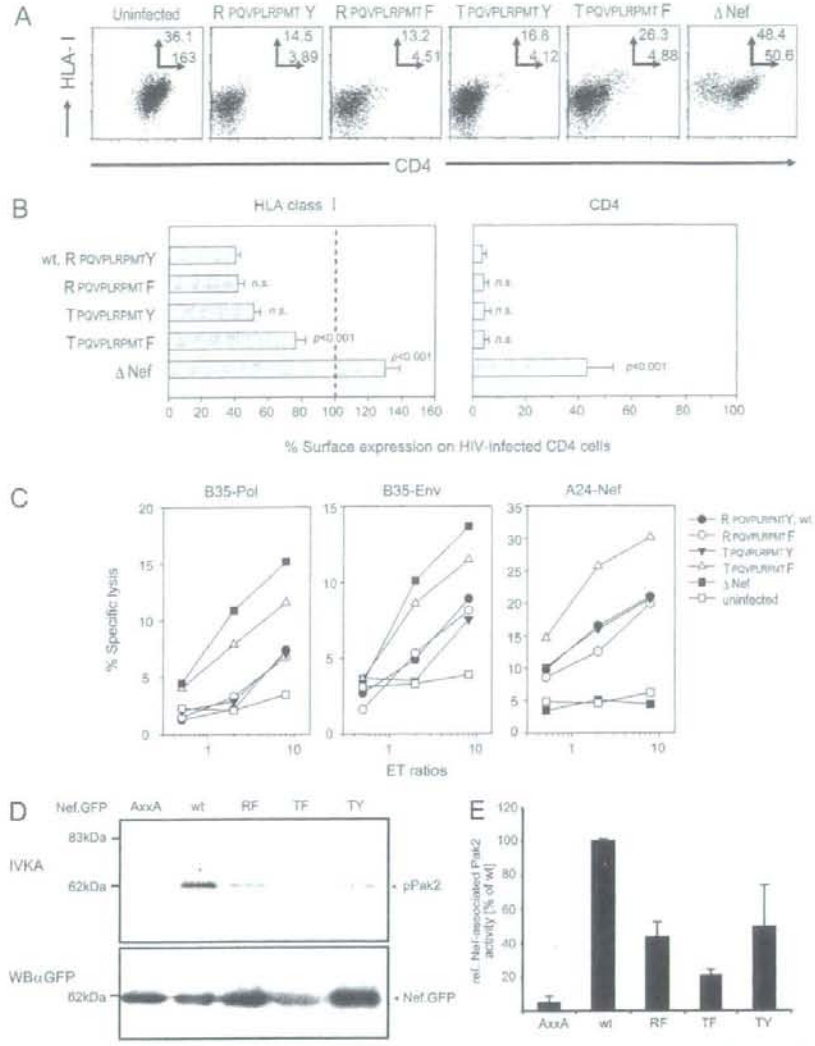

FIGURE 4. Functional consequences of CTL escape Nef mutations. *A*, Freshly isolated primary CD4$^+$ cells from an HIV-negative donor (HLA-B35$^+$) were activated by PHA for 3 days and then infected with wt or various variants for 5 days. The cells were stained with anti-HLA-Bw6 mAb (clone: SFR8-B6) and anti-CD4 mAb, and 7-AAD followed by intracellular staining for p24 Ag. In flow cytometric analysis, cells negative for 7-AAD and positive for p24 Ag were gated and analyzed for their fluorescence intensity for HLA-Bw6 and CD4. The frequency of infected cells was 29.6, 34.3, 30.5, 31.9, and 26.2% for HIV-1 wt, RF, TY, TF, and ΔNef variants, respectively. The mean fluorescence intensities (MFI) for HLA-Bw6 and CD4 are shown in the *right upper corner* of the dot plots. *B*, The same experiment as above was done by using three additional HIV-negative donors. The Ab specific for HLA-I allotypes used was either SFR8-B6 or A11,1M as appropriate for each donor. The MFI level of HLA-I and CD4 on uninfected cells was set to 100% and indicated by the dotted vertical line in the graph. Statistical analysis was performed by ANOVA with multiple comparisons vs wt. *n.s.*, not significant. *C*, Primary CD4$^+$ cells infected with wt or various variant HIV-1s as in Fig. 3 (the donor carries both HLA-A*2402 and HLA-B*3501) were used as target cells for cytolysis by CTL clones specific for HLA-B3501-restricted Pol ($Pol_{273-282}$: VPLDKDFRKY), Env (Env_{77-85}: DPNPQEVVL), or HLA-A2402-restricted Nef epitope ($Nef_{138-147}$: RYPLTFGWCF). An additional experiment using a different blood donor (positive for both HLA-A*2402 and HLA-B*3501) showed similar results. *D*, Nef-associated Pak2 activity. Jurkat cells were electroporated with plasmid DNAs encoding the indicated Nef-GFP fusion proteins. Total cell lysates were immunoprecipitated with anti-GFP Ab, and the resultant immunoprecipitates were analyzed by IVKA for Pak2 autophosphorylation (pPak) (*upper panel*). The same IVKA reactions were directly separated by SDS-PAGE and analyzed for immunoisolated Nef-GFP levels by Western blotting with anti-GFP Ab (*lower panel*). *E*, Quantification of Nef-Pak2 association. The indicated values represent the Nef-associated Pak2 activity after the levels of pPak2 had been normalized to the amounts of immunoisolated Nef-GFP. Values presented are the mean of at least three independent experiments with the indicated SEM expressed relative to the wt control that was arbitrarily set to 100%.

Effects of Nef mutations on the association of Nef with the cellular kinase Pak2

Given this reduced ability to down-modulate cell surface HLA-I, we also wanted to assess whether other Nef activities that depend on the interaction of the PxxP motif with SH3 domain-containing ligands are affected by the CTL escape mutations. To this end, we analyzed the association of Nef with cellular Pak2 kinase activity. This interaction is conserved among a variety of lentiviruses (39),

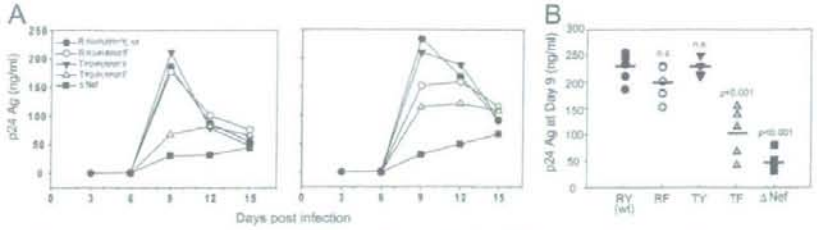

FIGURE 5. Effects of Nef mutations on viral replication in PBMC. *A*, Freshly isolated PBMC samples from two HIV-negative donors were first infected with wt or various variant HIV-1s and 3 days later cells were activated by PHA. For monitoring viral replication, culture supernatants were collected every 3 days and replaced with fresh medium containing rIL-2. *B*, The same experiment as above was done by using three additional HIV-negative donors. The level of p24 Ag obtained at day 9 postinfection was plotted and statistically analyzed based on ANOVA with multiple comparisons vs the wt. *n.s.*, not significant. Horizontal bars indicate means of data obtained for the five different PBMC donors.

strictly depends on the integrity of the PxxP motif and has multiple functional consequences that may optimize virus production (10, 40, 41). Expectedly (34, 39), wt Nef from HIV-1 SF2 (wt) showed robust association with phosphorylated Pak2 (pPak2) while the AxxA mutant (both Pro^{76} and ^{80} to Ala) did not show substantial association with pPak2 (Fig. 4*D*). Pak2 association was substantially reduced but not abrogated for the F85 (RF) and T75 (TY) single variants. According to phosphorimager quantification of the Nef-associated Pak2 signal and normalization to the levels of Nef present in the IVKA (Fig. 4*E*), Pak2 association was more than two-fold reduced for these two Nef variants relative to wt. The TF double mutant displayed an even stronger reduction to ~20% Pak2 association relative to wt Nef (Fig. 4, *D* and *E*). These data suggest that the T75 and F85 mutations in the PxxP region of Nef affect its ability to interact with SH3 domain-containing ligands.

Effects of Nef mutations on viral replication in PBMC

Nef significantly enhances virus replication in primary CD4 T cells, particularly if these cells are exposed to HIV-1 before activation with mitogens (42, 43). Because amino acid substitutions from prolines to alanines in the PxxP region have been shown to decrease this activity (25, 26, 32), we asked whether the T75 and F85 mutations would have similar effects.

Freshly isolated PBMC from two HIV-negative donors were first exposed to wt or various variant viruses for 3 days and subsequently activated by PHA. In both donors' PBMC, the wt and T75 variant viruses showed comparable replication kinetics, whereas the replication of the ΔNef virus was substantially delayed (Fig. 5*A*). The replication of the F85 variant virus was comparable to that of the wt virus in PBMC of a donor and was partially impaired with PBMC of another one (Fig. 5*A*). In contrast, the double TF variant virus showed delayed replication kinetics in PBMC from both donors (Fig. 5*A*).

To account for this donor variability, results from a total of five donors are summarized in Fig. 5*B*. As all PBMC samples showed a peak on day 9 after infection with the wt virus, the amounts of p24 Ag at day 9 after infection with the variant viruses were measured and statistically analyzed by multiple comparisons vs the wt (Fig. 5*B*). The ΔNef virus showed reproducibly the weakest replicative capacity under this assay condition, in good agreement with previous reports (26, 32). In addition, the TF double variant virus showed diminished capacity for viral replication compared with the wt; whereas each type of single variant virus did not show much difference in replication capacity (Fig. 5*B*). These data demonstrate that, even in the absence of HIV-specific CTL responses, the combination of T75 and F85 mutations is disadvantageous for Nef's ability to enhance virus replication.

Discussion

It is thought that the *nef* gene has higher levels of mutational plasticity in response to selective pressures compared with genes exhibiting structural or functional constraints (e.g., Gag, protease, reverse transcriptase, or integrase), because it exhibits considerable sequence diversity in vivo. In fact, some CTL escape variants of Nef, such as those with the mutations located in the CTL epitopes restricted by HLA-B*57 and HLA-A*24, have been suggested to have minimum fitness cost on the virus. This is because, in such a region, reversions are not often observed after transmission of the virus to new hosts who are negative for that particular *HLA-1* allele and the mutations are readily fixed in the population in the meantime (44, 45). In contrast, we show in the present study that the naturally arising mutations in the well-conserved PxxP region of HIV-1 Nef are selected under active CTL-mediated selective force at work and these mutations alone or in combination can modulate the pathogenic function by HIV-1 Nef including HLA-I down-regulation, enhancement of viral replication, and association with an activated cellular kinase, strongly suggesting that these mutations can impose functional constraints on the Nef activity and viral replication in vivo. Considering that various Nef activities substantially vary during the course of an infection at different stages of disease progression (4) and that there are substantial numbers of HLA-I-associated sequence variations in Nef (46–48), immunosurveillance by the Nef-specific CTLs plays additional roles in modulating the pathogenic potential of HIV-1 through selection of CTL-escape mutations in Nef particularly those in a well-conserved functional region.

It is obvious that HLA-B35-restricted CTL responses were shifted in patients during the early to chronic phase of an HIV-1 infection in our study, as the Nef VY8 epitope was dominantly recognized by CTLs relatively early in the infection, whereas the N-terminal extended RY11 epitope was recognized by CTLs in the chronic phase. This observation is in line with previous reports showing that CTL epitope specificity is different during the course of an HIV infection (23, 49, 50). Particularly, an immunodominant response directed against the HIV Gag p17-derived, HLA-A0201-restricted SL9 epitope (SLYNTVATL) was not detected early in an infection (50). Although the mechanisms underlying this phenomenon are not yet known, one possible explanation is that the responses detected in the early stage of an infection could have "mutated away," opening the field for a second wave of CTL specificities taking over in their place. The CTLs induced by a second or third waves of CTL specificities may have decreased antiviral effectiveness as predicted in the mathematical antigenic oscillation

model proposed by Nowak et al. (51). Our data support this scenario that the highly active VY8-specific CTLs elicited early in an infection were rendered ineffective apparently due to the acquisition of the F85 Nef mutation by the virus and that subsequently the cross-reactive RY11-specific CTLs, yet having moderate antiviral activity, became dominant. It is interest to note that the T75 variant, which had been selected by RY11-specific CTLs during the chronic phase, can induce de novo variant-specific CTLs with less effective Ag-specific proliferative capacity, further reducing antiviral activity of CTLs in vivo (29).

HLA-B*35 has been documented to be associated with rapid disease progression to AIDS (52). However, a further detailed study showed that individuals having HLA-B*35 allelic variants, including B3502/3503/3504, progress more rapidly to AIDS than do those with HLA-B*3501 (53). All the HLA-B35$^+$ subjects in this study were considered to carry HLA-B*3501, as the HLA-B*3501 is highly prevalent in the HLA-B35$^+$ Japanese population, though we have not yet done the genotypic analysis of *HLA-B* loci of all of the subjects. Further studies are needed to clarify whether CTL responses toward the PxxP region of Nef may be associated with the difference in the disease progression among HIV-infected patients having different HLA-B35 allelic variants.

Although the TF double mutation provided the best CTL escape of the Nef variants tested here, this variant was barely selected in HLA-B35$^+$ patients. This suggested that important functional constrains imposed by these combinatorial mutations precluded selection of these variants. The present study revealed at least two independent possible reasons for such a counterselection. First, the T75 and F85 double mutation in HIV-1 Nef significantly reduced the down-regulation activity of HLA-I and resulted in increased recognition by Pol- and Env-specific CTLs. Because down-regulation of MHC-I by SIV Nef in rhesus macaques limits CD8 T cell-mediated killing and contributes to the pathogenic effect of Nef in vivo (14), these results suggest that the sustained HLA-I down-regulation activity by HIV-1 Nef is required for efficient viral replication in vivo. This observation is in line with a previous report demonstrating that Nef mutations selected by Nef-specific CTLs in vitro, although most mutations disrupted *nef* reading frames in their study, leads to progeny virions that are increased in their susceptibility to CTLs with specificities for proteins other than Nef (54). However, the mutations in that report are different from representative naturally arising variations (54) as the *nef* reading frame is highly maintained intact in vivo (55) and large deletions or frame shifts are rarely observed. In contrast, the current study focused on the naturally arising mutations that are selected under Nef-specific CTL responses in vivo.

Second, the double mutation also affected PxxP-dependent activities of Nef in the absence of HIV-specific CTLs and significantly impaired Nef's ability to boost HIV-1 replication in primary human T lymphocytes. Because the individual mutations caused no significant impairment to HIV replication in the experimental system used, these results also help to explain why the double mutant is counterselected in HIV-infected patients. On the molecular level, Nef's effects on viral replication are likely mediated by a number of yet to be fully defined protein interactions. Among others, its association with Pak2 activity has also been implicated in the Nef-mediated enhancement of virus infectivity and replication (40, 56, 57). In this scenario, our results suggest that the reduction of Nef-Pak2 below a certain threshold activity may contribute to the reduction of Nef's ability to boost HIV spread. More importantly, the reduction of Pak2 association indicates that CTL escape Nef variants are impaired in their interaction with SH3 domains, which is expected to have select functional consequences in various cellular environments.

Together, these results demonstrate that CTL escape has severe consequences on the functionality of the PxxP motif in Nef, both for its role in immunoevasion and intrinsic replicative potential of the virus. Thus, a vaccine regimen that can elicit CTL responses targeting the regions involved in HLA-I down-regulation activity by Nef could be a potent candidate for future vaccine design.

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Disclosures

The authors have no financial conflict of interest.

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Different immunodominance of HIV-1-specific CTL epitopes among three subtypes of HLA-A*26 associated with slow progression to AIDS

Yuka Kawashima [a], Manami Satoh [a], Shinichi Oka [b], Takuma Shirasaka [c], Masafumi Takiguchi [a,*]

[a] Division of Viral Immunology, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan
[b] AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan
[c] AIDS Medical Center, National Hospital Organization, Osaka National Hospital, Japan

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Abstract

It is speculated that HLA-A*26-restricted HIV-1-specific CTLs can control HIV-1, since HLA-A*26 is associated with a slow progression to AIDS. In three major HLA-A*26 subtypes, HLA-A*2601-restricted, and HLA-A*2603-restricted HIV-1 epitopes have been identified, but HLA-A*2602-restricted ones have not. We here identified HLA-A*2602-restricted HIV-1 epitopes by using reverse immunogenetics and compared the immunodominance of the epitopes among the three subtypes. Out of 110 HIV-1 peptides carrying HLA-A*26 anchor residues, only the Gag169-177 peptide, which had been previously identified as an HLA-A*2601- and HLA-A*2603-restricted immunodominant epitope, induced Gag169-177-specific $CD8^+$ T cells from only two of six HLA-A*2602^+ HIV-1-infected individuals. No difference in affinity of this epitope peptide was found among these three HLA-A*26 subtypes, indicating that Gag169-177 was effectively presented by HLA-A*2602 but recognized as a subdominant epitope in HIV-1-infected HLA-A*2602^+ individuals. These findings indicate different immunodominance of Gag169-177 epitope among 3 HLA-A*26 subtypes.
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Keywords: HLA-A*26; HLA-A*2602; HIV-1; Epitope; CTL

Human immunodeficiency virus type-1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) responses play an important role in the control of HIV-1 infections [1–5]. However, it is thought that HIV-1 can escape from the host immune system, since it fails to completely eradiate HIV-1 from infected individuals. There are several proposed mechanisms that would allow HIV-1-infected cells to escape from being killed by HIV-1-specific $CD8^+$ T cells [6–11]. A mutation within the viral epitopes recognized by CTLs is one of these mechanisms [6,7]. Identification and characterization of HIV-1 CTL epitopes are therefore necessary for studies on the immunopathogenesis of AIDS. In addition, since HIV-1-specific CTLs are expected to suppress HIV-1 replication *in vivo*, characterization of these epitopes is also necessary for studies aimed at developing HIV-1 vaccines and immunotherapy to induce HIV-1-specific CTLs, either of which might be expected to prevent HIV-1 infection and the progression to AIDS.

HLA-A*26 is one of the alleles associated with a slow progression to AIDS [12]. Therefore, identification and characterization of HIV-1-specific epitopes presented by this allele are necessary for studies on the immunopathogenesis of AIDS and vaccine development. Three HLA-A*26 subtypes, i.e., HLA-A*2601, HLA-A*2602, and HLA-A*2603, are found at a gene frequency of 7.7%, 2.3%, and 1.5%, respectively, in the Japanese population [13]. We previously identified four HLA-A*2601- and two HLA-A*2603-restricted HIV-1 epitopes by using reverse immunogenetics [14,15]. Both HLA-A*2601 and -A*2603 presented one immunodominant epitope, Gag169-177

* Corresponding author. Fax: +81 96 373 6532.
E-mail address: masafumi@kumamoto-u.ac.jp (M. Takiguchi).

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(EVIPMFSAL), which overlaps with the HLA-B*57-restricted immunodominant epitope KF11 (KAFSPEVIPMF) [16]. Gag169-177-specific CTLs have been speculated to control HIV-1 replication.

In the present study, we sought to identify HLA-A*2602-restricted HIV-1 epitopes by using reverse immunogenetics and to compare them with HLA-A*2601- and HLA-A*2603-restricted ones [14,15].

Materials and methods

Cells. C1R and TAP-defective cells of mouse cell line RMA-S were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). C1R cells expressing HLA-A*2601, -A*2602 or -A*2603 (C1R-A*2601, C1R-A*2602 or C1R-A*2603, respectively) were generated by transfecting the C1R cells with the HLA-A*2601, -A*2602 or -A*2603 gene, respectively [17]. RMA-S transfectants expressing HLA-A*2602 (RMA-S-A*2602) were previously generated [18]. C1R-A*2601, -A*2602 and -A*2603 were maintained in RPMI 1640 medium supplemented with 10% FCS and 0.2 mg/ml neomycin; and RMA-S-A* 2602, in RPMI 1640 medium supplemented with 10% FCS and 0.15 mg/ml hygromycin B.

Synthetic peptides. Sequences derived from four proteins of the human immunodeficiency virus type-1 SF2 strain (HIV-1: Env, Gag, Pol, and Nef) were screened for HLA-A*2602-binding motifs. Peptides were prepared by utilizing an automated multiple peptide synthesizer, with the Fmoc strategy followed by cleavage. The purity of the synthesized peptides was examined by mass spectrometry. Peptides with more than 90% of purity were used in the present study.

HLA-stabilization assay. Binding of HIV-1-derived peptides to HLA-A*2602 was measured as previously described by using RMA-S-A*2602 cells [18]. RMA-S-A*2602 cells were cultured for about 16 h. Then they were incubated with peptides at 26 °C for 1 h and subsequently at 37 °C for 3 h. Peptide-pulsed cells were stained with the HLA class I α_3 domain-specific mAb TP25.99 [19] and the FITC-conjugated IgG fraction of sheep anti-mouse Ig (Silenius Laboratories, Hawthorn Victoria, Australia). The mean fluorescence intensity (MFI) was measured by using a FACS Calibur (BD Bioscience, San Jose, CA, USA). HLA-A*2602-binding peptides were defined as those which at a concentration of 10^{-3} M caused a >25% increase in MFI compared with the MFI of control RMA-S-A*2602 cells cultured at 26 °C. The peptide concentration that yielded the half-maximal levels of the MFI was calculated and was reported as the BL50 value.

Patients. Blood samples were collected with informed consent from six HIV-1 clade B-infected patients with HLA-A*2602 (KO-003, KI-021, KI-030, KI-082, KI-382, and KI-478), 11 those with HLA-A*2601, and eight those with HLA-A*2603 at the AIDS Medical Center, National Hospital Organization, Osaka National Hospital or the AIDS Clinical Center, International Medical Center of Japan. Clinical stage of all patients tested was chronic one. Significant difference of CD4 count was not found among three subtype groups (HLA-A*2601:458±257, HLA-A*2602:564±299, HLA-A*2603:314±109). This study was approved by the ethical committees of Kumamoto University, International Medical Center of Japan, and Osaka National Hospital. Informed consent was obtained from all subjects, according to the Declaration of Helsinki.

Intracellular cytokine staining (ICC assay). After C1R-A*2601, C1R-A*2602 or C1R-A*2603 cells had been incubated for 60 min with each peptide (1 μM) or each peptide cocktail (1 μM concentration of each peptide), they were washed twice with RPMI-1640 containing 10% FCS. These C1R-A*26 cells and cultured PBMCs were incubated at 37 °C for 6 h at an effector-to-stimulator ratio of 1:4 after the addition of Brefeldin A (10 μg/ml). Next, the cells were stained with anti-CD8 mAb (DAKO Corporation, Flostrup, Denmark), fixed with 4% paraformaldehyde at 4 °C for 20 min, and then permeabilized at 4 °C for 10 min with PBS supplemented with 0.1% saponin containing 20% NCS (permeabilizing buffer). The cells were resuspended in the permeabilizing buffer and then stained with anti-IFN-γ mAb (BD Bioscience Pharmingen, San Diego, CA). The cells were finally resuspended in PBS containing 2% paraformaldehyde, and then the percentage of $CD8^+$ cells positive for intracellular IFN-γ was determined by using the FACSCalibur.

*ICC assay using C1R-A*2602 cells infected with recombinant HIV-1 vaccinia.* C1R-A*2602 cells were infected for 1 h at 37 °C with 10 plaque-forming units (per target cell) of recombinant vaccinia virus expressing HIV-1 SF2 Gag protein or of WT vaccinia virus and cultured for 16 h. These infected cells were washed twice with RPMI 1640 containing 10% FCS and then incubated with cultured effector cells at 37 °C for 6 h after the addition of Brefeldin A (10 μg/ml). The ability of the effector cells to produce IFN-γ was tested at an E:S ratio of 1:4. The cells were then stained with anti-CD8 mAb and anti-IFN-γ mAb.

Results and discussion

HLA-A*2602-binding peptides have two anchor residues, Val, Phe, Ile, Leu or Thr at position 2 and Tyr, Phe, Met or Leu at the C-terminus [17]. A previous study demonstrated that acidic amino acids (Asp and Glu) and a broad range of amino acids with the exception of positively charge amino acids function as an anchor at position 1 and the C-terminus, respectively [18]. Therefore, to identify HLA-A*2602-binding HIV-1 peptides, 8-mer to 11-mer sequences containing the anchor residues Asp or Glu at position 1, Val, Thr, Ile, Leu or Phe at position 2, and any amino acids except positively charged ones at the C-terminus were selected from the sequence of Gag, Pol, Nef, and Env proteins in the HIV-1 SF2 strain; and then 110 peptides matching these sequences were synthesized. The binding affinity of these synthesized peptides for the HLA-A*2602 molecule was tested by using the HLA-stabilization assay [18,20]. Representative results are shown in Fig. 1. Thirty-two out of these 110 peptides bound to HLA-A*2602 (Table 1). The frequency of HLA-A*2602-binding HIV-1 peptides was similar to that of HLA-A*2601- or HLA-A*2603-binding HIV-1 peptides previously identified by using the same 110 peptides [14,15].

PBMCs from three $HLA\text{-}A^*2602^+$ HIV-1-infected individuals (KO-003, KI-030 and KI-082) were stimulated

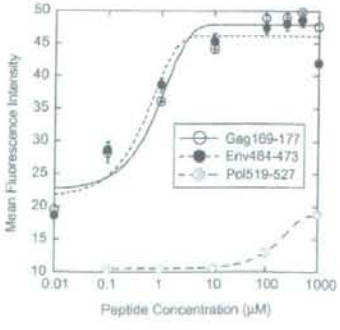

Fig. 1. Binding to HLA-A*2602 of HIV-1 peptides carrying HLA-A*2602 motif. Binding of the peptides carrying A*2602 anchors was measured by a stabilization assay using RMA-S-A*2602 cells. Representative results showing high-affinity peptides (Gag169-177 and Env464-473) and a very low-affinity peptide (Pol519-527) are given in this figure.

Table 1
HLA-A*2602-binding peptides

Sequence	Position	BL50[a]
EVFRPGGGDM	Env464-473	3.98×10^{-7}
EVIPMFSAL	Gag169-177	5.84×10^{-7}
ELKKIIGQV	Pol872-880	9.88×10^{-6}
EVNIVTDSQY	Pol647-656	1.21×10^{-5}
EVVIRSDNF	Env272-280	4.1×10^{-5}
ELNKRTQDF	Pol234-242	1.02×10^{-4}
EIKGEIKNCSF	Env147-157	1.62×10^{-4}
DIVIYQYMDDL	Pol332-342	1.94×10^{-4}
DTTNQKTEL	Pol626-634	2.78×10^{-4}
EIVASCDKCQL	Pol750-760	3.50×10^{-4}
ETVPVKLKPGM	Pol161-171	4.26×10^{-4}
ETWEAWWMEYW	Pol551-561	6.28×10^{-4}
ETWEAWWMEY	Pol551-560	$>1 \times 10^{-3}$
EICGHKAIGTV	Pol121-131	$>1 \times 10^{-3}$
EVVLGNVTENF	Env82-92	$>1 \times 10^{-3}$
DLRSLCLFSY	Env758-767	$>1 \times 10^{-3}$
DLWIYHTQGYF	Nef115-125	$>1 \times 10^{-3}$
EVIPLTEEA	Pol446-454	$>1 \times 10^{-3}$
ETPGIRYQY	Pol293-301	$>1 \times 10^{-3}$
EVYYDPSKDLV	Pol471-481	$>1 \times 10^{-3}$
EVYYDPSKDL	Pol471-480	$>1 \times 10^{-3}$
ETKLGKAGYV	Pol604-613	$>1 \times 10^{-3}$
EVHNVWATHAC	Env63-73	$>1 \times 10^{-3}$
EVQLGIPHPA	Pol244-253	$>1 \times 10^{-3}$
ELYPLTSLRS	Gag484-493	$>1 \times 10^{-3}$
DLNTMLNTV	Gag185-193	$>1 \times 10^{-3}$
DVKQLTEAV	Pol519-527	$>1 \times 10^{-3}$
ELYPLTSLRSL	Gag484-494	$>1 \times 10^{-3}$
DIQKLVGKL	Pol411-419	$>1 \times 10^{-3}$
DIAGTTSTL	Gag235-245	$>1 \times 10^{-3}$
ELRQHLLRW	Pol359-367	$>1 \times 10^{-3}$
DTKEALEKI	Gag96-104	$>1 \times 10^{-3}$

[a] The half maximal binding level was calculated as the peptide concentration yielding the half-maximal MFI.

in vitro for 14 days with a cocktail of HLA-A*2602-binding peptides containing 5–7 peptides (cocktail 1: Env464-473, Gag169-177, Pol647-656, Pol872-880, and Pol551-560; cocktail 2: Env272-280, Pol121-131, Env82-92, Pol551-561, Env758-767, Pol234-242, and Nef115-125; cocktail 3: Pol446-454, Pol293-301, Pol471-481, Pol471-480, Pol604-613, Env147-157, and Env63-73; cocktail 4: Pol244-253, Gag484-493, Gag185-193, Pol626-634, Pol519-527, Gag484-494, and Pol411-419; and cocktail 5: Gag235-243, Pol359-367, Pol161-171, Gag96-104, Pol750-760, and Pol332-342). IFN-γ production by each bulk culture in response to C1R-A*2602 prepulsed with the corresponding peptide cocktail was assessed by intracellular IFN-γ staining. All five cocktails failed to induce specific $CD8^+$ T cells among the cells in bulk culture obtained from patients KI-030 and KI-082. On the other hand, only cocktail 1 induced specific $CD8^+$ T cells among the cells in bulk culture obtained from patient KO-003 (Fig. 2A). To determine which peptides in the cocktail induced the specific $CD8^+$ T cells, we re-stimulated the cells of this bulk culture with C1R-A*2602 cells prepulsed with each single peptide in this cocktail to detect the specific $CD8^+$ T cells. Only the Gag196-177 peptide induced $CD8^+$ T cells producing IFN-γ (Fig. 2B).

To clarify whether Gag169-177 was a naturally occurring peptide, we investigated the ability of these peptide-specific $CD8^+$ T cells to produce IFN-γ after having stimulated them with C1R-A*2602 cells infected with recombinant HIV-1 vaccinia virus (r-HIV vaccinia). IFN-γ-producing cells were induced in the Gag169-177-specific $CD8^+$ T cell culture after stimulation with r-HIV vaccinia-infected C1R-A*2602 cells, whereas they were not detected in that stimulated with WT vaccinia-infected C1R-A*2602 cells or r-HIV vaccinia-infected C1R cells (Fig. 2C). These results indicate that Gag169-177 is indeed a naturally occurring HIV-1 epitope peptide presented by HLA-A*2602.

Gag169-177-specific $CD8^+$ T cells were induced from only 1 of the 3 HLA-A*2602^+ HIV-1-infected individuals; whereas two HLA-A*2601 epitopes, Pol647-656 and Env464-473, which were also HLA-A*2602-binding peptides, failed to induce specific T cells in these individuals. To address lower frequency of these peptide-specific $CD8^+$ T cells, we investigated the induction of the specific $CD8^+$ T cells by stimulating PBMC from three additional donors carrying HLA-A*2602 with these three peptides. Only Gag169-177 peptide induced the specific $CD8^+$ T cells in one donor. Thus, Gag169-177-specific $CD8^+$ T cells were induced in 2 of 6 HLA-A*2602^+ HIV-1-infected individuals whereas they were induced in 8 of 11 HLA-A*2601^+ and 7 of 8 HLA-A*2603^+ HIV-1-infected ones (Fig. 2D). These results indicate that Gag169-177 is a subdominant epitope in the HLA-A*2602^+ donors.

Gag169-177-specific $CD8^+$ T cells were previously detected in five of seven HLA-A*2601^+ donors and in all four HLA-A*2603^+ ones [14,15], suggesting that Gag169-177 is a dominant epitope in HIV-1-infected individuals carrying either of these HLA-A*26 alleles. Additional experiments in the present study confirmed the immunodominance of this epitope in HLA-A*2601^+ and HLA-A*2603^+ donors (Fig. 2D). In contrast, they were detected in only two of six HLA-A*2602^+ donors, indicating Gag169-177 to be a subdominant epitope in HLA-A*2602^+ individuals. Interestingly, they were elicited in only a long-term non-progressor and a controller having low viral load. HLA-A*1101-restricted Gag349-, Nef73-, and/or Nef84-specific $CD8^+$ T cells were induces in two of four HLA-A*1101^+/A*2602^+ donors who did not have Gag169-177-specific $CD8^+$ T cells (data not shown), supporting that these HLA-A*2602^+ donors maintain HIV-1-specific cellular immunity. The affinity of Gag169-177 for HLA-A*2602 was similar to that for HLA-A*2601 and -A*2603 (Table 2), indicating that Gag169-177 was effectively presented by HLA-A*2602 but recognized as a subdominant epitope in HIV-1-infected individuals carrying HLA-A*2602. Pol604-612 and Env63-72 are HLA-A*2601 and HLA-A*2603 epitopes, respectively [14,15]. These epitope peptides failed to bind to HLA-A*2602 (data not shown). Since only one amino acid, at residue 116, differs between HLA-A*2602 and the other two subtypes (Asp

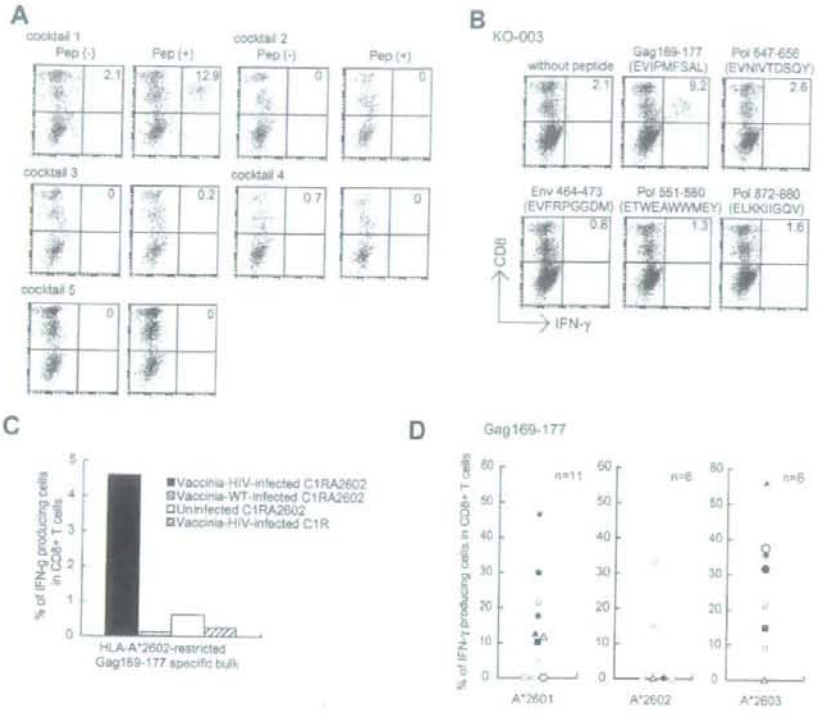

Fig. 2. Identification and recognition of Gag169-177-specific CD8$^+$ T cells. (A) PBMCs from HIV-1-infected individuals with HLA-A*2602 (KO-003) were cultured for 10–14 days after they had been stimulated with the indicated cocktails of HLA-A*2602-binding peptides. The cultured cells were then tested for IFN-γ production by CD8$^+$ T cells after stimulation with C1R-A*2602 cells prepulsed with the peptide cocktails. (B) Induction of Gag169-177-specific CD8$^+$ T cells. PBMCs from KO-003 were stimulated with peptide cocktail 1 and cultured for 10–14 days. The cultured cells were stimulated with C1R-A*2602 cells prepulsed with each single peptide included in cocktail 1. The population of IFN-γ-producing CD8$^+$ T cells was determined by using flow cytometry. The percentage of IFN-γ-producing CD8$^+$ T cells is presented at the right of the upper right-hand quadrant. (C) Presentation of Gag169-177 by HLA-A*2602 on r-HIV-1 vaccinia-infected cells. Bulk cultures containing Gag169-177-specific CD8$^+$ T cells were examined for IFN-γ production after they had been stimulated with C1R-A*2602 cells infected with wild-type vaccinia (Vaccinia-WT) or with C1R-A*2602 cells or with C1R cells infected with r-HIV-1Gag-vaccinia (Vaccinia-HIV-1), or uninfected C1R-A*2602 cells (Uninfected). The percentage of IFN-γ-producing CD8$^+$ T cells was measured by using flow cytometry. (D) Percentage of Gag169-177-specific CD8$^+$ T cells in HIV-1-infected individuals having three HLA-A*26 subtypes. The percentage of IFN-γ-producing cells among CD8$^+$ T cells from each individual was plotted in the graph. The percentage of IFN-γ-producing cells in the cultures was measured by using flow cytometry after they had been stimulated with the corresponding C1R-A*26 cells prepulsed with Gag169-177 peptide.

Table 2
Comparison of binding affinity of HLA-A*26 epitope peptides among three HLA-A*26 subtypes and induction of the peptide-specific CD8$^+$ T cells

	Binding affinity (BL50)			Comparison of binding affinity		Frequency[a]		
	A*2601	A*2602	A*2603	A*2602/A*2601	A*2602/A*2603	A*2601	A*2602	A*2603
Gag169-177	7.5×10^{-7}	5.8×10^{-7}	2.1×10^{-6}	0.77	0.28	8/11	2/6	7/8
Env63-72	1.1×10^{-4}	No binding	7.6×10^{-7}	—	—	0/11	NT	3/8
Pol604-612	6.5×10^{-5}	No binding	No binding	—	—	10/11	NT	NT
Pol647-656	6.3×10^{-5}	1.2×10^{-5}	6.6×10^{-4}	0.19	0.02	1/11	0/6	0/8
Env464-473	1.5×10^{-6}	4.0×10^{-7}	3.7×10^{-5}	0.27	0.01	1/11	0/6	0/8

NT, not tested.
[a] The number of individuals in whom peptide specific CD8$^+$ T cells were induced/the number of tested individuals.

for HLA-A*2601 and -A*2603, but Asn for HLA-A*2602), this substitution in the floor of the peptide binding groove is thought to affect the binding of these peptides.

In summary, Gag169-177 was not an HIV-1 immunodominant epitope in HIV-1-infected individuals carrying HLA-A*2602, whereas it was one in those carrying HLA-

A*2601 or -A*2603. These findings imply the possibility that HLA-A*2602 is not an allele associated with a slow progression to AIDS. However, it still remains unknown that Gag169-177-specific CTLs can control HIV-1. A further study using a cohort of a large number of subjects will clarify the association of these HLA-A*26 subtypes or Gag169-177-specific CTLs with the progression of AIDS.

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REVIEW

Pharmacogenetic information derived from analysis of *HLA* alleles

Hiroyuki Gatanaga†, Haruhito Honda & Shinichi Oka

†Author for correspondence AIDS Clinical Center, International Medical Center of Japan, 1–21–1 Toyama, Shinjuku-ku, Tokyo 162–8655, Japan Tel.: +81 332 027 181; Fax: +81 352 736 483; E-mail: higatana@imcj.acc.go.jp

A large amount of pharmacogenetic information has, in particular, accumulated on the association between human leukocyte antigen (*HLA*) alleles and hypersensitivity to certain drugs. Prospective *HLA* typing has dramatically reduced the risk of abacavir hypersensitivity because of its strong association with *HLA-B*5701*. Significant predisposition to nevirapine hypersensitivity has been reported in Caucasian Australians harboring *HLA-DRB1*0101* with high CD4+ T-cell counts, and Sardinians and Japanese harboring *HLA-Cw8*. A strong association between carbamazepine hypersensitivity and *HLA-B*1502* has been reported in Han Chinese. Most Han Chinese individuals with allopurinol-induced severe cutaneous adverse reactions are positive for *HLA-B*5801*. *HLA* typing can stratify risk of hypersensitivity to certain drugs and allow personalized treatment, although the patients should be monitored closely even if they are negative for *HLA* alleles associated with hypersensitivity.

Keywords: abacavir, allopurinol, carbamazepine, HIV, hypersensitivity, nevirapine

Hypersensitivity reactions can occur with most drugs, although their frequency, severity and clinical manifestations vary. They commonly involve the skin and mucosal surfaces, and in severe cases can result in Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). Other severe hypersensitivity reactions can affect other organs such as the liver (hepatitis), lung (pneumonitis) and digestive system (gastrointestinal bleeding), and show more generalized symptoms [1]. Rechallenge with the same drugs usually induces more severe reactions, even fatal reactions in some cases, suggesting that hypersensitivity reactions are immunological memory responses after sensitization. These reactions affect only a minority of patients taking the drug. However, hereditary forms of severe drug hypersensitivity and cases occurring in identical twins have been reported, implying the involvement of certain genetic factors in predisposing individuals to such hypersensitivity reactions [2,3]. Given the immunological basis of their mechanisms, it is not surprising that the associations between human leukocyte antigen (*HLA*) alleles and hypersensitivity to some drugs have been reported during the past decade. HLA is a key molecule in T-cell-mediated immune reactions. It presents antigens (usually eight or nine peptide residues) to T-cell receptors (TCRs), thereby selecting antigen-specific T cells and initiating immune responses. Such reactions usually occur in viral and bacterial infections, and microbe-derived peptides restricted by host HLA are targeted by antigen-specific immune responses [4]. Since drugs and their metabolites are small chemical compounds, they do not usually trigger immune reactions by themselves. However, they may conjugate or bind to intracellular proteins, where they are presented as antigens or haptens by MHC class I or class II molecules to CD8+ or CD4+ T cells, resulting in activation of drug-specific T cells [5,6].

We will review in this article the recent literature on the association between *HLA* allele and hypersensitivity reactions to abacavir, nevirapine, carbamazepine and allopurinol. We will also discuss the clinical implications of such associations, with a special focus on the association of *HLA-B*5701* with hypersensitivity to abacavir, an anti-HIV-1 agent, because it is the most well analyzed and reported. Widespread genetic screening of such association in HIV-1-infected individuals can be used to prevent hypersensitivity reactions.

Abacavir hypersensitivity & *HLA-B*5701*

The currently recommended anti-HIV-1 treatment is the use of a combination regimen. The initial regimen for treatment-naive infected individuals should contain two nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) and either a non-nucleoside reverse transcriptase (NNRTI) or an HIV protease inhibitor [7,10]. The action of the NRTI drug class is to inhibit viral replication through competitive inhibition of viral RNA-dependent DNA polymerase (reverse transcriptase) that allows the creation of a nascent DNA sequence from its own RNA template, whereas NNRTI drugs function by direct binding and inactivation of the polymerase. HIV protease

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inhibitors prevent the cleavage of the Gag protein and Gag–Pol protein precursors, thus inhibiting viral replication at a later stage in the replication cycle [8]. NRTIs have been prescribed since the late 1980s, and their advantages and disadvantages are well recognized. A major adverse effect of NRTI is mitochondrial toxicity, which can result in life-threatening lactic acidosis [9–11]. Two recently developed NRTIs, tenofovir disoproxil fumarate (TDF) and abacavir, have low mitochondrial toxicity and both can be prescribed with once-daily dosing [12,13]. However, only TDF is listed as a preferred NRTI in the guideline of the Department of Health and Human Services. On the other hand, abacavir is listed as an alternative NRTI because of its potential for serious hypersensitivity reactions in 5–8% of Caucasians [14,101].

The safety data for abacavir are well described and based on approximately 200,000 patients who received abacavir in clinical trials. The most important limitation to continuous use of this drug is hypersensitivity reactions [15,16]. Such reactions are multi-organ clinical syndromes, which generally occur within the first 6 weeks of abacavir treatment, and typically present with fever, skin rash, malaise/fatigue, gastrointestinal symptoms (e.g., nausea, vomiting and diarrhea) and/or respiratory symptoms (e.g., dyspnea, cough and pharyngitis) [15]. It is important to make a correct diagnosis of abacavir-related hypersensitivity reactions, since a rechallenge with abacavir after an initial reaction can evoke a more rapid reappearance of more severe symptoms within hours of re-exposure, which could result in death in some cases [17–19]. Unfortunately, abacavir hypersensitivity reactions are sometimes difficult to distinguish from systemic viral illness or similar drug reactions caused by other concurrently administered antiretroviral drugs or antibiotics [20].

Meta-analysis of clinical trials indicating a low risk of abacavir hypersensitivity reactions in black people, as well as a case report of familial hypersensitivity, are strong indicators of a genetic basis of this idiosyncratic syndrome [21,22]. Two independent studies identified a strong association between abacavir hypersensitivity and *HLA-B*5701*, which can assist clinicians in predicting those individuals who could develop hypersensitivity reactions and to make a correct diagnosis of hypersensitivity reactions in abacavir-treated individuals, although the association was observed only in Caucasians but not in the black people originally (Table 1) [23,24]. In addition to *HLA-B*5701*, the possession of *HLA-DR7* and *HLA-DQ3*, which are markers of the 57.1 ancestral haplotype, is associated with an increase in the odds ratio of hypersensitivity risk, suggesting that another causative genetic region is linked to *HLA-B*5701* [23]. Fine recombinant genetic mapping has identified a significant linkage disequilibrium of the haplotypic M493T polymorphism of heat shock protein-Hom (Hsp70-Hom; Hsp1AL) and *HLA-B*5701* in abacavir hypersensitive cases, which simplified and enhanced the discrimination of hypersensitive subjects from tolerant controls when compared with the *HLA-B*5701* test alone (Table 1) [25]. The Hsp70-Hom M493T polymorphism may facilitate loading of abacavir- or its metabolite-haptenated endogenous peptides onto *HLA-B*5701* [26]. High intracellular and extracellular levels of TNF are

Table 1. Drug hypersensitivity and associated *HLA* alleles.

Study	Drug	HLA	Population	OR	Pc	Ref.
Mallal *et al.* (2002)	Abacavir	*B*5701*	Australian	117	$<10^{-4}$	[23]
Hetherington *et al.* (2002)	Abacavir	*B*5701*	British	24	$<10^{-4}$	[24]
Martin *et al.* (2004)	Abacavir	*B*5701*	Australian	960	$<10^{-4}$	[25]
Martin *et al.* (2005)	Nevirapine	*DRB1*0101* and high CD4	Caucasian Australian	18	0.0006	[58]
Littera *et al.* (2006)	Nevirapine	*Cw8-B14(65)*[‡]	Sardinian	15	0.05	[59]
Gatanaga *et al.* (2007)	Nevirapine	*Cw8*	Japanese	6.2	0.03	[60]
Chung *et al.* (2004)	Carbamazepine	*B*1502*	Han Chinese	2504	$<10^{-4}$	[68]
Hung *et al.* (2006)	Carbamazepine	*B*1502*	Han Chinese	1357	$<10^{-4}$	[69]
Hung *et al.* (2005)	Allopurinol	*B*5801*	Han Chinese	580	$<10^{-4}$	[75]

[‡]Cw*0802 and B*1402 are in strong linkage equilibrium in Sardinians.

present in abacavir-stimulated peripheral blood mononuclear cells (PBMCs) of abacavir-hypersensitive patients, relative to those of abacavir-tolerant individuals, and depletion of $CD8^+$ T cells results in reduction of TNF levels [25]. Considering that marked infiltration of $CD8^+$ T cells is observed in cutaneous abacavir patch testing of hypersensitive patients and that higher $CD8^+$ T-cell count is a risk factor of hypersensitivity reactions, *HLA-B*5701*-restricted $CD8^+$ T cells must play a major pathogenic role in abacavir hypersensitivity reactions [27–29].

Prospective *HLA-B*5701* genetic screening has been instituted in clinical practice in Western Australia, the UK and Paris for abacavir-naive patients, and this had markedly reduced the risk of developing abacavir hypersensitivity (Table 2) [30–32]. This strategy unexpectedly reduced the proportion of patients who stopped their treatment after the appearance of symptoms that were otherwise unrelated to hypersensitivity reactions, suggesting that genetic screening seems to prevent overestimation of hypersensitivity reactions with subsequent discontinuation of abacavir in *HLA-B*5701*-negative individuals [30,32]. The PREDICT-1 study randomized patients either to receive abacavir according to standard of care or to be prospectively screened for *HLA-B*5701* before starting abacavir (to exclude *HLA-B*5701* carriers) [33]. The incidence of hypersensitivity reactions was significantly lower in the prospective screening arm compared with the control arm. However, most of the screened patients described above were Caucasian, and the utility and cost–effectiveness of the genetic screening largely depends on the prevalence of *HLA-B*5701* in the targeted population [34]. The prevalence of *HLA-B*5701* among Hispanics and black people is lower than Caucasians, and the relationship between *HLA-B*5701* and abacavir hypersensitivity was described as weak in Hispanics and nonexistent in black patients [35,36]. The SHAPE study corroborated the low rate of abacavir hypersensitivity immunologically confirmed by skin patch testing in black patients, but it also reported high sensitivity of *HLA-B*5701* in immunologically validated cases in both whites and blacks, suggesting the importance of supplementing a clinical definition of abacavir hypersensitivity by immunological assessment [37]. In our study, none of the 669 Japanese HIV-1-infected patients had *HLA-B*5701*, yet hypersensitivity reactions occurred in seven (all *HLA-B*5701*-negative, not immunologically confirmed) of 536 Japanese patients exposed to abacavir [38]. Thus, genetic screening of *HLA-B*5701* does not seem cost-effective in Japanese populations. Close monitoring of patients after abacavir prescription without HLA typing may be a more reasonable approach in the populations that do not carry *HLA-B*5701*.

Interestingly, strong reponses of *HLA-B*57*-restricted cytotoxic T lymphocytes can ocur against multiple HIV-1 epitopes, which is considered to result in slow disease progression of *HLA-B*57*-positive HIV-1-infected individuals [39,40]. One of the major *HLA-B*57*-restricted epitopes is located in codons 244–252 of HIV-1 reverse transcriptase, which is routinely sequenced as a part of drug-resistance testing [7,41,101]. Furthermore, cytotoxic T lymphocytes escape mutations (wild-type V to E, M and L) are commonly observed at codon 245 in *HLA-B*57*-positive patients, which may serve as an indirect marker for the presence of *HLA-B*5701* [40,42]. In one study [43], the negative predictive value was over 99% (meaning that the presence of wild-type amino acid V at codon 245

Table 2. Reduced frequencies of abacavir hypersensitivity reactions after *HLA-B*5701* genetic screening.

Study	Country	n (%)‡		p-value	Ref.
		Before screening	*After screening*		
Rauch *et al.* (2006)	Australia	16/199 (8.0)	3§/151 (2.0)	0.01	[30]
Reeves *et al.* (2006)	UK	20/321 (6.2)	1¶/155 (0.6)	0.002	[31]
Zucman *et al.* (2007)	France	11#/49 (22.4)	0/128 (0)	$<10^{-4}$	[32]

‡*Number (%) of hypersensitive patients/abacavir-treated patients.*

§*All three individuals were* HLA-B*5701 *positive; two inadvertently exposed to abacavir because of a lack of review of HLA results, and one on the basis of his own content.*

¶HLA-B*5701 *negative; non-HIV-expert physician discontinued therapy because of possible hypersensitivity reactions.*

#*Included five* HLA-B*5701 *negative cases of possible hypersensitivity based on wide-range clinical criteria.*

excludes the possibility of *HLA-B*5701* in >99% of cases), while the positive predictive value was low (20%). These results suggest that abacavir can be safely prescribed to most HIV-1-infected patients harboring wild-type V at codon 245 in reverse transcriptase [43]. This method can save the cost of HLA typing by utilizing the HIV-1 sequence data, which are obtained from routine resistance testing approved by the public and private health insurance industries of many developed countries. However, it may result in inadequate withholding of abacavir in a significant number of *HLA-B*5701*-negative patients infected with escape HIV-1 variants, because these escape mutations are often observed and probably able to persist over long periods even in the absence of *HLA-B*5701*-restricted cytotoxic T lymphocyte pressure. Another problem is differences among HIV-1 subtypes. The wild-type amino acid at codon 245 in reverse transcriptase is V only in HIV-1 subtype B, which is most prevalent in developed countries, but is another amino acid such as Q or E in non-B subtypes. Therefore, this method is not suitable when the obtained HIV-1 sequence in phylogenetic analysis belongs to non-B subtypes, which decreases its utility in African and Asian countries where non-B subtypes are prevalent. Considering that practical and accurate HLA typing has already been implemented and is effectively identifying *HLA-B*5701* carriers [44], direct HLA typing is a more simple and better approach to stratify the risk of abacavir hypersensitivity than speculating HLA type from HIV-1 sequences.

Nevirapine hypersensitivity & associated *HLA* alleles

Nevirapine is also a well-tolerated anti-HIV-1 agent, which is listed as an alternative NNRTI in the HIV-1 treatment guideline of the Department of Health and Human Services [45,101]. The most common adverse event associated with the use of nevirapine is hypersensitive reactions (observed in 4.9% of recipients), which are characterized by a combination of rash, fever or hepatitis, and typically occurs within the first 6 weeks of initiation of treatment and can be more rapid and severe with re-challenge [46,47]. Woman with high $CD4^+$ T-cell counts appear to be at higher risk of hypersensitivity reactions [48,49]. The HIV-1 treatment guidelines do not recommend the use of nevirapine for female patients with $CD4^+$ T cell counts over 250 cells/mm^3 and male patients with $CD4^+$ T-cell counts over 400 cells/mm^3 [7,50–53,101]. A higher incidence of hypersensitivity reactions was reported in non-HIV-infected individuals who received nevirapine as part of post-exposure prophylactic treatment, probably associated with a high CD4 count [54]. Usually, cutaneous diseases, including drug hypersensitivity to sulfamethoxazole, dapsone and antituberculous agents, are extremely common in patients with HIV infection, and their incidence increases as immune function deteriorates [55]. However, conversely, in the case of nevirapine hypersensitivity, normal and relatively maintained immune function is a risk factor for unknown reasons [56].

The description of nevirapine-induced SJS in a Ugandan mother and her son suggests a genetic basis for nevirapine hypersensitivity [57]. The possession of *HLA-DRB1*0101* is associated with increased risk of nevirapine hypersensitivity involving multisystemic or hepatotoxic reactions, and which was abrogated by low $CD4^+$ T-cell counts, in the Western Australian HIV Cohort (Table 1) [58]. Littera *et al.* reported that the *HLA-Cw*0802-B*1402* haplotype is associated with nevirapine hypersensitivity in Sardinian patients [59]. We also reported a significant association between *HLA-Cw8* and nevirapine hypersensitivity in Japanese patients, suggesting that nevirapine or its metabolite coupled with *HLA-Cw8* antigen may be expressed on the cell surface and may induce hypersensitivity reactions (Table 1) [60]. In this regard, there was no significant association between *HLA-DRB1*0101* and hypersensitivity in the Sardinian and Japanese cohorts described above, implying that primarily determining HLA alleles may be different among populations. Isolated mild rash and simple hepatotoxicity often occur within 6 weeks of nevirapine treatment initiation. It is possible that this reaction is pathologically different from the severe hypersensitivity reactions, making the definition of hypersensitivity confusing and comparison of different studies difficult [58,61,62]. Establishment of a standardized definition and accurate diagnosis of hypersensitivity seems indispensable for further study of the linkage between HLA alleles and nevirapine hypersensitivity.

Carbamazepine-induced SJS/TEN & *HLA-B*1502*

Carbamazepine is one of the most widely used anticonvulsants, and is also used in bipolar depression and trigeminal neuralgia. Carbamazepine is generally well tolerated but can cause dose-dependent adverse reactions such as dizziness and nystagmus [63]. It is also associated with idiosyncratic hypersensitivity reactions, most

commonly skin rashes such as SJS and TEN, accompanied with fever, lymphadenopathy, and multiorgan-sysem abnormalities [64]. A high frequency of carbamazepine-related hypersensitivity reactions was reported in South-East Asian countries compared with 0.01–0.1% in Caucasians [64–67]. Furthermore, carbamazepine hypersensitivity was reported in identical twins [3]. These studies suggest that susceptibility to such reactions may be genetically determined.

A Taiwanese study reported a strong association between carbamazepine-induced SJS/TEN and the *HLA-B*1502* allele in Han Chinese [68]. The finding was confirmed later by the same group in another study that included patients who were Han Chinese or Chinese descendants from Taiwan, Hong Kong, China and the USA (Table 1) [69]. The allele frequency of *HLA-B*1502* is 3–12% in South-East Asians and less than 0.1% in Caucasians, which may explain the higher incidence of carbamazepine-induced SJS/TEN in South-East Asia. In one European study, 15 patients with carbamazepine-induced SJS/TEN were analyzed and five patients who had a parent of Asian origin were positive for the *HLA-B*1502* allele. The remaining ten patients, who were Caucasians, were *HLA-B*1502*-negative [70]. Another European study of Caucasians did not find any *HLA-B*1502*-positive patients who were hypersensitive to carbamazepine [71]. Considered together, *HLA-B*1502* does not seem to be associated with carbamazepine hypersensitivity in the Caucasian population and ethnicity seems important. While it seems conceivable that the causative genetic region of carbamazepine hypersensitivity is linked to *HLA-B*1502*, especially in the Han Chinese population, fine recombinant genetic mapping confirmed the susceptibility gene is *HLA-B*1502* itself [69].

Allopurinol-induced severe cutaneous adverse reactions & *HLA-B*5801*

Allopurinol is widely used for hyperuricemia and recurrent urate kidney stones [72]. However, it is also one of the most frequent causes of severe cutaneous adverse reactions including SJS and TEN [73]. Familial predisposition has been reported and susceptibility to such idiosyncratic reactions is thought to be genetically determined [74]. One Taiwanese study reported a strong association between allopurinol hypersensitivity and *HLA-B*5801* in a Han Chinese population and recombinant genetic mapping further identified *HLA-B*5801* itself as the major susceptibility gene (Table 1) [75]. In support of these results, a Japanese group reported three cases with different manifestations of allopurinol hypersensitivity and all of them were positive for *HLA-B*58* [76].

Conclusion

We reviewed here the HLA association with hypersensitivity to abacavir, nevirapine, carbamazepine and allopurinol. Considering that hypersensitivity reactions to abacavir can be life-threatening and even fatal, abacavir prescription to *HLA-B*5701* should be avoided. The following prescriptions should be followed by close monitoring of the patients: nevirapine to patients positive for *HLA-DRB1*0101* or *Cw8*, carbamazepine to *HLA-B*1502* holders and allopurinol to *HLA-B*5801*-positive patients, even if the patient is from a population with no described allele association, because one cannot exclude possible association. It is noteworthy that pharmacogenetic studies are more likely to yield negative results when conducted in populations with low frequencies of the possibly associated allele [77]. More importantly, patients treated with any of these drugs should be monitored closely even if they are negative for *HLA* alleles that are known to be associated with hypersensitivity. Hypersensitivity reactions can potentially occur in any patient as they may hold *HLA* alleles that have yet unreported associations with hypersensitivity. Application of genetic screening should not substitute appropriate clinical vigilance and patient management.

Before abacavir-containing treatment is introduced for HIV-infected patients, HLA analysis should be performed to exclude *HLA-B*5701*, unless the patient is from a population which does not carry *HLA-B*5701*. Such exclusion of *HLA-B*5701* would markedly reduce the possibility of hypersensitivity reactions and prevent overestimation of hypersensitive reaction that could otherwise result in excessive discontinuation of treatment [29–31].

HLA associations with nevirapine hypersensitivity have been reported, but the odds ratios are not high [58–60]. According to the HIV-1 treatment guidelines, avoiding nevirapine prescription is reasonable for female patients with $CD4^+$ T-cell counts over 250 cells/mm^3 and male patients with $CD4^+$ T-cell counts over 400 cells/mm^3, without HLA typing [7,53,101].

Strong associations between carbamazepine hypersensitivity and *HLA-B*1502*, and between allopurinol hypersensitivity and *HLA-B*5801* have been reported in Han Chinese population [68,69,75].

Analysis of these associations in different ethnic populations is urgently needed before it is widely applied in clinical practice.

Future perspective

Current pharmacogenetic information is limited in relation to the genes of HLA, metabolizing enzymes and drug transfer proteins. Considering that the technology to identify genetic variants across the whole genome is advancing rapidly, many more significant genetic factors for drug efficacy and adverse reactions are likely to be identified in the future. Identification of such factors is important not only to discover new pharmacological mechanisms, but also to improve the currently available drugs and to develop novel drugs. In such whole-genome analysis, drug-induced phenotypes should be carefully observed in genetically variable populations, which will be feasible only through international collaboration.

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Executive summary

- Human leukocyte antigen (HLA) information can help predict risk of some drug hypersensitivity.

Abacavir hypersensitivity & HLA-B*5701

- Abacavir hypersensitivity is strongly associated with *HLA-B*5701*.
- Prospective HLA screening can markedly reduce the risk of abacavir hypersensitivity.

Nevirapine hypersensitivity & associated HLA alleles

- Significant predisposition to nevirapine hypersensitivity has been reported in Caucasian Australians harboring *HLA-DRB1*0101* with high $CD4^+$ T-cell counts, and Sardinians and Japanese harboring *HLA-Cw8*.

Carbamazepine-induced SJS/TEN & HLA-B*1502

- Carbamazepine hypersensitivity is frequent in *HLA-B*1502*-positive Han Chinese.

Allopurinol-induced severe cutaneous adverse reactions & HLA-B*5801

- Most Han Chinese individuals with allopurinol-induced severe cutaneous adverse reactions are positive for *HLA-B*5801*.

Conclusion

- Prospective HLA screening can stratify the risk of hypersensitivity to abacavir, nevirapine, carbamazepine and allopurinol, and allows personalized medicine.
- Application of genetic screening should not substitute appropriate clinical vigilance and patient management.

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