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Multilayered Defense in HLA-B51–Associated HIV Viral Control

YongHong Zhang,†,‡,§,1 YanChun Peng,†,1 HuiPing Yan,*, Keyi Xu,‡, Masumichi Saito,§,1 Hao Wu,*, XinYue Chen,*, Srinika Ranasinghe,†, Nozomi Kuse,§, Tim Powell,‡,1 Yan Zhao,*, WeiHua Li,*, Xin Zhang,*, Xia Feng,*, Ning Li,*, Aleksandra Legidowicz,†, XiaoNing Xu,†, Mina John,§, MasaTak Tamiguchi,§, Andrew McMichael,†, and Tao Dong†,1

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


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

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

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

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

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

We selected 22 HLA-B51* individuals from the cohort, and the immunodominance hierarchy of B51-restricted CTL responses was mapped using 17 known HLA-B51–restricted epitopes from the Los Alamos Molecular Immunology database. Three epitopes—Gag 327-345(N19) NAPPDCKTI, Pol 743-751(L19) LPPVYAKEI, and Pol 283-289 (TIB) TATFIPTPI—were found to be the most dominant epitopes among HLA-B51+ individuals. Disease progression was found to be associated with the sequential selection of mutations within the epitope and the T cell responses generated in each patient. We postulate that HLA-B51* HIV-infected patients might benefit from having several effective immunodominant epitopes, which enable them to form multiple layers of defense against virus escape, starting with L19 responses, followed by TV8 and N19, making it much harder for the virus to escape immune control by HLA-B51–restricted T cell responses.

Materials and Methods

Patient cohort

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

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

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

Human IFN-γ ELISPOT assay

A total of 200,000 PBMCs with 10 µg/ml peptide or 400 T cell clones with 20,000 peptide-pulsed B cell lines were used in a standard Human IFN-γ ELISPOT assays as described elsewhere (19). In brief, assays were carried out in 96-well MultiScreen filter plates (Millipore) coated with 15 µg/ml anti-IFN-γ mAb (1-DIK, Mabtech). A total of 5 µg/ml PHA (final concentration, 1 µg/ml) were used as positive control. Plates were incubated for 16 h at 37°C, 5% CO2. Spot enumeration was performed with an AID ELISPOT reader system (Autoimmun Diagnostika). To quantitatively assess specific responses, mean spots of the control wells were subtracted from the positive wells, and results were expressed as spot-forming units (SFUs) per 106 PBMCs. Responses were regarded as positive if results were at least three times the mean of the quadruplicate negative control wells and >50 SFUs/106 PBMCs. If background wells were >30 SFUs/106 PBMCs or positive control wells (PHA or Flu, EBV, and CMV epitope pool stimulation) were negative, the assay was excluded from further analysis.

CTL clones and HIV-1 permissive target cells

CTL clones specific for HLA-B51 epitopes were generated by limiting dilution from the PBMCs of HIV-infected patients responding to the N19, L19, and TV9 epitopes and maintained as described by Dong et al. (20). The human T cell leukemia MT2 line stably expressing CD4+ and HLA-B51 was maintained in R10 media (RPMI 1640 with 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM l-glutamine, and 10% heat-inactivated FCS from Sigma-Aldrich).

Live virus ELISPOT

Differing HIV-1 strains (IIB and MN) were used to infect MT2 cells. Infected cells were washed twice and then resuspended in 2 ml R10 at a concentration of 1 × 106 cells/ml and cultured in a 25-ml flask for a period of 72 h postinfection at 37°C 5% CO2. Cells were then washed, counted, and cocultured with the panel of HLA-matched CTL clones in triplicate at one E:T ratio of 400:20,000 on the precoated IFN-γ ELISPOT plates at a final volume of 100 µl/well. Negative controls included the individual CTL clones cocultured with uninfected target cell line in triplicate, and positive control included each CTL clone cocultured with uninfected target cells pulsed with 2 µM specific peptide. ELISPOT plates were incubated for 6 h at 37°C 5% CO2 and subsequently washed and developed as described previously. SFUs were counted using the ELISPOT reader system AID ELISPOT 4.0.

Generating HIV mutant virus

Molecular clones of NL4-32 Pol TIB and pNL4-32 Pol L19 mutant virus were generated by PCR-based point mutagenesis of pNL4-32 by using PrimeSTAR HS DNA Polymerase (Takara, Japan) as described previously (7). To obtain pNL4-32 wild-type (WT) and mutant viruses, 293T cells were transfected with WT and mutant plasmids using Lipofectamine 2000 (Invitrogen). Supernatants from transfected 293T cell cultures were stored at −80°C.

Viral suppression assay

High-titer HIV-1 strains (1000 tissue culture-infective dose 50) were used to infect MT2 cell as target cells for T cell clones, and cell pellets were resuspended in a total volume of 200 µl R10 and then incubated for 90 min at 37°C. Infected cells were subsequently washed (twice) to remove free virus. A total of 5 × 104 infected cells were cocultured with HLA-matched HIV-1–specific CTL clones at differing E:T ratios of 1:1, 1:2, 1:4, 1:8, and 1:16 in H10-II-2 (RPMI 1640 + 10% human serum + 200 µl IL-2) on a flat-bottom 96-well plate, in a final volume of 200 µl/well, at 37°C for 4 d. Each condition was performed in triplicate, including one HLA-mismatched clone as a negative control and virus-infected cells in the
absence of CTL as a positive control. The extracellular p24 content in the supernatant was assayed on day 4 by quantitative p24 Ag ELISA (Immunodiagnostics) in accordance with the manufacturer's protocol (21, 22).

**Statistical analysis**

Correlation with HIV viral load was analyzed using nonparametric Mann-Whitney U tests, and correlation with CD4+ T cell counts was analyzed using the unpaired Student t test. Mutations associated with HLA-B51 were analyzed using χ² test. Statistical test differences were considered significant if p < 0.05.

**Results**

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

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

Twenty-two HLA-B51+ individuals selected from this cohort were studied in detail. The clinical details and HLA typing of those individuals are shown in Supplemental Table I. Seventeen of the 22 HLA-B51+ individuals showed a mutation of L243I at position 1 of the LI9 epitope, 17 of 22 showed a mutation of V289X at position 8 of the TV8 epitope, and 6 of 22 showed a mutation of A328S at position 2 of the NI9 epitope. Interestingly, we found a pattern of mutation suggesting that there may have been a sequential selection hierarchy of mutations in these three epitopes (Table II): individuals with the A328S mutation in the NI9 epitope also showed mutations in the other two Pol epitopes (LI9 and TV8) and individuals with the V8L mutation in the TV8 epitope also showed the LI1 mutation in the LI9 epitope. These results strongly suggest that L743I in position 1 of the LI9 epitope seems to mutate the most readily and is likely to be the first to mutate, followed by V289L in position 8 of the TV8 epitope, and then A328S in position two of the NI9 epitope.

**HLA-B51–restricted N9 and TV8 T cell responses are associated with viral control and a better clinical outcome when epitopes are not mutated**

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

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

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**Bold and italic formatting indicates the variant amino acid within the epitope. Asterisks indicate these mutations have significant associations with HLA-B51.**
hierarchy of epitopes are combined (Table II), it is evident that if there is only one epitope mutation in the patient, the mutation is more likely to be that of LI9, and if two epitope mutations are detected, they are likely to be LI9 and TV8. Therefore, the results suggest that, when mutations developed in TV8 and NI9, this is associated with loss of viral control in HLA-B51+ patients; moreover, an S328A mutation developing in the NI9 epitope is significantly associated with low CD4+ counts.

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

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

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

![Figure 2](http://www.jimmunol.org/)  
**Figure 2.** Increased numbers of mutated epitopes are significantly associated with lower CD4$^+$ counts and higher viral load in this cohort. The HIV viral load (**A**) and CD4$^+$ T cell counts (**B**) were compared among people with mutations developed in none or one (LI9 only), two (LI9 + TV8), or three epitopes (LI9 + TV8 + NI9).
T cell clones specific for the TV8 and LI9 epitopes were used (Fig. 4B, 4C); those results were further confirmed by ex vivo ELISPOT assay using HLA-B51+ patient's PBMCs stimulating with both WT and variant peptides (Supplemental Fig. 1). Interestingly, when we tested the recognition of virus-infected target cells, using HIV-1 containing the 289L variant, which is the major variant occurring in B51+ individuals (59%), TV8-specific clones failed to recognize the 8L mutant virus (Fig. 5A), strongly suggesting that this mutation had affected Ag processing and presentation of the epitope. Although we didn’t observe escape recognition of the L19 epitope through the 743I mutation, we found that 82% (14 of 17) of B51+ patients with the 743I mutation in the L19 epitope also developed mutations in the downstream flanking region (I or V to L mutation in position 760) and 41% developed a mutation in position 754 (C to S) (Supplemental Table II), whereas none of the B51– individuals with WT LI9 sequences showed these mutations. Mutant virus was made with the single mutation L743I as well the combinations L743I/L760I and L743/L760I/S754C. We tested the ability of L9-specific CTL clones to inhibit viral replication and found significantly reduced antiviral efficacy against the L743I virus (2-fold increment of p24 level when compared with WT) (Fig. 5B), suggesting that these mutations have an impact on Ag processing and presentation, therefore affecting the antiviral efficacy of LI9-specific T cell responses against HIV-1 expressing those mutations. Comparable levels of P24 production were observed between WT and mutant virus-infected MT2 cells in the absence of clones.

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

We tested the ability of NI9-, LI9-, and TV8-specific T cells to recognize IIIB and MN virus-infected target cells in live virus ELISPOT assays (Fig. 6A). NI9 CTL showed the most effective recognition of virus-infected cells, followed by TV8 CTL. LI9 CTL showed very poor recognition of virus-infected target cells, which could be due to a mutation detected at position 4 (V4I) of the MN virus (Supplemental Fig. 1). Pol and Gag proviral DNA sequences were examined by extracting genomic DNA from HIV-1 IIIB- and MN-infected MT2 cells. NI9, TI8, and LI9 WT sequences were confirmed in IIIB virus-infected cells, whereas an
intraepitope mutation, I746V, at position 4 of LI9 epitope was detected in MN virus-infected cells (data not shown), which explains the weak recognition by LI9-specific T cell clones.

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

### Discussion

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

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

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

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

Live virus ELISPOT assays and suppression assays showed that NI9, LI9, and TV8 T cells could recognize MN or IIIB virus.
infected cells efficiently when the WT epitope was presented, with no clear hierarchical ranking in terms of antiviral efficacy observed among these three epitope-specific clones, suggesting that these T cells could function efficiently if the infecting virus were to be processed and presented properly in target cells.

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

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

Our data are consistent with other studies suggesting that the emergence of escape mutations coincides with increased viral replication (9); moreover, these mutations do not occur at random but develop in the same position in different patients, consistent with T cell driven selection pressure. However, without detailed longitudinal studies, we could not exclude the possibility that increased viral replication may lead to more mutations, rather than escape mutations preceding the rise in viral load. De novo CTL responses may also develop in response to new viral variants (27), which merits future investigation.
In conclusion, our results provide an example of the constant battle between the human immune system and HIV. In this case, the presence of HLA-B*51 and T cells targeting these three immunodominant epitopes could be advantageous, and more importantly, having an immunodominant response to the slow mutating epitope N9 is beneficial for long-term control of viral replication.

Disclosures

The authors have no financial conflicts of interest.

References


