Multilayered HIV-1 gag-specific T-cell responses contribute to slow progression in HLA-A*30-B*13-C*06-positive patients

Hui Zhang\textsuperscript{a,b,*}, Xiaoxu Han\textsuperscript{a,b}, Bin Zhao\textsuperscript{a,b}, Minghui An\textsuperscript{a,b}, Zhe Wang\textsuperscript{c}, Fanming Jiang\textsuperscript{a,b}, Junjie Xu\textsuperscript{a,b}, Zining Zhang\textsuperscript{a,b}, Tao Dong\textsuperscript{d} and Hong Shang\textsuperscript{a,b,*}

**Objective:** The HLA-A*30-B*13-C*06 haplotype is reported to be associated with slow disease progression in the HIV-1-infected Northern Han Chinese population, but the mechanism remains unknown.

**Design:** Gag-specific T-cell responses and gag sequencing were performed in nine B' clade HIV-1-infected HLA-A*30-B*13-C*06-positive slow progressors to understand HLA-associated viral control.

**Methods:** Interferon-\( \gamma \) ELISPOT assays were performed to determine the Gag-specific T-cell responses and cross-reactivity to variant peptides. Longitudinal HIV-1 gag sequencing was performed at the clonal level.

**Results:** The overlapping peptides (OLP)-48: RQANFLGKIVPSHKGRPGNF (RL42 Gag\textsubscript{434-453}); OLP-2: GQLDRWEKIRLRPGGKKYR (RL42 Gag\textsubscript{11-30}); OLP-15: VQNLQGQMVHQPISRTLNA (RL42 Gag\textsubscript{135-154}); and OLP-16: HQPISRTLNAWVKVEEKA (RL42 Gag\textsubscript{144-163}) were dominant in HLA-A*30-B*13-C*06-positive patients. A new epitope [HQPISRTL (Gag\textsubscript{144-152}, HL9)] within OLP-15 and OLP-16 was identified. Results showed that strong cross-reactive responses to multiple immunodominant peptides were associated with better clinical outcomes. In addition, efficient cross-recognition of HL9 autologous variants developed in patients was associated with high CD4\textsuperscript{+} T-cell counts. However, two patients who had developed mutations to their dominant responses during the follow-up experienced decrease in CD4\textsuperscript{+} T-cell counts. It appears that Gag-specific T-cell responses against one or more unmutated epitopes or cross-recognition of autologous epitope variants contribute to slow disease progression in HLA-A*30-B*13-C*06-positive patients.

**Conclusion:** We conclude that a single ‘appropriate’ Gag-specific T-cell response appears to be sufficient to protect patients from disease progression. HLA-A*30-B*13-C*06-positive individuals benefited from having a choice of numerous immunodominant gag epitopes for T cells to react. The study offers new insight for future design of T-cell-based HIV-1 vaccine.

**Keywords:** Gag, HIV-1, slow progressors, T-cell response

\textsuperscript{aKey Laboratory of AIDS Immunology of National Health and Family Planning Commission, Department of Laboratory Medicine, The First Affiliated Hospital, China Medical University, Shenyang, \textsuperscript{b}Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Hangzhou, \textsuperscript{c}Center for Disease Control and Prevention of Henan Province, Zhengzhou, China, and \textsuperscript{d}Medical Research Council Human Immunology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford University, Oxford, UK.}

Correspondence to Hong Shang, MD, PhD, Key Laboratory of AIDS Immunology of National Health and Family Planning Commission, Department of Laboratory Medicine, The First Affiliated Hospital, China Medical University, Shenyang, China. Tel: +86 24 83282634; fax: +86 24 83282634; e-mail: hongshang100@hotmail.com

\textsuperscript{*}Hui Zhang and Hong Shang contributed equally to the writing of this article.

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Background

Virus-specific cytotoxic T-lymphocyte (CTL) responses have been shown to play an important role in the control of HIV-1 infections [1–3]. Growing studies suggest that Gag-specific CTL responses are strongly associated with slow disease progression in HIV-1 infection [4–7]. Particular human leukocyte antigen (HLA) alleles – such as HLA-B*57, B*27 in whites, B*13 in South African populations, and B*51 in Chinese populations – in HLA-restricted Gag-specific CTL responses have demonstrated an association with slow disease progression [7–12]. So far, several immunodominant Gag epitopes associated with better clinical outcomes have been defined for HIV-1 – for example, B*57-restricted TSTLQEQIAW (Gag260–279), TW10) epitope [12,13]; B*27-restricted KRWILGLNK (Gag250–269, KK10) epitope [8]; and B*51-restricted NANPDCKTI (Gag327–345, NP9) [7]. However, virus-specific CTL responses cannot completely eliminate HIV-infected cells because the viral variants within targeted epitopes or flanking regions can evade CTL responses, which may result in the loss of viral suppression [14–18]. It has also been reported that CTLs that recognize escape mutations are elicited after the emergence of an escape mutant selected by wild-type-specific CTLs [19–22]. Thus, the interaction between HIV-1 and the host immune response is believed to relate to disease progression.

Our previous study on a cohort of 321 HIV-1-infected typical progressors and 105 long-term nonprogressors from Northern China found that the HLA-A*30-B*13-C*06 haplotype was associated with slow disease progression [23]. The mechanism underlying this association remains unknown. An inverse correlation between Gag-specific CTL responses and viral loads was reported in the B* clade HIV-1-infected HLA-A*30-B*13-C*06-positive Chinese population [24]. In this study, we performed a comprehensive analysis of Gag-specific immune responses and epitope evolution in B* clade HIV-1-infected HLA-A*30-B*13-C*06-positive slow progressors who maintain high CD4⁺ T-cell counts for more than 10 years without antiretroviral treatment. Results showed that four peptides – OLP-48: RQANFLGKWPISHKGRPGNF(RL42 Gag344–353); OLP-2: GQDLRWEKIRLPGGKKYYR(RL42 Gag111–120); OLP-15: VQNLOQQMVHPISPRTLNA(RL42 Gag135–144); and OLP-16: HQPISPRTLNAWKVVEEKA(RL42 Gag144–163) – were immunodominant among HLA-A*30-B*13-C*06-positive individuals who were infected by HIV-1 through paid blood donations in the 1990s were recruited from the Henan province, China (Table 1) [25]. At the time of recruitment, these patients had been seropositive for at least 10 years with a CD4⁺ T-cell count above 500 cells/µl. None of the patients received any antiretroviral therapy, except for patient 510099, who had taken nevirapine twice to reduce the risk of maternal–fetal HIV-1 transmission from August to December in both 2006 and 2011. Blood samples were collected between 2009 and 2012. Every patient received at least three follow-ups. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque Plus (GE Healthcare Biosciences AB, Sweden) density gradient centrifugation and cryopreserved until used. PBMCs from patient 510099 were collected in October 2010 – 46 months after the patient’s first treatment. The mean CD4⁺ T-cell count was 653 cells/µl at the time of recruitment and then 684 cells/µl at the time of enzyme-linked immunospot (ELISPOT) assays. All patients provided informed consent for donating blood for the purposes of this study. Methods

Study participants

In this study, nine B* clade HIV-1-infected HLA-A*30-B*13-C*06-positive individuals who were infected by HIV-1 through paid blood donations in the 1990s were recruited from the Henan province, China (Table 1) [25]. At the time of recruitment, these patients had been seropositive for at least 10 years with a CD4⁺ T-cell count above 500 cells/µl. None of the patients received any antiretroviral therapy, except for patient 510099, who had taken nevirapine twice to reduce the risk of maternal–fetal HIV-1 transmission from August to December in both 2006 and 2011. Blood samples were collected between 2009 and 2012. Every patient received at least three follow-ups. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque Plus (GE Healthcare Biosciences AB, Sweden) density gradient centrifugation and cryopreserved until used. PBMCs from patient 510099 were collected in October 2010 – 46 months after the patient’s first treatment. The mean CD4⁺ T-cell count was 653 cells/µl at the time of recruitment and then 684 cells/µl at the time of enzyme-linked immunospot (ELISPOT) assays. All patients provided informed consent for donating blood for the purposes of this study.

Table 1. Clinical characters of study participants.

| PID   | Sex | Age | Infection year | HLA class I HLA-A | HLA-B | HLA-C | Baseline CD4⁺ (cells/µl) | Viral loads (copies/ml) | ELISPOT assay time CD4⁺ (cells/µl) | Viral loads (copies/ml) |
|-------|-----|-----|----------------|-------------------|-------|-------|------------------------|--------------------------|--------------------------|------------------------|
| 510096 | M   | 45  | 1995          | 30,33,13,38       | 03,06 |       | 743                    | 0.00                     | 510                      | 1.90                   |
| 510097 | F   | 37  | 1994          | 30,33,13,35       | 04,06 |       | 641                    | 0.00                     | 706                      | 2.44                   |
| 510099 | F   | 32  | 1994          | 24,30,13,48       | 06,08 |       | 760                    | 4.49                     | 1225                     | 4.86                   |
| 510082 | F   | 35  | 1995          | 02,30,13,30       | 06,06 |       | 556                    | 4.47                     | 803                      | 4.85                   |
| 510084 | M   | 42  | 1994          | 30,30,13,15       | 03,06 |       | 535                    | 4.75                     | 583                      | 4.55                   |
| 510110 | M   | 40  | 1995          | 02,30,13,67       | 06,07 |       | 640                    | 3.61                     | 447                      | 3.55                   |
| 510109 | F   | 46  | 1994          | 02,30,08,13       | 06,07 |       | 504                    | 2.08                     | 511                      | 2.79                   |
| 510121 | M   | 51  | 1990          | 30,32,13,44       | 04,06 |       | 795                    | 4.61                     | 878                      | 4.43                   |
| 510013 | M   | 43  | 1990          | 02,30,13,15       | 01,06 |       | 704                    | 3.67                     | 492                      | 3.93                   |

F, female; M, male.

*Patient 510099 had taken nevirapine (NVP) twice to reduce the risk of mother-to-infant HIV-1 transmission from August to December in 2006 and 2011. PBMCs used in ELISPOT assays were collected in October 2010. Time off therapy prior to ELISPOT assays was 46 months.
This study was approved by the Medical Research Ethics Committee of the First Affiliated Hospital of China Medical University.

**Immunological and virological measurements**

CD4⁺ T-cell counts were measured with a FACS Calibur flow cytometer (Becton-Dickinson, USA). HIV-1 RNA levels in plasma (viral loads) were determined using a COBAS AmpliPrep/COBAS TaqMan HIV-1 Test assay (Roche, Germany), which detects between 25 and 100 000 RNA copies/ml.

**Sequencing of viral RNA**

Viral RNA was extracted from plasma using the QIAamp Viral RNA Mini-kit (Qiagen, UK) according to the manufacturer’s instructions. The entire gag gene was amplified with the SuperScript Polymerase One-Step RT-PCR System (Takara, Dalian, China). The first round of PCR with outer primers 172A (5’-ATCTCTAG-CAGTGCGGCCCCAGACG-3’ 628–648 nt of HIV-1 HXB2) and Gag-6 (5’-TAATGCTTTTTATT-TYTCTTCTGTCAATGGC-3’) was performed with the following cycling parameters: 56°C for 30 min; 94°C for 5 min; followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 2.5 min; and a final extension step at 72°C for 10 min. The second round of PCR with inner primers Gag-763 (5’-TGACTAGCGGAGGCTAGAAGG-3’) 763–783 nt of HIV-1 HXB2 and Gag-5 (5’-TTCYCCCTAT-CATTGGTTTCC-3’) 2377–2400 nt of HIV-1 HXB2) was performed with the following cycling parameters: 94°C for 5 min; followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 2.5 min; and a final extension step at 72°C for 10 min. The PCR products were confirmed through 1.0% agarose gel electrophoresis. PCR products were purified using the QIAquick gel extraction kit (Qiagen) and cloned using a TOPO TA cloning kit (Invitrogen, USA). The fragments were sequenced by Huada Genomics Company (China). Individual sequence fragments were assembled and edited using the Sequencher program (version 4.9).

**Synthetic HIV-1 Gag overlapping peptides**

The 18–20 mer peptides with 10 or 11 overlapping amino acids and spanning the first isolated full-length B’ clade RL42 Gag sequence (GenBank U71182.1) were synthesized by Meilian Company (China). In total, 54 peptides were synthesized, and 15 pools were made by mixing 7–8 peptides per pool in a 7 x 8 matrix design. The peptide responsible for any observable reaction in a matrix cell could be named by identifying the common peptide present in both the pools that were mixed. When more than one peptide was identified, we confirmed the identity of the responsible peptide by individually testing all peptides common to the relevant mixed pools. Nine additional 9-mer peptides and 1320-mer peptides presenting amino acid variants which were different from RL42 sequence and were observed in virus from our study population were synthesized by Sigma–Aldrich (USA).

**Interferon-γ ELISPOT assay**

Interferon (IFN)-γ ELISPOT assays were performed according to the manuscript (BD ELISPOT, USA) to detect the HIV-1-specific IFN-γ-secreting cells among the PBMCs. PBMCs were plated at 100 000 per well with peptides at a final concentration of 10 μg/ml for peptide pools and 5 μg/ml for single peptides in 96-well plates. Phytohemagglutinin at a concentration of 10 μg/ml was used as a positive control, and medium alone was used as a negative control. Spots were quantified using the ImmunoSpot Analyzer (Cellular Technology Ltd, USA). The number of specific IFN-γ-secreting T cells was expressed as spot-forming unit (SFU) per 10⁶ PBMC inputs. A response was considered positive if there were at least 50 SFCs per 10⁶ PBMCs, as well as activity that was at least three times greater than the mean background activity.

**Statistical analysis**

Data were analyzed and graphical presentation was performed using GraphPad Prism version 5.0 software (GraphPad Software, San Diego, Californis, USA).

**Results**

**Immunodominant responses in nine B’ clade HIV-1-infected HLA-A*30-B*13-C*06-positive patients**

Gag-specific CTL responses were performed for nine B’ clade HIV-1-infected HLA-A*30-B*13-C*06-positive slow progressors. The clinical details and HLA typing of the nine patients were shown in Table 1. Results showed that the immunodominant peptides for this study population were OLP-48: RQANFLGKIWPShKGRGPNF (RL42 Gag114–143); OLP-2: GQLRDKWEIRLRPGKKKyr (RL42 Gag11–30); OLP-15: VQNLQGQVMHQPIS-PRTLNA (RL42 Gag163–182); and OLP-16: HQPISPRTLNAWVKVVEEKA (RL42 Gag144–163). Responses to these peptides were present in 100.0, 77.8, 55.6, and 55.6% of the study population, respectively (Fig. S1A, http://links.lww.com/QAD/A672). The magnitude of responses to OLP-15 and OLP-16 were strongest, followed by responses to OLP-2 and OLP-48 (Fig. S1b, http://links.lww.com/QAD/A672).

**Identification of a new B’13-restricted epitope**

According to our previous study on a cohort of 95 B’ clade HIV-1-infected patients, variations at Gag residues 146 and 147 were identified in association with expression of HLA-B*13 [26]. In this study, we found that these two residues were located in the overlapping region of the adjacent peptides OLP-15 and OLP-16. Moreover, the frequency and magnitude of CTL responses to both peptides were similar. Therefore, we
predicted that the overlapping region HQPISPRTLNA might contain a CTL epitope. In addition, the results of epitope prediction by SYFPEITHI (available at http://www.syfpeithi.de/home.htm) supported the inference that a potential B*13-restricted HQPISPRTL (Gag144–152) HL9 epitope is located in this overlapping region. The prediction score for the HL9 epitope was 21 points and ranked second only to the prediction score of the VQLNQGQMV (Gag135–143, VV9, 22 points) epitope—the well known B*13-restricted epitope. The HL9 epitope also contains the B*13 binding motif (Q at position 2; and L/I at the C-terminal end). On the basis of the above analysis, we inferred that OLP-15 and OLP-16 might contain a new B*13-restricted HL9 epitope.

In order to assess whether HL9-specific CTL responses could have attributed to the responses to OLP-15/16, samples from patients 510013, 510096, 510097, and 510099 were selected for further study. Results showed that all four patients who responded to OLP-15 and OLP-16 had strong responses to the HL9 epitope (Table S1, http://links.lww.com/QAD/A672). Furthermore, two published optimal epitopes—B*13-restricted VV9 and B*57/C*06-restricted ISPRTLNAW (Gag147–155, ISW9)—were located in OLP-15 and OLP-16, respectively. However, the magnitude of responses to the VV9 and ISW9 epitopes was weak (Table S1, http://links.lww.com/QAD/A672). Hence, HL9 could be considered an immunodominant epitope for HLA-A*30-B*13-C*06-positive patients.

### HLA-B*13-associated selection pressure on HL9 in HLA-A*30-B*13-C*06-positive patients

In order to investigate the importance of immunodominant responses in virus evolution, a total of 202 longitudinal clonal sequences were obtained to analyze the amino acid variants within immunodominant peptides (Table S2, http://links.lww.com/QAD/A672). Patients 510096 and 510097 were excluded as their viral loads were too low to obtain longitudinal clonal sequences. At the time of ELISPOT assays, we found that three patients (510013, 510096, and 510097) carried the wild-type sequence (HQPISPRTL). The HL9 epitope showed (P146S, I147L) variants in viruses from patient 510099, who showed immunodominant responses to OLP-15/16, but relatively weak responses to OLP-2 and OLP-48 (Fig. 1a). In patients 510096 and 510097, whose viral isolates represented the wild-type HL9 epitope, positive IFN-γ responses were generated against autologous epitope (4270 SFU/10^6 PBMCs and 3370 SFU/10^6 PBMCs at 10 μg/ml peptide, respectively). Patients 510096 and 510097 exhibited a highly efficient cross-recognition of HL9 variants. Our previously observed variants P146A (P146A/S, I147L) and a novel P149A variant abrogated CTL recognition to a large extent. I147L alone and S148A variants slightly reduced the HL9-specific response. Strong cross-reactive T-cell responses against HL9 autologous variants in patients 510096 and 510097 might help to maintain high CD4^+ T-cell counts and low viral loads. However, we observed a less efficient cross-recognition of HL9 variants in patient 510099, in which case the predominant viral isolates represented the (P146S, I147L) variants (Fig. 1b). Decreased responses to HL9 autologous epitope variants might explain the decreased CD4^+ T-cell counts and high viral loads in patient 510099 (Fig. 1c). These data strongly suggest that HL9-specific CTL response contributed to the slow disease progression in HLA-A*30-B*13-C*06-positive patients.

### Strong cross-reactive T-cell response against HL9 epitope containing autologous sequences in HLA-A*30-B*13-C*06-positive patients is associated with better clinical outcomes

To examine the influence of amino acid variants within the HL9 epitope on CTL recognition, HL9 epitope containing the wild-type RL42 sequence and other autologous sequences observed in the study population were synthesized. IFN-γ ELISPOT assays were performed on PBMCs from patients 510096, 510097, and 510099, who showed immunodominant responses to OLP-15/16, but relatively weak responses to OLP-2 and OLP-48 (Fig. 1a). In patients 510096 and 510097, whose viral isolates represented the wild-type HL9 epitope, positive IFN-γ responses were generated against autologous epitope (4270 SFU/10^6 PBMCs and 3370 SFU/10^6 PBMCs at 10 μg/ml peptide, respectively). Patients 510096 and 510097 exhibited a highly efficient cross-recognition of HL9 variants. Our previously observed variants P146A (P146A/S, I147L) and a novel P149A variant abrogated CTL recognition to a large extent. I147L alone and S148A variants slightly reduced the HL9-specific response. Strong cross-reactive T-cell responses against HL9 autologous variants in patients 510096 and 510097 might help to maintain high CD4^+ T-cell counts and low viral loads. However, we observed a less efficient cross-recognition of HL9 variants in patient 510099, in which case the predominant viral isolates represented the (P146S, I147L) variants (Fig. 1b). Decreased responses to HL9 autologous epitope variants might explain the decreased CD4^+ T-cell counts and high viral loads in patient 510099 (Fig. 1c). These data strongly suggest that HL9-specific CTL response contributed to the slow disease progression in HLA-A*30-B*13-C*06-positive patients.

### Strong responses to autologous OLP-2 in HLA-A*30-B*13-C*06-positive patients are associated with high CD4^+ T-cell counts

Despite no detectable T-cell responses to OLP-15/OLP-16 in four patients 510082, 510084, 510110, and 510109, these patients have shown immunodominant responses to OLP-2 (Fig. 2a). We analyzed the evolution in the RLP.PGKXKKY (Gag20–29, RY10) epitope within OLP-2. Patients 510109 and 510110 carried the wild-type RY10 epitope. A mixture of K28R and (K26N, I28R) variants stimulated IFN-γ responses against HL9 autologous variants (Table S2, http://links.lww.com/QAD/A672). Then the influence of amino acid variants within OLP-2 on CTL recognition was examined (Fig. 2b). Surprisingly, (K26N, K28R) variants stimulated IFN-γ responses that were stronger than responses to wild-type OLP-2 in patient 510082, in which case (K26N, K28R) variants were the dominant viral quasispecies. However, patients 510084,
510110, and 510109 had a diminished IFN-γ response to the (K26N, K28R) variants of OLP-2, and viral isolates from these three patients showed no (K26N, K28R) variants. Furthermore, patient 510084 showed a strong response to wild-type OLP-2 and a slightly diminished response to the K28R and (R20Q, K28R) variants, but responded weakly to the G24M, K26N, and K28Q variants (Fig. 2b). Interestingly, patient 510109 showed strong responses to autologous OLP-48 in HLA-M and OLP-2. This patient had nearly equivalent responses to the wild-type OLP-48 and to various OLP-48 variants. Patient 510121 showed immunodominant responses to OLP-48, but relatively weak responses to OLP-15/16 and OLP-2. This patient had nearly equivalent responses to the wild-type OLP-48 and to various OLP-48 variants. Immunodominant responses to OLP-48 were also detected in patient 510013, followed by OLP-15/16 and OLP-2-specific T-cell responses. Patient 510013 had strong responses to autologous viral peptide and moderately diminished responses to I437L and (K436R, I437V) variants. Hence, highly efficient recognition of autologous OLP-48 in patients 510121 and 510013 may have played a role in their slow disease progression.

**Strong responses to autologous OLP-48 in HLA-A*030-B*13-C*06-positive patients associated with slow disease progression**

OLP-48 contained the B*13-restricted RQANFLGKI (Gag429-437, R9) epitope. R9-specific CTL responses may have selected for the K436R, I437L, I437V, and (K436R, I437V) variants in seven out of the nine patients (Table S2, http://links.lww.com/QAD/A672). CTL recognition of OLP-48 variants was detected in the remaining two patients – 510121 and 510013 (Fig. 3). Patient 510121 showed immunodominant responses to OLP-48, but relatively weak responses to OLP-15/16 and OLP-2. This patient had nearly equivalent responses to the wild-type OLP-48 and to various OLP-48 variants. Immunodominant responses to OLP-48 were also detected in patient 510013, followed by OLP-15/16 and OLP-2-specific T-cell responses. Patient 510013 had strong responses to autologous viral peptide and moderately diminished responses to I437L and (K436R, I437V) variants. Hence, highly efficient recognition of autologous OLP-48 in patients 510121 and 510013 may have played a role in their slow disease progression.

![Fig. 1. Responses to a panel of HL9 variants in patients 510096, 510097, and 510099.](image)

(a) CTL responses to immunodominant peptides. (b) Responses to various variants within the HL9 epitope. Epitopes in boxes indicate autologous viral clonal sequences at the time of ELISPOT assays. The number in parentheses indicates the number of clones in the reported sequence. Responses to variant HQSLPPRTL (1/13) in patient 510099 were not detected due to sample limitations. (c) Clinical outcomes during the follow-up. Arrows denote the time points by ELISPOT assays. CTL, cytotoxic T-lymphocyte.
T-cell counts. Hence, clade HIV-1 strain [27], which showed a 13-C 0 13-C 2010–3 06 2012–1 2011–3 13-restricted optimal C clade T-cell epitopes have been 30-B strains 2012–1 2010–3 30-B 2010–3 13-C clade HIV-1-infected 13-C-positive slow progressors from 06-positive patients. 06 is one of the main three-loci study is associated with slow disease progression in B 30-B immunodominant autologous peptides identified in this Gag-specific T-cell response that targets any one of the W e previously reported that the HLA-A 30-B 06-positive patients. As described above, we divided all patients into three groups according to the immunodominant peptides detected at the time of ELISPOT assays (Table 2). W e hypothesized that the patients who are able to generate a response toward at least one of the three immunodominant peptides in which the targeted peptides remain unmutated should maintain a state of slow disease progression. Results showed that strong HL9-specific responses in patients 510096 and 510097 (group 1), OLP-2-specific responses in patients 510084 and 510110 (group 2), and OLP-48-specific responses in patient 510013 (group 3) might contribute to their slow disease progression. In addition, patients 510082 and 510121 generated strong responses to autologous OLP-2 and OLP-48, respectively. However, patient 510099 who had three mutated peptides, and patient 510109 who lost OLP-2-specific T-cell response during the follow-up experienced decrease in CD4+ T-cell counts. Hence, Gag-specific T-cell response that targets any one of the immunodominant autologous peptides identified in this study is associated with slow disease progression in B’ clade HIV-1-infected HLA-A-30-B-13-C-06-positive patients.

**Discussion**

We previously reported that the HLA-A-30-B-13-C-06 haplotype was associated with slow disease progression [23]. Here, we have extended this study to the analysis of the T-cell responses that were associated with slow disease progression in HLA-A-30-B-13-C-06-positive patients. We discuss a unique population that was infected with very similar viral strains within a narrow period through contaminated plasma in the early 1990s. The RL42 strain, identified among injection drug users in the Yunnan province in the 1990s, is the earliest full-length genome of a primary B’ clade HIV-1 strain [27], which showed a close evolutionary relationship with other B’ strains widely transmitted among the paid plasma donors across China, including the Henan province. Therefore, RL42 is an ideal representative strain of the B’ clade in China. In this study, we designed a set of overlapping peptides based on the RL42 Gag sequence, and we performed ELISPOT assays of samples from nine B’ clade HIV-1-infected HLA-A-30-B-13-C-06-positive slow progressors from the paid plasma donor cohort in the Henan province. Results showed that HL9, OLP-2, and OLP-48 peptides were immunodominant. Strong responses that targeted a single immunodominant peptide were associated with slow disease progression.

HLA-A-30-B-13-C-06 is one of the main three-loci haplotypes in the Han Chinese population, with a frequency of 4.6% [28]. HLA-B’13, which is a component allele of this haplotype, was previously shown to be associated with successful HIV disease control [9,29]. B’13-restricted optimal C clade T-cell epitopes have been
well defined. Unlike C clade-infected B\(^+\)13-positive patients in Durban, studied by Honeyborne et al. [9], our study cohort patients responded weakly to GQMREPRGSDI (Gag\(^{226-236}\), GI11, OLP-25) and VQNLQGQMV (Gag\(^{135-143}\), VV9, OLP-14), but responded strongly to HQPISRTL (Gag\(^{144-152}\), HL9, OLP-15/16) and RQANFLGKI (Gag\(^{429-437}\), RI9, OLP-48). The discrepancy in responses to HL9 might be due to sequence heterogeneity in different viral clades. Deng et al. [30] reported that residue 146 in the B\(^+\)0 clade was proline, which was different from other viral clades, whose 146th residue was alanine or serine. P146 appeared to be characteristic of the B\(^+\)0 clade virus [30]. Furthermore, the variants P146A and (P146A/S, I147L) in HL9 epitope can escape CTL recognition in our study population. Collectively, our results suggest that the B\(^+\)13-restricted HL9-specific CTL response might be unique to the B\(^+\) clade HIV-1-infected Chinese population.

Newly identified HL9-specific CTL response might have helped to maintain better clinical outcomes in our study cohort, particularly in patients 510096 and 510097. However, patient 510099, who carried escape mutations, exhibited a less efficient functional cross-recognition of HL9 epitope variants and experienced rapid disease progression. Hence, T-cell responses showing efficient variant cross-recognition might be associated with delayed disease progression in HIV infection, which has been reported in several studies [20,31–33]. One factor contributing to this observed association may be that CTL responses to broad variant cross-reactivities have the ability to limit the outgrowth of the escape variants, which may help to restrict viral replication for longer periods [32].

Differences in the recognition of the (K26R, K28N) variants in OLP-2 between patients 510084 and 510082
were observed, which varied according to the dominant viral species in the individuals. We can speculate that there might be a diverse T-cell receptor repertoire targeting OLP-2 wild-type and (K26R, K28N) variants. Although we do not know whether patient 510082 had already been infected with HIV-1 variants containing the (K26N, K28R) variants, or whether their HIV-1 strains produced (K26N, K28R) variants via immune selection, responses to (K26N, K28R) variants may have been an important mechanism for delaying disease progression in this patient. Furthermore, patient 510121 also showed efficient recognition of autologous OLP-48 variants. Similarly, the development of responses to some mutations within the B/C357-restricted epitopes had been reported to be associated with the maintenance of viral suppression in HIV-1-infected B/C357+ elite suppressors [20,22].

HL9, OLP-2, and OLP-48 were the immunodominant peptides in HLA-A30-B13-C06-positive patients. It has been reported that Gag and Pol-specific effector CD8+ T cells targeting conserved epitopes can control HIV-1 replication in vitro [34,35]. Our data also suggested that T-cell responses to conserved peptides (e.g. patients 510096, 510097, and 510013) or highly cross-reactive T-cell responses to variant peptides (patients 510082 and 510121) were associated with slow disease progression in HLA-A30-B13-C06-positive patients. Moreover, patient 510099, who had three mutated peptides, and patient 510109, who lost OLP-2-specific T-cell response during the follow-up, experienced decrease in CD4+ T-cell counts. Hence, it appears that a single optimal immunodominant Gag-specific T-cell response was sufficient to prevent disease progression. Our results were further supported by previous studies, for example, the unique epitope KK10 (Gag263–272)-specific response in HIV-infected individuals with B27 [8], B51-restricted N9 (Gag327–343)-specific response in the HIV-infected Chinese population [7], and B51-restricted TAFTIPS (RT126–135, TI8)-specific response in the HIV-1-infected Japanese population [36]. However, without detailed analysis of other potentially confounding host genetic factors [37,38] and T-cell responses restricted by other HLA alleles, we could not exclude their role in disease progression. This might be the limitation of our study, and merits future investigation.

The present study suggests that T-cell response against one of the immunodominant gag autologous peptides containing epitopes HL9, OLP-2, or OLP-48 is associated with slow disease progression in B' clade HIV-1-infected HLA-A30-B13-C06-positive patients. We speculate

| Group | patients | HL9+++ | OLP-2+++ | OLP-48+++ | Last follow-up |
|-------|----------|---------|----------|---------|---------------|
| 1     | 510096   | +       | 19/19    | +       | 19/19         |
|       | 510097   | +       | 18/18    | +       | 13/13         |
|       | 510099   | +       | 12/13    | +       | 11/13         |
| 2     | 510084   | +       | 9/9      | +       | 9/9           |
|       | 510110   | +       | 6/6      | +       | 6/6           |
|       | 510082   | +       | 6/6      | +       | 1/6           |
|       | 510109   | +       | 5/5      | +       | 0/5/5         |
| 3     | 510121   | +       | 7/7      | +       | 7/7           |
|       | 510013   | +       | 7/7      | +       | 7/7           |

Patients were grouped according to immunodominant T-cell responses to HL9, OLP-2, and OLP-48, respectively. Gag-specific T-cell responses (solid boxes) play an important role in slow disease progression. T-cell responses (dashed boxes) may be of some protection. +++, the magnitude of response >1000 SFU/10^6 PBMCs; +++, 500–1000 SFU/10^6 PBMCs; +, <500 SFU/10^6 PBMCs; —, no response. M, mutation detected in peptides. The numbers of clones with the amino acid variations in peptides are shown. *Responses to OLP-15/OLP-16. †Patient 510082 showed strong response to OLP-2 autologous peptide and patient 510121 showed strong response OLP-48 autologous peptide. ‡Patient 510109 developed K28Q variant which was an escape mutation during the follow-up.
possessing numerous ‘beneficial’ T-cell epitopes in HIV gag protein restricted by HLA-A*30-B*13-C*06 haplotype would provide patients with multilayered defense against HIV-1 infection and therefore would contribute to slow disease progression. Our data suggested that such ‘optimal’ epitopes could be considered as candidate immunogens used for T-cell-based vaccine design.

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Conflicts of interest

There are no conflicts of interest.

References

1. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol 1994; 68:6103–6110.
2. Kouk RA, SattFF, Cao Y, Andrews CA, McLeod G, Borkowsky W, et al. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 1994; 68:4630–4635.
3. Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S, et al. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. Science 1998; 279:2103–2106.
4. Kiepiela P, Ngumbela K, Thobakgale C, Ramduth D, Honeyborne I, Moodley E, et al. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat Med 2007; 13:46–53.
5. Riviere Y, McCchesney MB, Porrot F, Tanneau-Salvadori F, Sansonetti P, Lopez O, et al. Gag-specific cytotoxic responses to HIV type 1 are associated with a decreased risk of progression to AIDS-related complex or AIDS. AIDS Res Hum Retroviruses 1995; 11:903–907.
6. Edwards BH, Bansal A, Sahij A, Bakari J, Mulligan MJ, Gompert PA. Magnitude of functional CD8+ T-cell responses to the gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma. J Virol 2002; 76:2298–2305.
7. Zhang Y, Peng Y, Yan H, Xu K, Saito M, Wu H, et al. Multi-layered defense in HLA-B*51-associated HIV viral control. J Immunol 2011; 187:684–691.
8. Goulder PJ, Phillips RE, Colbrett RA, McAdam S, Ogg G, Nowak MA, et al. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat Med 1997; 3:212–217.
9. Honeyborne I, Prendergast A, Pereyra F, Leslie A, Crawford H, Payne R, et al. Control of human immunodeficiency virus type 1 is associated with HLA-B*13 and targeting of multiple gag-specific CD8+ T-cell epitopes. J Virol 2007; 81:3667–3672.
10. Goulder PJ, Bunce M, Krausa P, McIntyre K, Crowley S, Morgan B, et al. Novel, cross-restricted, conserved, and immunodominant cytotoxic T lymphocyte epitopes in slow progressors in HIV type 1 infection. AIDS Res Hum Retroviruses 1996; 12:1691–1698.
11. Miura T, Brockman MA, Schneidewind A, Lobrizt M, Pereyra F, Rathod A, et al. HLA-B57/B5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte [corrected] recognition. J Virol 2009; 83:2743–2755.
12. Crawford H, Lum W, Leslie A, Schaefer M, Boera D, Prado JC, et al. Evolution of HLA-B*5703 HIV-1 escape mutations in HLA-B*5703-positive individuals and their transmission recipients. J Exp Med 2009; 206:909–921.
13. Brockman MA, Schneidewind A, Lahaye M, Schmidt A, Miura T, Desouza I, et al. Escape and compensation from early HLA-B*5703-mediated cytotoxic T-lymphocyte pressure on human immunodeficiency virus type 1 Gag alter capsid interactions with cyclophilin A. J Virol 2007; 81:12608–12618.
14. Kelleher AD, Long C, Holmes EC, Allen RL, Wilson J, Conlon C, et al. Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B*27-restricted cytotoxic T lymphocyte responses. J Exp Med 2001; 193:375–386.
15. Allen TM, Allfeldt M, Yu XC, S’Oullivan KM, Lichterfeld M, Le Gall S, et al. Selection, transmission, and reversion of an antigen-processing cytotoxic T-lymphocyte escape mutation in human immunodeficiency virus type 1 infection. J Virol 2004; 78:7069–7078.
16. Draenert R, Le Gall S, Pfafferott KJ, Leslie AJ, Chetty P, Brander C, et al. Immune selection for altered antigen processing leads to cytotoxic T-lymphocyte escape in chronic HIV-1 infection. J Exp Med 2004; 199:905–915.
17. Yokomaku Y, Miura H, Torniyama H, Kawana-Tachikawa A, Takuguchi M, Kojima A, et al. Impaired processing and presentation of cytotoxic-T-lymphocyte (CTL) epitopes are major escape mechanisms from CTL immune pressure in human immunodeficiency virus type 1 infection. J Virol 2004; 78:1314–1312.
18. Ransinghbe SR, Kramer HB, Wright C, Kessler BM, di Gloria K, Zhang Y, et al. The antiviral efficacy of HIV-specific CD8+ T-cells to a conserved epitope is heavily dependent on the infecting HIV-1 isolate. PLoS Pathog 2011; 7:e1001341.
19. Allen TM, Yu XC, Kafie ET, Rorey LL, Lichterfeld M, John M, et al. De novo generation of escape variant-specific CD8+ T-cell responses following cytotoxic T-lymphocyte escape in chronic human immunodeficiency virus type 1 infection. J Virol 2005; 79:12952–12960.
20. Bailey JR, Williams TM, Siliciano RF, Blankson JN. Maintenance of viral suppression in HIV-1-infected HLA-B*57*+ elite suppressors despite CTL escape mutations. J Exp Med 2008; 203:1357–1369.
21. Fujiwara M, Tanuma J, Koizumi H, Kawashima Y, Honda K, Masutani-Aizawa S, et al. Different abilities of escape mutant-specific cytotoxic T cells to suppress replication of escape mutant and wild-type human immunodeficiency virus type 1 in new hosts. J Virol 2008; 82:138–147.
22. O’Connell KA, Hegarty RW, Siliciano RF, Blankson JN. Viral suppression of multiple escape mutants by de novo CD8+ T-cell responses in a human immunodeficiency virus-1 infected elite suppressor. Retrovirology 2011; 8:63.
23. Zhang H, Zhao B, Han X, Wang Z, Liu B, Lu C, et al. Associations of HLA class I antigen specificities and haplotypes with disease progression in HIV-1-infected Hans in Northern China. Hum Immunol 2013; 74:1636–1642.
24. Jia M, Hong K, Chen J, Ruan Y, Wang Z, Su B, et al. Preferential CTL targeting of Gag is associated with relative viral control in long-term surviving HIV-1 infected former plasma donors from China. Cell Res 2012; 22:903–914.
25. Zhang H, Zhao B, Han X, Wang Z, Liu B, Lu C, et al. Associations of HLA class I antigen specificities and haplotypes with disease progression in HIV-1-infected Hans in Northern China. Hum Immunol 2013; 74:1636–1642.
26. Han XX, Zhang H, Zhao B, Chen O, Zhang C, Ji YT, et al. [Association between amino acid mutations in Gag protein and human leukocyte antigen class I alleles in human immunodeficiency virus-1 B' infected Han Chinese people]. Zhonghua Yi Xue Za Zhi 2012; 92:1159–1164.

27. Graf M, Shao Y, Zhao Q, Seidl T, Kostler J, Wolf H, et al. Cloning and characterization of a virtually full-length HIV type 1 genome from a subtype B'-Thai strain representing the most prevalent B-clade isolate in China. AIDS Res Hum Retroviruses 1998; 14:285–288.

28. Yuan F, Sun YY, Luo Y, Liang F, Liu N, Jin L, et al. [Haplotype and linkage analysis of HLA-I classical genes in Chinese Han population]. Zhongguo Shi Yan Xue Ye Xue Za Zhi 2007; 15:1084–1089.

29. Prado JG, Honeyborne I, Brierley I, Puertas MC, Martinez-Picado J, Goulder PJ. Functional consequences of human immunodeficiency virus escape from an HLA-B M13-restricted CD8+ T-cell epitope in p1 Gag protein. J Virol 2009; 83:1018–1025.

30. Deng X, Liu H, Shao Y, Rayner S, Yang R. The epidemic origin and molecular properties of B': a founder strain of the HIV-1 transmission in Asia. AIDS 2008; 22:1851–1858.

31. Gillespie GM, Kaul R, Dong T, Yang HB, Rostron T, Bwayo JJ, et al. Cross-reactive cytotoxic T lymphocytes against a HIV-1 p24 epitope in slow progressors with B57. AIDS 2002; 16:961–972.

32. Turnbull EL, Lopes AR, Jones NA, Cornforth D, Newton P, Aldan D, et al. HIV-1 epitope-specific CD8+ T cell responses strongly associated with delayed disease progression cross-recognize epitope variants efficiently. J Immunol 2006; 176:6130–6146.

33. Mother B, Llano A, Ibarrondo J, Zamarreno J, Schiaulini M, Miranda C, et al. CTL responses of high functional avidity and broad variant cross-reactivity are associated with HIV control. PLoS One 2012; 7:e29717.

34. Borthwick N, Ahmed T, Ondondo B, Hayes P, Rose A, Ebrahimsa U, et al. Vaccine-elicited human T cells recognizing conserved protein regions inhibit HIV-1. Mol Ther 2014; 22:464–475.

35. Letournou S, Im EJ, Mashishi T, Brereton C, Bridgeman A, Yang H, et al. Design and preclinical evaluation of a universal HIV-1 vaccine. PLoS One 2007; 2:e984.

36. Kawashima Y, Pfaafferott K, Frater J, Matthews P, Payne R, Addo M, et al. Adaptation of HIV-1 to human leukocyte antigen class I. Nature 2009; 458:641–645.

37. Soria A, Guerini FR, Bandera A, Bolognesi E, Uglietti A, Fusco C, et al. KIR-HLA genotypes in HIV-infected patients lacking immunological recovery despite effective antiretroviral therapy. PLoS One 2011; 6:e27349.

38. Zaunders J, van Bockel D. Innate and adaptive immunity in long-term non-progression in HIV disease. Front Immunol 2013; 4:95.