

RESEARCH PAPER



## HLA-DQB1\*06 and breadth of Nef core region-specific T-cell response are associated with slow disease progression in antiretroviral therapy-naive Chinese HIV-1 subtype B patients

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### ABSTRACT

Vaccines still are an important way to prevent and treat acquired immunodeficiency syndrome (AIDS).<sup>1</sup> For developing an effective T cell-based AIDS vaccine, it is critical to define the human leukocyte antigen (HLA) type and epitope that elicit the most potent responses. This study involved 29 antiretroviral therapy-naive and chronic human immunodeficiency virus (HIV)-1 subtype B-infected individuals. A polymerase chain reaction-sequence-specific primer was used to detect the HLA typing, and the enzyme-linked immunospot assay to quantify the T-cell immune function. The results showed that the HLA-DQB1\*06-positive group had higher CD4 counts and lower viral load (VL) compared with the HLA-DQB1\*06-negative group; A higher magnitude of HIV-1-specific T-cell response and breadth were observed in the HLA-DQB1\*06-positive group; the T-cell response was proportional to VL ( $R^2 = 0.488$ ,  $P = 0.0368$ ) in the HLA-DQB1\*06-positive group. The total T-cell responses to HIV-1 Nef core region were quantified at the single-peptide level. Nine (90%) peptides were recognized in 18 (62.1%) individuals. The breath of Nef core region-specific T-cell response was correlated positively with CD4<sup>+</sup> T cell count and inversely with VL, which improved disease outcomes. These data revealed that HLA-DQB1\*06 had a protective effect on the course of HIV-1 and T-cell targeting of certain specific Nef epitopes, contributing to HIV-1 suppression. The results suggested the potential use of HLA-DQB1\*06 and Nef core region in HIV-1 T-cell vaccine design.

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### KEYWORDS

HIV-1; HLA; NEF T-cell response; Vaccine

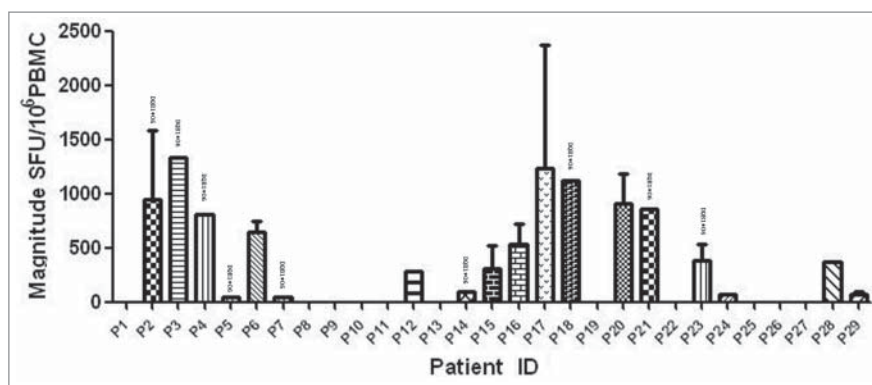
### Introduction

It is crucial to understand accurately the influence of sequence variation among human immunodeficiency virus (HIV) subtypes and human leukocyte antigen (HLA) diversity among ethnic groups for developing a more effective vaccine candidates.<sup>2,3</sup> The HLA-restricted T-lymphocyte immune response is one of the major factors determining the genetic diversity of HIV-1.<sup>4,5</sup> This has been reported more frequently in the HLA-I type, but less in the HLA-II type.<sup>6</sup> The HIV-1 Nef gene encodes 200–215 amino acids in length.<sup>1,7</sup> Nef recognition dominates in acute HIV-1 infection, but the function of Nef T-cell responses in cohort studies of chronically infected subjects is still not clear.<sup>8</sup> At the same time, Nef core protein is substantially more conserved.<sup>9</sup> It has a high density of overlapping CD8<sup>+</sup> T-cell epitopes (see Nef epitope map at [www.hiv.lanl.gov](http://www.hiv.lanl.gov)). Therefore, the Nef region was chosen as the target gene for the study. HIV-1 subtype B is the most prevalent HIV-1 subtype both in China and globally. HIV viral load (VL) and CD4<sup>+</sup> T-cell count are the key markers of HIV-1 disease progression. The emergent ascendancy of HIV-1 subtype B indicates a need for a comprehensive analysis of HIV-1 subtype B-specific immune responses, dealing with both CD4 and CD8, in the context of designing a vaccine.

### Results

#### *The Nef-core peptides of HIV-1 can cause T cell response in most HIV-1 patients*

Peptide is amide combining the amino group of one amino acid with the carboxyl group of another, usually obtained by partial hydrolysis of viral protein. Activation of a T cell is initiated by the interaction of TCR with a peptide displayed by major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APCs). Among 29 individuals, 18 (62.1%) were found to have at least 1 peptide. The average magnitude was 973.7 SFU/10<sup>6</sup> cells (range 47.2–3783.3) (Fig. 1 Magnitude of human immunodeficiency virus (HIV)-1 subtype B Nef core specific T cell responses in 29 HIV-1 subtype B-infected individuals). Out of 10 overlapping peptides (OLPs), 9 (90%) were recognized in at least 1 individual: only 1 OLP was recognized in 1 subject, and the other 8 OLPs were recognized in more than 1 volunteer. N14 (KRQDILDWVYHTQGYFP) was the highest ratio sum of all the responses from various individuals, with 5870 SFU/10<sup>6</sup> cells. The highest frequently recognized peptide was N12 (HFLKEKGGLEGLIYSQKR), which was recognized in 6 individuals (Fig. 2 Magnitude and Frequency of 10 HIV-1 subtype B Nef core OLPs). To visually see all peptides and patient responses, the heatmap was made out (Fig. 3 The



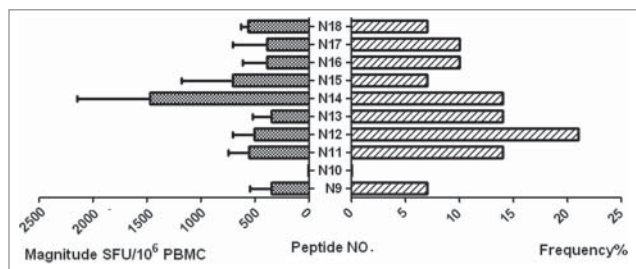
**Figure 1.** Magnitude of human immunodeficiency virus (HIV)-1 subtype B Nef core specific T cell responses in 29 HIV-1B-infected individuals. Measured as interferon (IFN)- $\gamma$  enzyme-linked immunospot (ELISPOT), represented as spot-forming units (SFU)/ $10^6$  peripheral blood mononuclear cells.

heatmap of peptides and patient responses). These peptides can be used as a reference for the design of vaccines.

### ELISPOT magnitude, breadth, and clinical outcome

The correlations of ELISPOT magnitude with the CD4<sup>+</sup> T-cell count and VL were first examined to investigate the relationship between HIV-1 Nef core region-specific T-cell function and clinical outcome. The results showed no significant correlations with the total magnitude of responses and the CD4<sup>+</sup> T-cell count ( $R^2 = 0.0216$ ,  $P = 0.6161$ ) (Fig. 4a ELISPOT magnitude is associated with CD4<sup>+</sup> T cell count and viral load); the same was found with the plasma VL ( $R^2 = 0.0093$ ,  $P = 0.7031$ ) (Fig. 4b).

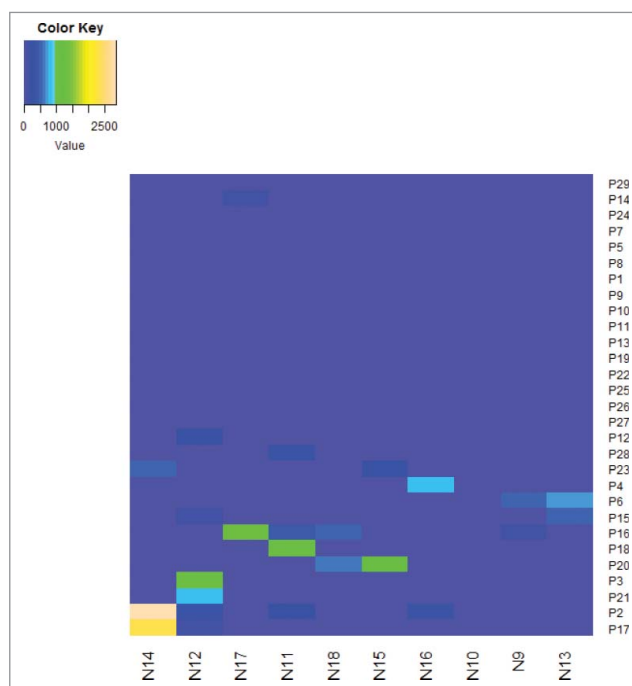
Next, the relationship between the ELISPOT breadth and CD4<sup>+</sup> T-cell count and VL was investigated. The CD4<sup>+</sup> T-cell median count was found to be significantly higher [499 cells/mL (range 475–523)] in individuals with 4 responses compared with 329 cells/mL (range 159–543) among those with a greater range of responses ( $P = 0.0258$ ), but not in other sites (Fig. 5a ELISPOT breadth is associated with CD4<sup>+</sup> T cell count and viral load). The VL median was significantly higher in individuals with 4 responses [2.81 log copies/mL (range 2.17–3.46)] compared with 4.55 log copies/mL (range 3.34–5.73) individuals with a greater range of responses ( $P = 0.0397$ ), but not in other sites (Fig. 5b). The data showed that the more the number of reacting Nef core peptides, the higher the CD4 cell count and the lower the VL. Therefore, The Nef core region-specific immune responses could partially reduce the level of viral load.



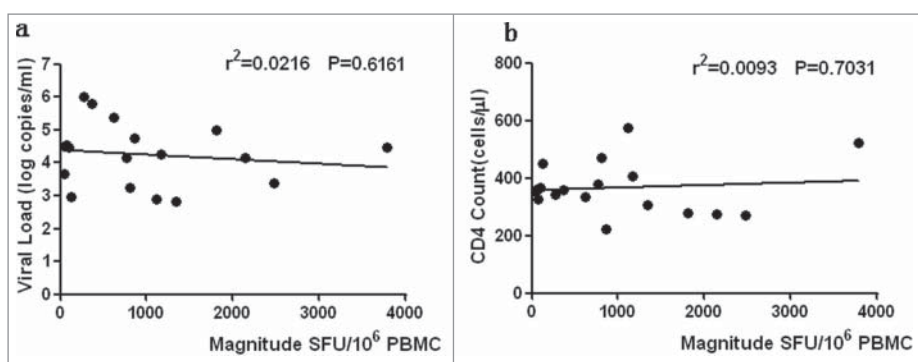
**Figure 2.** Magnitude and frequency of 10 HIV-1 subtype B Nef core OLPs. The total magnitude of T cell responses specific for each tested peptide is shown as bars on the left part of the graph. The frequency of each tested peptide is shown as bars on the right part of the graph.

### HLA-DQB1\*06 and clinical outcome

The relationship between HLA-DQB1\*06 and clinical outcome was examined to further clarify the role of HLA-DQB1\*06 in the progression of HIV-1. The results showed that the CD4 count of the HLA-DQB1\*06-positive group was higher than that of the HLA-DQB1\*06-negative group, and the difference was not statistically significant ( $P > 0.05$ ) (Fig. 6a Comparison of CD4 count, VL, Magnitude and Breadth between DQB1\*06 positive and negative group). VL in the HLA-DQB1\*06-positive group was significantly lower than that in the HLA-DQB1\*06-negative group, with a statistically significant difference ( $P = 0.0415$ ) (Fig. 6b). The quantitative T-cell responses were significantly higher in the HLA-DQB1\*06-positive group than in the HLA-DQB1\*06-negative group ( $P = 0.0297$ ) (Fig. 6c). The number of reacting peptides wasn't significant difference in the HLA-DQB1\*06-positive group and negative group ( $P > 0.05$ ) (Fig. 6d). The correlation between



**Figure 3.** The heatmap for all patients and all peptides provide full picture with the ELISPOT responses. The right ordinate is the patient NO., The bottom abscissa is the peptide NO. The heatmap should define the cut-off as the detection limit.



**Figure 4.** ELISPOT magnitude is associated with CD4<sup>+</sup>T cell count and viral load. The associations between ELISPOT magnitude (total spot forming units per 1 Million PBMCs and CD4<sup>+</sup>T cell count or viral load) were analyzed by Spearman's correlation. The Nef core-specific response was compared with CD4<sup>+</sup>T cell count (a) and viral load (b). P value is corrected by FDR.

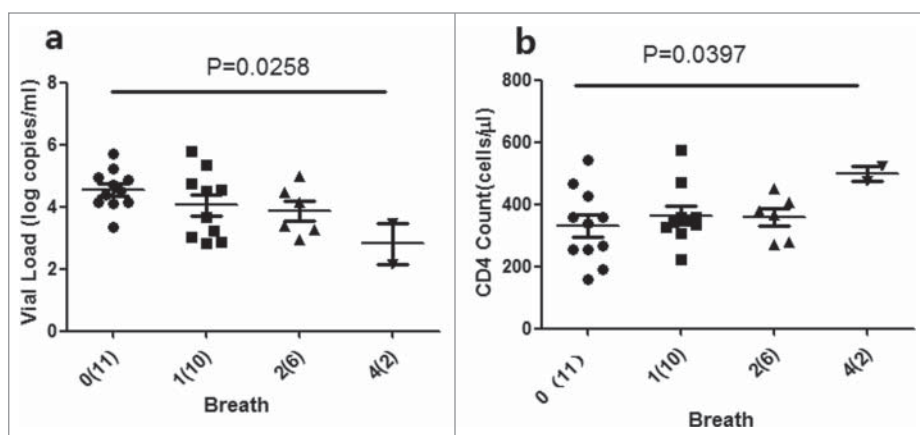
HIV-1-specific T-cell responses and CD4<sup>+</sup> T-cell count and plasma VL were analyzed. Numerical counts in the HLA-DQB1\*06-positive group had a significant correlation with clinical outcome in VL ( $R^2 = 0.4881$ ,  $P = 0.0368$ ) (Fig. 7a) DQB1\*06 allele-specific enzyme-linked immunospot (ELISPOT) magnitude were compared with viral load (VL) and CD4<sup>+</sup> T cell count) and CD4<sup>+</sup> T-cell count ( $R^2 = 0.2131$ ,  $P = 0.3121$ ) (Fig. 7b). No statistically significant correlation was observed between either plasma VL or CD4<sup>+</sup>T-cell count and T-cell responses in the HLA-DQB1\*06-negative group (Fig. 6c and d).

## Discussion

HIV-1 vaccine is essential in combating current HIV-1 pandemics. The HIV-1 Nef gene encodes 200–215 amino acids in length.<sup>10,11</sup> Nef has a complex role in HIV-1 pathogenicity via several mechanisms, including downregulation of host CD4 and major histocompatibility complex (MHC) cell surface expression, modulation of T-cell function, and altering of macrophage signaling.<sup>12–14</sup> Thus, Nef is the most versatile of all HIV-1 accessory proteins.<sup>15</sup> This study demonstrated multiple immunodominant regions in Nef core protein. The dominant epitope peptide was screened using an HIV-1 Gag region whole-gene overlapping peptide. About 38% peptides were recognized in Gag protein.<sup>16</sup> However, the overlap polypeptide in

the Nef core region caused a higher immune response (90%) compared with the Gag region, leading to response in 62.1% individuals. These results suggested that the ability to target Nef epitopes with sufficient strength might serve as a correlate of protection. At the same time, 2 dominant epitope peptides (N12 and N14) were found in the Nef core region. Therefore, Nef core protein had extremely high immune efficiency.

This study showed statistically significant correlations between the breadth of Nef core region-specific T-cell responses and plasma VL or CD4<sup>+</sup> T-cell count. It suggested that Nef core region-specific CD8<sup>+</sup> T-cell responses might mediate highly effective immune control of viremia. Several further lines of evidence pointing toward the potential immunological benefit of targeting HIV-1 Nef have been reported.<sup>6,17,18</sup> Nef has a high density of overlapping CD8<sup>+</sup> T-cell epitopes (see Nef epitope map at [www.hiv.lanl.gov](http://www.hiv.lanl.gov)), and multiple copies of Nef are produced early in the HIV-1 life cycle.<sup>10,19</sup> Nef is the most targeted protein in acute infection,<sup>11–13</sup> accounting for 50–90% of CD8<sup>+</sup> T-cell responses in acutely infected subjects,<sup>8,20</sup> as well as having the maximum CD8<sup>+</sup> T-cell responses and the highest-magnitude IFN- $\gamma$  responses in chronic infection.<sup>21</sup> HIV-1 vaccine-design efforts have, in large part, centered on T-cell priming immunizations, as the pivotal role of CD8<sup>+</sup> T cells in controlling viral replication has been demonstrated in both HIV-1<sup>22–24</sup> and experimental Simian immunodeficiency virus (SIV)



**Figure 5.** ELISPOT breadth is associated with CD4<sup>+</sup>T cell count and viral load. The associations between ELISPOT breadth [the number of reacting OLPs] and CD4<sup>+</sup>T cell count (a) or viral load (b) were analyzed using the Kruskal-Wallis test. (n) Refers to the number of individuals. P value is corrected by FDR.

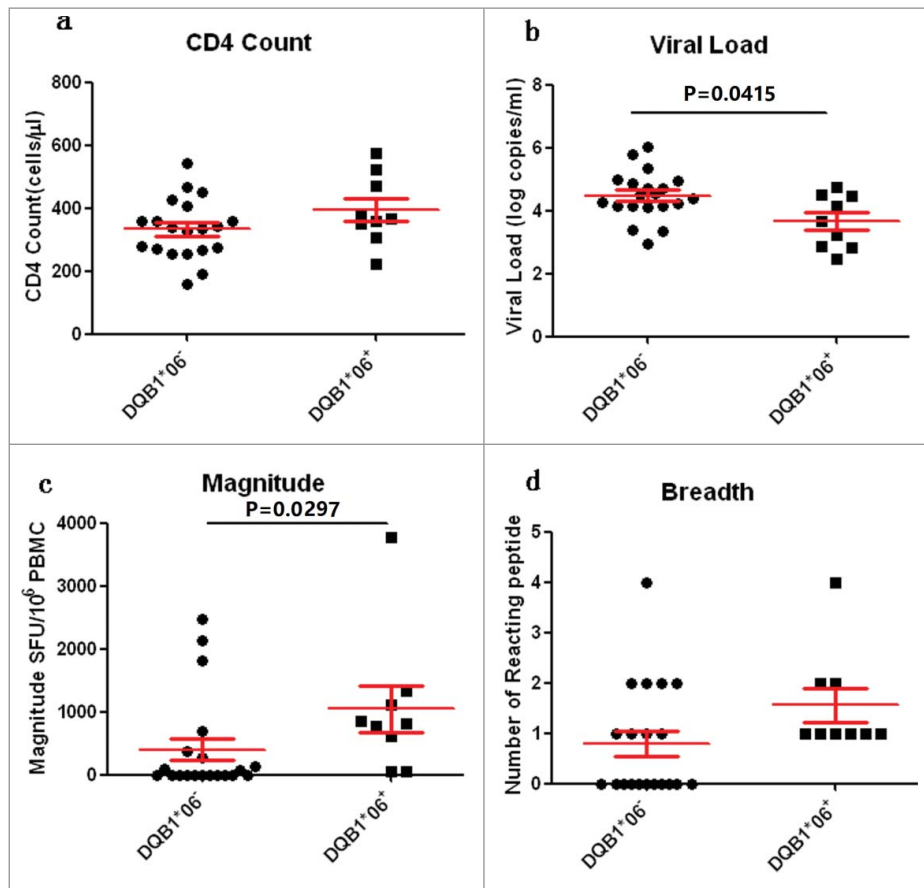


Figure 6. Comparison of CD4 count, VL, Magnitude and Breadth between DQB1\*06 positive and negative group. P value is corrected by FDR.

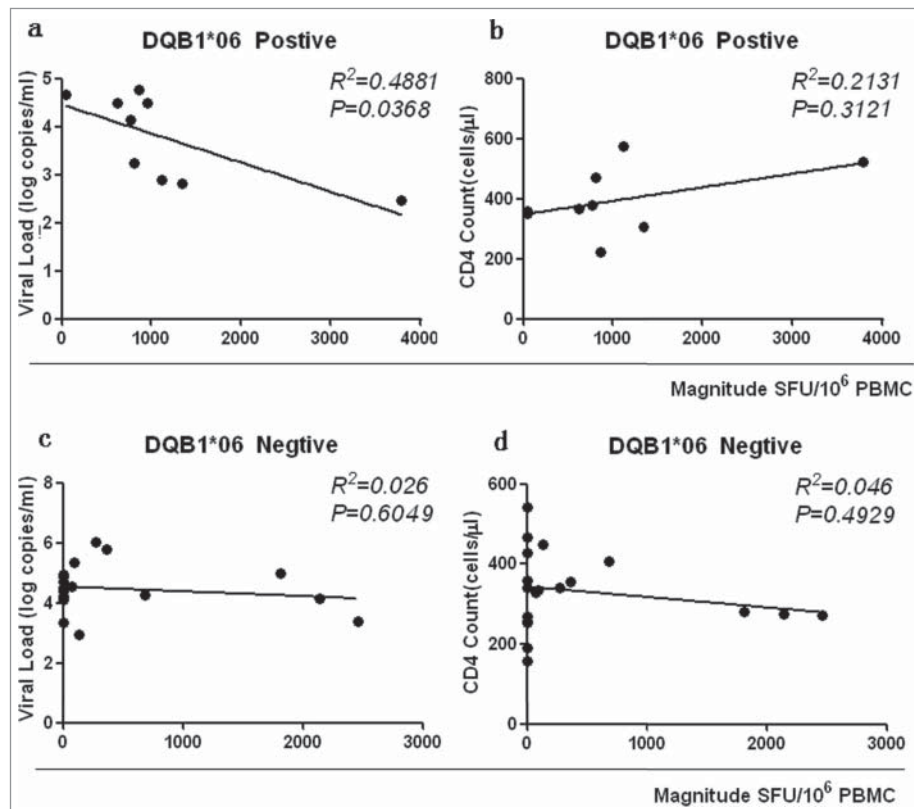


Figure 7. DQB1\*06 allele-specific enzyme-linked immunospot (ELISPOT) magnitude were compared with viral load (VL) and CD4+ T cell count. P value is corrected by FDR.

infections.<sup>25,26</sup> The structure of Nef core region is relatively conservative. Therefore, the vaccine targeted at the Nef core region could improve T-cell activity against infected cells and improve immune control.

This study further strengthened the evidence regarding the involvement of DQB1\*06 haplotype in the control of HIV-1 infection. Host genetic factors play an important role in mediating resistance to HIV-1 infection and may modify the course of virus infection. Many reports have shown that class I HLA alleles are closely related to disease progression. Less is known about the association of class II HLA alleles with disease progression. A few studies have cited a relationship between HLA-DRB1\*13 and/or HLA-DQB1\*06 and HIV-1 control.<sup>27,28</sup> The present study showed that the DQB1\*06 haplotype-positive group had a higher CD4<sup>+</sup> T-cell count and lower VL compared with the DQB1\*06 haplotype-negative group. This raised the possibility of previous observations reporting a similar protective effect.<sup>27</sup> It should be noted that most of the subjects with HLA-DQB1\*06 do not have class I alleles associated with HIV-1 control: HLA-B\*57, HLA-B\*27, Bw4/Bw4, HLA-B\*81, and so forth (Table 1 Characteristics of the study patient cohort). Therefore, the interference of other genotypes was excluded. Further exploration of mechanisms showed that the magnitude and breath of HIV-1-specific T-cell response differed among individuals with or without DQB1\*06. The DQB1\*06-positive group had higher magnitude and breath of HIV-1-specific T-cell response compared with the DQB1\*06 haplotype-negative group. At the same time, the magnitude of HIV-1-specific T-cell response had a significant correlation with clinical outcome in the DQB1\*06-positive group, suggesting that the presence of DQB1\*06 haplotype influenced the T-cell immune

responses to HIV-1 infection. This was consistent with data from Vyakarnam et al., who showed that HLADQB1\*06 alleles had a potential protective effect on the course of HIV-1 disease.<sup>27</sup> One of the possible explanations is that specific structural features that are shared by different HLA molecules and influence peptide-binding sites confer a protective effect. HLA-DQB1\*06 is prone to combine with peptide-binding sites, mounting an efficient immune response against HIV-1. Another explanation is that patients with HLA-DQB1\*06 have been reported to have strong mucosal CD4 T-cell responses.<sup>28</sup> The last explanation is that HLA-DQB1\*06 may be resistant to escape mutation if the epitope is in a region of the HIV-1 genome, the detailed structure of which is essential for viral function, and the peptide-binding groove of the allele can tolerate limited mutations in the peptide.<sup>27</sup>

As differences in correlation patterns are related to the control of viremia, differences between diverse MHC class II HLA supertypes should be taken into account in vaccine design to elicit optimal T-cell responses within HLA supertypes, as well as to design vaccine efficacy trials for participants that represent the population in which such a vaccine would be used in the future.

## Materials and methods

### Study subjects and clinical features

Samples were taken from participants in a previously well-characterized HIV-1-infected cohort, established in 2004, of 236 HIV-1-infected patients attending clinics in Beijing, China. The cohort consisted of chronically infected individuals with confirmed serodiagnosis of HIV-1. The HIV-1 infection was

**Table 1.** Characteristics of the study patient cohort.

Laboratory ID	Age	Sex	Route of transmit	CD4+ T cell count	VL (Log)	HLA typing									
						A1	A2	B1	B2	Bw1	Bw2	DRB1-1	DRB1-2	DQB1-1	DQB1-2
P1	33	Male	Sex	358	4.7	A*03	A*31	B*51	B*51	Bw4	Bw4	DRB1*14	DRB1*04	DQB1*05	DQB1*03
P2	27	Male	Sex	523	4.5	A*01	A*02	B*57	B*39	Bw4	Bw6	DRB1*07	DRB1*08	DQB1*06	DQB1*03
P3	30	Male	Sex	306	2.8	A*02	A*11	B*40	B*51	Bw4	Bw6	DRB1*08	DRB1*09	DQB1*06	DQB1*03
P4	39	Female	Blood	470	3.2	A*03	A*24	B*07	B*15	Bw6	Bw6	DRB1*15	DRB1*04	DQB1*06	DQB1*06
P5	27	Male	Sex	359	3.7	A*11	A*11	B*07	B*52	Bw4	Bw6	DRB1*01	DRB1*15	DQB1*05	DQB1*06
P6	24	Male	Sex	406	4.3	A*02	A*11	B*39	B*15	Bw6	Bw6	DRB1*09	DRB1*09	DQB1*03	DQB1*03
P7	31	Male	Sex	357	6	A*02	A*02	B*13	B*15	Bw4	Bw6	DRB1*15	DRB1*12	DQB1*06	DQB1*03
P8	29	Male	Sex	190	4.4	A*33	A*33	B*44	B*58	Bw4	Bw4	DRB1*13	DRB1*03	DRB1*01	DRB1*08
P9	25	Male	Sex	255	4.9	A*02	A*11	B*07	B*52	Bw4	Bw6	DRB1*01	DRB1*08	DRB1*01	DRB1*08
P10	28	Male	Sex	358	3.7	A*02	A*11	B*46	B*51	Bw4	Bw6	DRB1*04	DRB1*09	DQB1*03	DQB1*03
P11	27	Male	Sex	256	2.7	A*02	A*02	B*46	B*15	Bw6	Bw6	DRB1*09	DRB1*12	DQB1*03	DQB1*03
P12	36	Male	Sex	342	6	A*03	A*30	B*13	B*15	Bw4	Bw6	DRB1*07	DRB1*12	DQB1*02	DQB1*03
P13	30	Male	Sex	340	4.9	A*03	A*30	B*07	B*52	Bw6	Bw6	DRB1*09	DRB1*12	DQB1*03	DQB1*03
P14	22	Male	Sex	335	1.4	A*02	A*24	B*27	B*46	Bw4	Bw6	DRB1*08	DRB1*11	DQB1*06	DQB1*03
P15	31	Male	Sex	569	2.5	A*02	A*24	B*07	B*52	Bw4	Bw6	DRB1*09	DRB1*12	DQB1*03	DQB1*03
P16	25	Male	Sex	276	4.2	A*02	A*29	B*40	B*35	Bw6	Bw6	DRB1*03	DRB1*11	DQB1*02	DQB1*03
P17	29	Male	Sex	271	3.4	A*02	A*29	B*39	B*15	Bw6	Bw6	DRB1*09	DRB1*12	DQB1*03	DQB1*03
P18	25	Male	Sex	573	2.9	A*02	A*31	B*15	B*15	Bw6	Bw6	DRB1*15	DRB1*04	DQB1*06	DQB1*06
P19	28	Male	Sex	429	4.2	A*02	A*33	B*58	B*46	Bw4	Bw6	DRB1*03	DRB1*09	DQB1*02	DQB1*03
P20	35	Male	Sex	280	5	A*02	A*31	B*40	B*46	Bw6	Bw6	DRB1*09	DRB1*11	DQB1*03	DQB1*03
P21	27	Male	Sex	250	4.6	A*24	A*24	B*40	B*15	Bw6	Bw6	DRB1*15	DRB1*14	DQB1*06	DQB1*03
P22	39	Male	Sex	159	3.3	A*02	A*24	B*40	B*51	Bw4	Bw6	DRB1*09	DRB1*09	DQB1*03	DQB1*03
P23	32	Male	Sex	679	2.1	A*02	A*26	B*08	B*40	Bw6	Bw4	DRB1*07	DRB1*08	DQB1*06	DQB1*03
P24	27	Male	Sex	328	4.5	A*02	A*30	B*13	B*15	Bw4	Bw6	DRB1*15	DRB1*07	DQB1*02	DQB1*03
P25	27	Male	Sex	224	4.8	A*02	A*11	B*40	B*15	Bw6	Bw6	DRB1*04	DRB1*14	DQB1*04	DQB1*05
P26	50	Male	Sex	467	4.5	A*02	A*02	B*48	B*52	Bw4	Bw6	DRB1*11	DRB1*14	DQB1*05	DQB1*03
P27	26	Male	Sex	543	4.2	A*02	A*24	B*40	B*15	Bw6	Bw6	DRB1*16	DRB1*04	DQB1*05	DQB1*05
P28	49	Male	Sex	350	4.5	A*02	A*02	B*40	B*51	Bw4	Bw6	DRB1*01	DRB1*08	DQB1*05	DQB1*03
P29	22	Male	Sex	449	3	A*02	A*24	B*40	B*38	Bw4	Bw6	DRB1*01	DRB1*08	DQB1*05	DQB1*03

diagnosed using the solid-phase method with enzyme-linked immunosorbent assay (ELISA) kits, and infections were confirmed further by Western blot test, which was provided by the National AIDS Control Organization, located in China. Most of the subjects (95%) were homosexual men in the cohort. A total of 56 patients who remained antiretroviral treatment-naïve were identified to study the potential impact of genotype on disease progression in patients with an extended follow-up. HIV-1 subtypes were detected in 56 subjects, of which 29 patients had B subtype. Moreover, individuals who were screened for acute illnesses and infections requiring treatment were also included in the present study, although pregnant females and patients with blood dyscrasias were excluded. This study was approved specially by Beijing Youan Hospital Ethics Committee (No. 20061109V1) and conducted according to the set guidelines for research. Written informed consent was obtained from the patients after explaining the purpose and expected consequences of the study. The characteristics of the patients in the present study are detailed in Table 1.

### Peripheral blood mononuclear cell isolation

Fresh blood was obtained by phlebotomy. Peripheral blood mononuclear cells (PBMCs) were then isolated using ethylenediamine tetraacetic acid, treated and heparinized. The sample blood was then placed onto a Ficoll-Hypaque density gradient (Sigma, MO, USA) and finally cryopreserved [90% fetal calf serum (FCS), 10% dimethyl sulfoxide] at  $-196^{\circ}\text{C}$  liquid nitrogen until needed for testing.

### Plasma VL and CD4<sup>+</sup> T-cell counts

The HIV-1 RNA plasma VL and CD4<sup>+</sup>T-cell counts were quantified using the HIV-1 RNA 3.0 bDNA assay (Bayer, Leverkusen, Germany) and fluorescence activating cell sorter count (Becton Dickinson, CA, USA), respectively, according to the manufacturer's instructions. The threshold for RNA detection was at 50 copies/mL. All plasma VLs were presented as log<sub>10</sub> transformed data.

### Synthetic HIV-1 Nef core overlapping peptides

HIV-1 subtype B Nef core whole-gene polypeptide was obtained from the National Institute of Health, AIDS Research and Reference Reagent program (<https://www.aidsreagent.org/>). The peptides were synthesized by Sigma and the Medical Research Council Human Immunology Unit (WIMM, Oxford, UK); 18-mer peptides overlapped by 10 nucleotides and representing the consensus clade B proteome were pooled into the Nef core protein (Table 2 Nef Core OLPs sequence, magnitude and breath), as described previously.<sup>8,9,16</sup>

### HLA typing

Genomic DNA was extracted from the patient's PBMCs using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Genotypes, including HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1, were determined by sequence-specific primer (SSP) PCR (performed at the Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford University, UK).

**Table 2.** Nef Core OLPs sequence, magnitude and breath.

Nef core Peptide NO	Peptide sequence	Average magnitude (SFU/10 <sup>6</sup> PSMC)	Number of positive patients
N9	EVGFPVRPQVPLRPMTYK	346.1	1
N10	QVPLRPMTYKALDLSHF	0.0	0
N11	YKGALDLSHFLKEKGGLE	553.5	4
N12	HFLKEKGGLEGLIYSQKR	504.4	6
N13	LEGLIYSQKRQDILDWV	346.4	4
N14	KRQDILDWVYHTQGYFP	1467.5	4
N15	WVYHTQGYFPDQWNYTPG	707.8	2
N16	FPDQWNYTPGPGIRYPLT	386.8	3
N17	PGPGIRYPLTFGWCFKLV	387.0	3
N18	LTFGWCFKLVPEPEKVE	565.1	2

### Interferon- $\gamma$ enzyme-linked immunospot assay

The PBMCs were thawed and cultured at  $37^{\circ}\text{C}$  for 18 h in Roswell Park Memorial Institute (RPMI)-1640 medium (Hyclone, UT, USA) with 10% FCS (Hyclone). The cultured cells ( $10^5$ ) were then added to each well in a 96-well microtiter high-affinity plate (MAIP54510; Millipore, MA, USA), precoated with an anti-interferon (IFN)- $\gamma$  antibody (10 mg/mL; Mabtech, Nacka Strand, Sweden). The single peptide was pooled, and the final concentration of each peptide was measured at 4 mg/mL. The plate was incubated for 16 h at  $37^{\circ}\text{C}$  in 5% CO<sub>2</sub>. The IFN- $\gamma$ -producing cells were then made visual with a biotinylated anti-IFN- $\gamma$  secondary antibody (Mabtech) and an enzyme-substrate complex. The spots with decreasing density radiating from the center were counted using the ELISPOT reader (CTL). The results were expressed as the number of spot-forming units (SFUs) per million PBMCs. More than 50 SFU/million PBMCs and 3 times more spot-forming cells compared with the negative control were considered positive.

### Statistical analysis

The statistical analysis was performed using SPSS 17.0 (SPSS, IL, USA). The number of individuals with positive responses to Nef core proteins was compared using the Fisher's exact test. Then, the association between the breadth in terms of cell count and clinical outcomes (CD4<sup>+</sup>T-cell count and VL) was analyzed using the Mann-Whitney *U* test and the association between the magnitude of T-cell response and clinical outcomes (CD4<sup>+</sup> T cell count and VL) was analyzed using the Spearman's correlation test. Since this study was conducted in a small subset, adjust the p-value with matlab 7.0 False Discovery Rate (FDR).

### Disclosure of potential conflicts of interest

The authors declare no conflicts of interest.

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