Complete Human Immunodeficiency Virus-1 Specific T Lymphocyte Response to Chinese Human Immunodeficiency Virus-1 B/C Chronic Infectors

ZHE WANG[#], HONG-WEI LIU[+], KUN-XUE HONG[*], ZU-JIANG YU[#], JIAN-PING CHEN[*], YU-HUA RUAN[*], QUAN-CHENG KAN[#,1], AND YI-MING SHAO[*,1]

[#]The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan, China; [+]Center for AIDS/STD Control and Prevention, Henan Center for Disease Control and Prevention, Zhengzhou 450016, Henan, China; [*]Division of Virology and Immunology, National Center for Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 100052, China

Objective To characterize the human immunodeficiency virus (HIV) -specific T lymphocyte responses and identify the immunodominant regions in Chinese HIV-1 recombinant subtype B/C chronic infectors at complete genome level. **Methods** Twenty-five HIV-1 B/C recombinant chronic infectors were screened for their specific T lymphocyte responses to a panel of peptides corresponding to the complete HIV-1 subtype B genome by gamma interferon ELISPOT assay. Kruskal-Wallis nonparametric analysis of variance was used to test significant differences across gene regions, and Tukey pairwise analysis was used to identify differences between gene regions. Spearman rank correlation was used to assess the relation between responses. **Results** The order of recognized frequencies of specific T lymphocyte responses to HIV proteins was Nef>Vpr>Gag>Pol>Vpu>Env>Rev>Vif>Tat. When adjusted for protein length, Nef, Vpr, Gag, and Pol were the most intensely targeted proteins and the central region of Nef, Gag p24, Pol RT, and Vpr was most frequently recognized. No significant correlation was observed between the magnitude of IFN-γ production of HIV-1-specific T lymphocyte responses and plasma viremia, breadth of response and CD_4 counts. **Conclusion** The central region of Nef, Gag p24, Pol RT, and Vpr is most frequently targeted in HIV-1 B/C recombinants chronic infectors. HIV-1-specific T lymphocyte responses and plasma viremia or CD_4 counts play no protective role at complete genome level in these infectors.

Key words: Human immunodeficiency virus (HIV); Specific T lymphocyte response; Elispot; IFN-γ

INTRODUCTION

The emergence and preservation of specific T lymphocytes are fundamental in host defense against human immunodeficiency virus type 1 (HIV-1) infection[1-2]. Thus, the characterization of HIV-1 specific T lymphocyte immune responses in genetic diverse individuals infected with locally prevalent HIV-1 strains is essential in elucidating the mechanism of HIV-1 pathogenesis and in developing an immunogen which can induce potent and broad anti-HIV T-cell immunity. It has been shown that HIV-1 subtype B/C epidemic is speeding[3], but limited data are available on T cell responses covering the whole genome at single peptide level in HIV-1 subtype B/C infection. This study was to use the overlapping peptides spinning the whole HIV-1 B consensus genome to determine the scope and specificity of specific T lymphocyte immunity in Chinese HIV-1 subtype B/C infectors.

MATERIALS AND METHODS

Subjects

Twenty-five HIV-1 infected individuals with untreated chronic infection were enrolled from Xinjiang, China. All individuals in this study were infected with HIV-1 B/C recombinant, as determined by gag, nef, and pol sequencing. Relevant clinical and demographic data about the study subjects are summarized in Table 1. There was a significant correlation between the number of CD_4 T cells and

[1]Correspondence should be addressed to Quan-Cheng KAN and Yi-Ming SHAO. Tel: 86-371-66989940. Fax: 86-371-66910907. E-mail: johnyue70@yahoo.com (Quan-Cheng KAN); Tel:86-10-63166184. Fax: 86-10-63154638. E-mail: yshao@bbn.cn (Yi-Ming SHAO)

Biographical note of the first author: Zhe WANG, male, born in 1960, professor, doctor, fellow at the first affiliated hospital, Zhengzhou University, majoring in HIV/AIDS.

0895-3988/2009
CN 11-2816/Q

plasma viral loads (P=0.0011 r^2=0.3628). The study was approved by the respective institutional review boards and all subjects gave their written informed consent.

TABLE 1

Clinical and Demographical Information about the Study Subjects*

	Ethnicity	Gender	Risk Factor	Age (Yr)	Log_{10} Plasma Viral Load (RNA copies/mL)**	CD_4 Count (per mm^3)**	CD_8 Count (per mm^3)**
Mean	H:3 U:22	m:16 f:9	IDU	32.12	5.61	324.12	1060.44
Median				32	4.83	321	967
Range				17-44	<LDL-6.38	603	2619

Note: *H, Han; V, Uyigur; m, male; f, female; IDU; IV drug use. **Measured during HIV-1-specific T-cell analysis, there were six infectors whose plasma viral load was below the lowest detection level.

CD_4 Cell Count

CD_4 cells from EDTA anticoagulated whole blood were calculated using FITC-conjugated CD3 antibody, PE-conjugated CD_4 antibody, PC5-conjugated CD_{45} antibody and Beckman-Coulter elite flow cytometer (Beckman-Coulter, USA) equipped with argon ion laser (488 nm) according to the manufacturer's instructions.

HIV-1 Viral Load

Plasma viral load was detected by fluorescence real time PCR (LightCycler, Roche, USA; PCR Fluorogence Diagnostic, PG BioTech, China) according to the manufacturer's instruction, the detection limit of PG assay was 100 HIV-1 RNA copies per mL.

Preparation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMC) were prepared from whole blood by density-gradient centrifugation on Ficoll-Hypaque (Sigma, USA). After washed twice with Hank's solution, pellet was resuspended in a R10 medium (RPMI 1640 containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μg /mL streptomycin, and 2 mmol L-glutamine/L) and the final concentration of PBMC was adjusted to 1.0×10^6 cells/mL.

Design of Peptide Matrix

Peptides, obtained from the National Institute of Health AIDS Research and Reference Reagent Program (USA), are consisted of 770 overlapping peptides spanning the HIV-1 genes encoding the Gag (123 15-mers), Pol (249 15-mers), Env (212 15-mers), Nef (49 15-mers), Tat (23 15-mers), Vpr (22 15-mers), Rev (27 15-mers), Vif (46 15-mers), and Vpu (six 9-mers and 13 15-mers). All 15-mers are overlapped by 11 amino acids, and 9-mers are overlapped by 5 amino acids. The of the Env peptides were sequenced based on HIV-1MN, and the others were sequenced based on the HIV-1 clade B consensus sequence. All the 770 overlapping peptides were included in one-peptide matrix systems including 28 peptide pools, respectively, at two different axes. Each peptide was represented in two different peptide pools, allowing for identification of the respective peptide by responses in the two corresponding pools. This is exemplified for the identification of Pol5491 peptide in Table 2 (e.g., Pool BY2=peptides Pol5489 to Pol5516, aggregately 28 peptides; Pool BX3= Pol5463, Pol5491, Vpu5979, and other peptides, aggregately 28 peptides). The final concentration of each peptide within a peptide pool was 50 μg/mL.

TABLE 2

Example of Peptide Matrix Setup for Complete Genome*

	Pool BX1	Pool BX2	**Pool BX3**	Pool BX4	Pool BX28
Pool BY1	Pol5461	Pol5462	**Pol5463**	Pol5464	Pol5488
Pool BY2	**Pol5489**	**Pol5490**	**Pol5491**	**Pol5492**	**Pol5516**
Pool BY3	Pol5517	Pol5518	**Pol5519**	Pol5520	Pol5544
Pool BY28	Vpu6428	Vpu5978	**Vpu5979**	Vpu5980	

Note. *Example, shown in bold face: a positive response to peptide Pol5491 would be reflected in positive responses in pools BX3 and BY2.

Characterization of HIV-1 Specific T Cell Responses by Elispot Assay

HIV-1 specific T lymphocyte responses were quantified by Elispot assay, performed according to the Manual of human IFN-γ ELISPOT kit (U-CyTech, Netherland). Briefly, fresh PBMC were plated onto 96-well plates precoated with 0.5 g of anti-IFN-γ monoclonal antibody, PBMC were added at a concentration of 100 000 cells per well in a volume of 100 μL R10 medium (RPMI 1640 containing 10% fetal calf serum, 10 mmol/L HEPES buffer) with antibiotics (50 U of penicillin-streptomycin/mL). The final concentration of peptides in the well was 5 μg/mL. Plates were incubated overnight at 37 ℃ in an atmosphere containing 5% CO_2 and developed. Wells containing PBMC and R10 medium were used as negative controls. Wells containing PBMC and phorbol 12-myristate 13-acetate (PMA) and inomysin served as positive controls. Duplicate experimental wells and quadruple control wells were used. The number of spots per well was counted on an automated Elispot plate reader (BioReader 4000, Germany), and the number of specific T cells was calculated by subtracting the negative control values. A response was considered positive when the mean number of spot forming cells in the experimental wells was at least three times the mean number of SFC in the negative control wells and the mean number of SFC/106 cells in the experimental wells was more than 50 SFC/106 PBMC.

Statistical Analysis

Statistical analysis and graphical presentation were done using SigmaPlot 5.0 (SPSS). Results are given as $\bar{x} \pm s$ or median with range. Kruskal-Wallis nonparametric analysis of variance was used to test significant differences across gene regions, and Tukey pairwise analysis was used to identify differences between gene regions. Spearman rank correlation was used to assess the relation between responses.

RESULTS

HIV-1 Specific T Lymphocyte Responses across Complete Genome

Using the above-described peptide matrix approach, we screened 25 HIV-1-infected individuals for HIV-1 specific T lymphocyte responses to the entire expressed HIV-1 genome in order to assess the total breadth and magnitude of virus-specific responses at single peptide level. The mean number of IFN-γ-secreting cells per subject was 694 SFC/106 PBMC and ranged from undetectable to 2799 (median, 566 SFC/106 PBMC). The mean number of positive peptides per subject was 68 and ranged from undetectable to 425 (median, 40), revealing that all HIV-1 proteins and protein subunits can serve as targets for HIV-1 specific T lymphocyte responses (Fig. 1). Of the 25 subtype B/C HIV-1-infected individuals, 20 (80%) responded to one or more of the 770 peptides used in this study. Figure 3 shows the distribution of positive peptides in the 20 responders across the expressed genome, with 85% recognizing Gag, 80% recognizing Nef, 75% recognizing Pol, 60% recognizing Env, 55% recognizing Vpu, 45% recognizing Vpr, 20% recognizing Vif, 20% recognizing Tat and 15% recognizing Rev. When the order of positive peptides in the 20 individuals who responded to the peptide pools was Pol>Gag>Env>Nef>Vpr>Vpu>Vif> Rev>Tat. When adjusted for protein length, the order was Nef > Vpr> Gag> Pol > Vpu> Env> Rev> Vif> Tat (Fig. 2). No significant difference was found in Nef, Gag, Pol, Env, Vpu, and Vpr across different regions of the genome. However, the number of Nef and Gag was significantly greater than that of Vif, Tat, and Rev across different regions of the genome (Fig. 2).

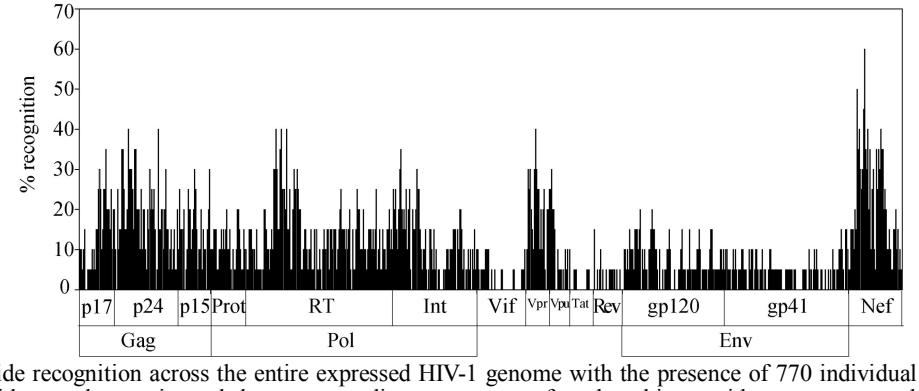

FIG. 1. Peptide recognition across the entire expressed HIV-1 genome with the presence of 770 individual overlapping peptides on the *x*-axis, and the corresponding percentage of study subjects with a response to the individual peptide on the *y*-axis. The horizontal bar indicates the corresponding regions of individual peptides.

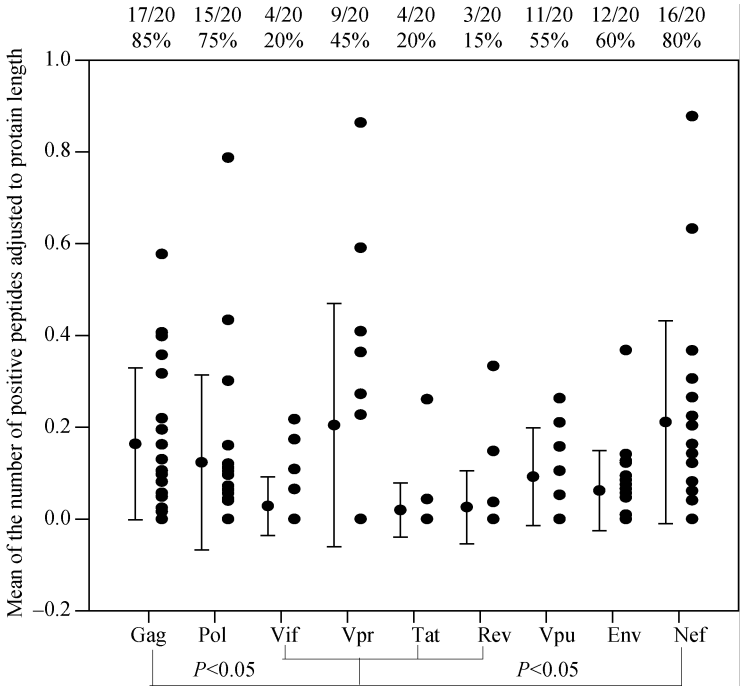

FIG. 2. Distribution of cross responses across complete peptide pool sets, showing the mean and individual plots. The proportion of individuals responding to each gene is shown above the plot, and the significance level between regions is shown below the plot. Significance was measured by Kruskal-Wallis nonparametric analysis of variance and Tukey pairwise analysis.

Identity of Most Frequently Recognized Peptides and Region within Gag, Pol, Nef, and Vpr

The individual peptides were targeted at different frequencies. While some peptides were only recognized by one study subject, others were targeted by several individuals. Seven, five, thirteen, and one most frequently recognized peptides were found within Gag, Pol, Nef, and Vpr, respectively. The most frequently recognized peptides within Gag, Pol, Nef, and Vpr are summarized in Table 3. Seven most frequently recognized peptides were found in Gag, most of them (6/7) lie in p24 except for one in p17. P24 is the main region frequently recognized by individuals in Gag. When the overlaps were removed, 2 relatively long amino acid stretches, ^{aa25}KVVEEK AFSPEVIPMFSAL^{aa43} and ^{aa73}INEEAAEWDRLHPV HAGPI^{aa91}, and another two peptides, ^{aa49}PQDLNTM LNTVGGHQ^{aa63} and ^{aa161}FRDYVDRFYKTLRAE^{aa175} within p24, were identified, which contain multiple epitopes frequently targeted by individuals. Within p17, only one peptide, ^{aa97}TKEALEKIEEEQNKS^{aa111} was considered highly immunodominant. Within Pol, 5 most frequently recognized peptides were found. Most of them (4/5) lie in RT except for one in RNase. RT is the main region frequently recognized by individuals in Pol. When the overlaps were removed, a relatively long amino acid stretch, ^{aa241}DFWEVQL GIPHPAGLKKKKSVTVLDVGDAYFSVPLDKDF RKYTAFTIPSINNETP^{aa295} within RT, was identified, which contains multiple epitopes frequently targeted by individuals. One peptide ^{aa709}AGIRKVLFLDGID KA^{aa723}, was found in Rnase. Within Nef, 13 most frequently recognized peptides were found. When the overlaps were removed, two long amino acid stretches, ^{aa25}PAADGVGAVSRDLEKHGAITSSNT AANNADCAWLEAQEEEEVGFPVRPQVPLRPM TYKAAVDL^{aa87} and ^{aa97}LEGLIYSQKRQDILDLW VYHTQGYFPDWQNYTPGPGIRY^{aa135}, were identified, which contain multiple epitopes frequently targeted by individuals. One peptide, ^{aa33}HFPRIWLHSLGQH IY^{aa47}, was found in Vpr. Nef, Gag p24, Pol RT, and Vpr were most frequently recognized.

TABLE 3

Most Frequently Recognized Peptides in HIV-1 B/C Recombinant Chronic Infectors

Peptide	Amino Acid Position	Sequence	Study Subjects with Response (%)
NEF5152	Nef 53-67	ADCAWLEAQEEEEVG	60
NEF5145	Nef 25-39	PAADGVGAVSRDLEK	50
NEF5151	Nef 49-63	AANNADCAWLEAQEE	45
POL5521	Pol 241-255 = RT 86 ->100	DFWEVQLGIPHPAGL	40
POL5526	Pol 261-275 = RT 106 ->120	VTVLDVGDAYFSVPL	40
POL5531	Pol 281-295 = RT 126 ->140	KYTAFTIPSINNETP	40
GAG7917	Gag 181-195 = p24 49 ->63	PQDLNTMLNTVGGHQ	40
GAG7945	Gag 293-307 = p24 161 ->175	FRDYVDRFYKTLRAE	40
NEF5147	Nef 33-47	VSRDLEKHGAITSSN	40
NEF5155	Nef 65-79	EVGFPVRPQVPLRPM	40
NEF5167	Nef 113-127	WVYHTQGYFPDWQNY	40
VPR6072	Vpr 33-47	HFPRIWLHSLGQHIY	40
POL5525	Pol 257-271 = RT 102 ->116	KKKSVTVLDVGDAYF	35
POL5638	Pol 709-723 = RNase 114 ->Integrase 8	AGIRKVLFLDGIDKA	35
GAG7896	Gag 97-111 = p17 97 ->111	TKEALEKIEEEQNKS	35
GAG7911	Gag 157-171 = p24 25 ->39	KVVEEKAFSPEVIPM	35
GAG7912	Gag 161-175 = p24 29 ->43	EKAFSPEVIPMFSAL	35
GAG7923	Gag 205-219 = p24 73 ->87	INEEAAEWDRLHPVH	35
GAG7924	Gag 209-223 = p24 77 ->91	AAEWDRLHPVHAGPI	35
NEF5146	Nef 29-43	GVGAVSRDLEKHGAI	35
NEF5153	Nef 57-71	WLEAQEEEEVGFPVR	35
NEF5157	Nef 73-87	QVPLRPMTYKAAVDL	35
NEF5163	Nef 97-111	LEGLIYSQKRQDILD	35
NEF5165	Nef 105-119	KRQDILDLWVYHTQG	35
NEF5168	Nef 117-131	TQGYFPDWQNYTPGP	35
NEF5169	Nef 121-135	FPDWQNYTPGPGIRY	35

Relation between HIV-1 Specific T-cell Responses and Plasma Viremia or Number of CD_4 Cells

No significant correlation (Fig. 3a, P=0.282, r^2=0.795) was found between magnitudes of specific T lymphocyte responses and plasma viral loads in 16 of the 25 participants whose plasma viral load was higher than the lowest detection level at complete genome level. A more detailed analysis of the magnitude of gene specific T lymphocyte responses and viral loads revealed the same result of GAG (Fig. 3b, P=0.458, r^2=0.0484), Pol (Fig. 3c, P=0.0843, r^2=0.2601), Vif (Fig. 3d, P=0.750, r^2=0.160), Vpr (Fig. 3e, P=0.217, r^2=0.250), Tat (Fig. 3f, P=0.333, r^2=0.010), Rev (Fig. 3g, P=1.000, r^2=1.000), Vpu (Fig. 3h, P=0.931, r^2=0.0006), Env (Fig. 3i, P=0.844, r^2=0.0044), and Nef (Fig. 3j, P=0.493, r^2=0.0412). When the breadth of HIV-1 specific responses was correlated with CD_4 count, there was also no significant correlation (P=0.283, r^2=0.0444, Gag: P=0.173, r^2=0.0666, Pol: P=0.465, r^2=0.0375, Vif: P=0.917, r^2=0.0568, Vpr: P=0.948, r^2=0.0009, Tat: P=0.917, r^2=0.0045, Rev: P=1.000, r^2=0.1247, Vpu: P=0.776, r^2=0.0377, Env: P=0.974, r^2=0.0055 and Nef: P=0.167, r^2=0.0874, respectively).

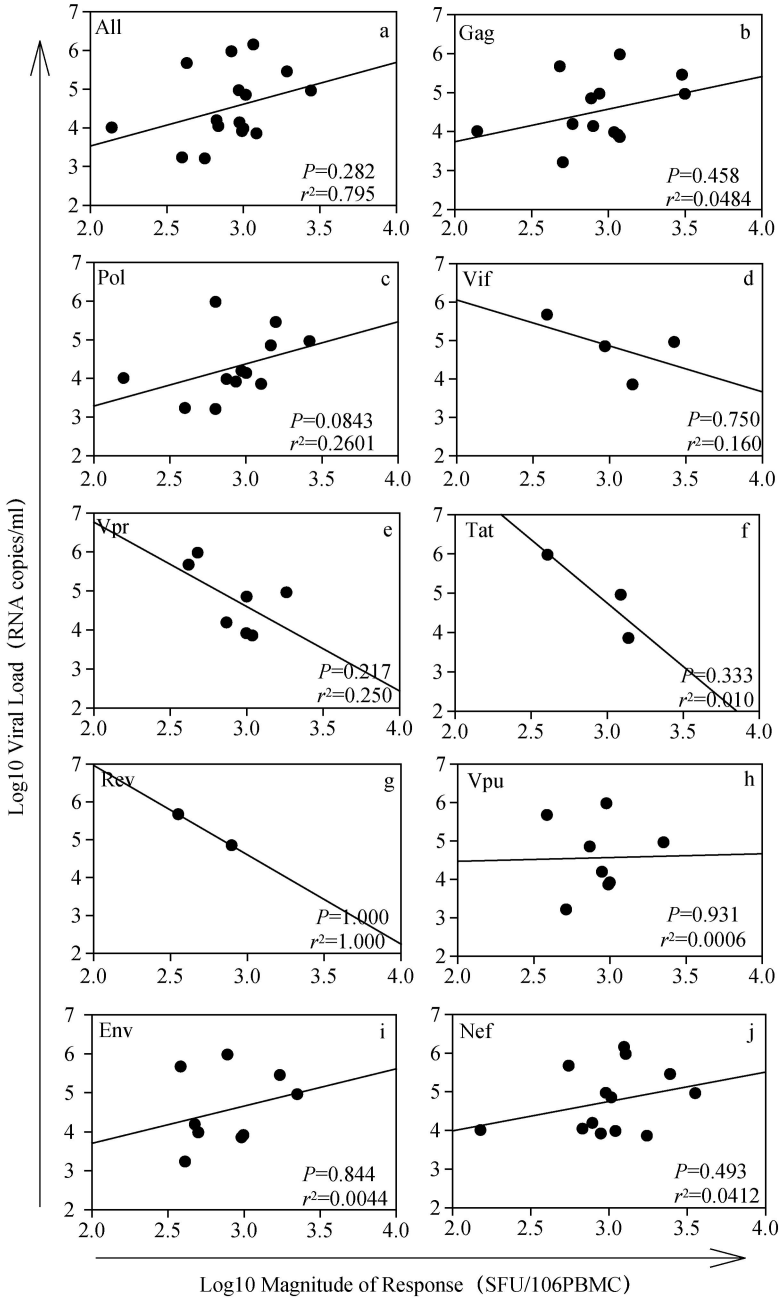

FIG. 3. Correlations between log10 SFU/106 PBMC values and plasma viral loads showing total responses to complete genome (A), Gag (B), Pol (C), Vif (D), Vpr (E), Tat (F), Rev (G), Vpu (H), Env (I), and Nef (J).

DISCUSSION

In this study, most individuals responded to at least one peptide. However, 5 of the 25 HIV-1-infected individuals did not respond to any of the peptides, suggesting that these individuals may have developed HIV-1 specific T lymphocytes targeting variant regions of the sequences in the peptide set. The order of recognized frequency T lymphocyte responses to HIV-1 proteins was Nef>Vpr>Gag>Pol>Vpu>Env>Rev>Vif>Tat when adjusted for the protein length. Nef, Vpr, Gag, and Pol were the most intensely targeted proteins. It was recently reported that HIV-1 specific T lymphocytes mainly target the conserved regions in different clades[4-5]. In our study, the central region of Nef was highly targeted and spanned 63 and 39 aa, which is highly conserved across subtypes[6]. The more conserved regions in Gag p24, Pol RT were also frequently targeted. Responses were less frequent to

Env, Vif, Tat, Rev, and Vpu than to Gag, Pol, and Nef, which may reflect more variabilities in these genes[7]. Responses were more frequent to Vpr than to other HIV proteins, indicating that Vpr is the least variable of accessory proteins. Recent data suggest that Vpr is preferentially targeted by CD_8^+ T cells[8]. The conserved central region of Nef, P24, and Vpr regions may be included in design of T cells-based immunogen.

The correlation between viral load and HIV-1 specific T cell responses still remains controversial. Studies on HIV-1 infected individuals showed that positive[9], negative[10] or no correlation[11] between viral load and HIV-1 specific T cell responses may be partly due to the different stages of infection or measured parameters. The comprehensive analysis using overlapping peptides in our study showed no significant correlation between the magnitude of total IFN-γ secretion to HIV-1 and the viral load or breadth of responses and CD_4 counts, and correlation at individual protein level showed similar results, supporting the hypothesis that the frequency of IFN-γ production by HIV-1-specific T lymphocytes is not a determining factor for viral control, and is independent of subtypes.

It has been shown that the expression of granzyme B is not associated with that of interferon-γ of CD_8^+ T cells in HIV infection[12]. It was reported that IFN-γ-based screening techniques cannot reflect the functional impairment of CD_8^+ T cell responses in late-stage infection[13]. Collectively, the absence of a negative correlation between HIV-1-specific CD_8^+ T cell responses and viral load suggests that IFN-γ production by HIV-1-specific T cells is not the main mechanism underlying the control of viral replication in chronic infection[14-15]. Further study on HIV-1-specific T cell responses is needed for the control of HIV-1 replication.

REFERENCES

1. Ueno T, Motozono C, Dohki S, *et al*. (2008). CTL-mediated selective pressure influences dynamic evolution and pathogenic functions of HIV-1 Nef. *J Immunol* **180**, 1107-1116.
2. Wang Y E, Li B, Carlson J M, *et al*. (2008). Protective HLA class I alleles restricting acute-phase CD_8^+ T cell responses are associated with viral escape mutations located in highly conserved regions of HIV-1. *J Virol* **83**(4), 1845-1855.
3. Database of Chinese HIV molecular epidemiological investigation (http://shgy.jhgl.org/shownews.asp?newsid=893).
4. Yu X G, Lichterfeld M, Perkins B, *et al*. (2005). High degree of inter-clade cross-reactivity of HIV-1-specific T cell responses at the single peptide level. *AIDS* **19**, 1449-1456.
5. Thakar M R, Bhonge L S, Lakhashe S K, *et al*. (2005). Cytolytic T lymphocytes (CTLs) from HIV-1 subtype C-infected Indian patients recognize CTL epitopes from a conserved immunodominant region of HIV-1 Gag and Nef. *J Infect Dis* **192**, 749-759.
6. Mashishi T, Loubser S, Hide W, *et al*. (2001). Conserved domains of subtype C nef from South African HIV type 1-infected individuals include cytotoxic T lymphocyte epitope-rich regions. *AIDS Res Hum Retroviruses* **17**, 1681-1687.
7. Betts M R, Yusim K, Koup R A (2002). Optimal antigens for HIV vaccines based on CD_8^+ T response, protein length, and sequence variability. *DNA Cell Biol* **21**, 665-670.
8. Altfeld M, Addo M M, Eldridge RL, *et al*. (2001). Vpr is preferentially targeted by CTL during HIV-1 infection. *J Immunol* **167**, 2743-2752.
9. Northfield J W, Loo C P, Barbour J D, *et al*. (2007). Human immunodeficiency virus type 1 (HIV-1)-specific CD_8^+ T(EMRA) cells in early infection are linked to control of HIV-1 viremia and predict the subsequent viral load set point. *J Virol* **81**, 5759-5765.
10. Novitsky V, Gilbert P, Peter T, *et al*. (2003). Association between virus-specific T-cell responses and plasma viral load in human immunodeficiency virus type 1 subtype C infection. *J Virol* **77**, 882-890.
11. Bartovska Z, Beran O, Rozsypal H, *et al*. (2007). HIV-1-specific CD_8^+T cells do not correlate with viral load in HIV-1-positive patients. *Acta Virol* **51**, 59-61.
12. Kleen T O, Asaad R, Landry S J, *et al*. (2004). Tc1 effector diversity shows dissociated expression of granzyme B and interferon-gamma in HIV infection. *AIDS* **18**, 383-392.
13. Draenert R, Verrill C L, Tang Y, *et al*. (2004). Persistent recognition of autologous virus by high-avidity CD_8 T cells in chronic, progressive human immunodeficiency virus type 1 infection. *J Virol* **78**, 630-641.
14. Addo M M, Draenert R, Rathod A, *et al*. (2007). Fully differentiated HIV-1 specific CD_8^+ T effector cells are more frequently detectable in controlled than in progressive HIV-1 infection. *PLoS ONE* **2**, e321.
15. Jiang Y, Karita E, Castor D, *et al*. (2005). Characterization of CD_8^+ T lymphocytes in chronic HIV-1 subtype A infection in Rwandan women. *Cell Mol Biol (Noisy-le-grand)* **51**(Suppl), OL737-743.

(*Received February* 19, 2009 *Accepted October* 12, 2009)