CHAPTER IV

MATERIALS AND METHODS

1. Population and subjects

The study of HLA-A11-restricted HIV-specific CD8+ T cell responses in HIV-1-infected Thai patients has not been systematically studied. We conducted a pilot study by enrolling total of 18 the HIV-1-infected patients with HLA-A11 alleles. For control group (n=10), there are 5 HLA-A11 negative HIV-infected patients and 5 HLA-A11 positive healthy donors.

The HIV-infected Thai patients attended the Anonymous Clinic of the Thai Red Cross AIDS Research Centre and the Immune Clinic of the King Chulalongkorn Memorial Hospital. The selected HIV-1-infected patients were clinically asymptomatic and their CD4 cell counts were equal to or more than 300 cells/mm^3. All patients were antiretroviral therapy naïve. Patients who had active current infection and/or opportunistic infections were excluded from the study.

2. HLA typing

HLA typing information was provided by Professor Sarah L. Rowland-Jones, MRC Human Immunology Unit, University of Oxford, UK. HLA alleles were identified by polymerase chain reaction amplification using sequence-specific primers (PCR-SSP).

3. HLA-A11 restricted HIV-specific CTL epitopes

The 9-to11-amino acid peptides study have been reported in the Los Alamos Immunology Database (http://hiv-web.lanl.gov) and the other previous studies that were reported in the other journals (Table 1). These peptides were generously provided by Professor Sarah L. Rowland-Jones, MRC Human Immunology Unit, University of Oxford, UK.

No	Protein	Hxb2 location	Peptide sequence	Abbreviation	HLA restriction	References
1	Gag	82-91	IATLWCVHQR	IATL	A11	http://hiv-web.lanl.gov
2	Pol	93-102	GIPHPAGLKK	GIPH	A11	http://hiv-web.lanl.gov
3	Pol	117-126	SVPLDESFRK	SVPL	A11	http://hiv-web.lanl.gov
4	Pol	158-166	AIFQSSMTK	AIFQ	A11, A3, A68, A33	http://hiv-web.lanl.gov
5	Pol	520-528	QIIEQLIKK	QIIE	A11	http://hiv-web.lanl.gov
6	Pol	269-277	QIYAGIKVK	QIYA	A11	(124)
7	Pol	340-350	QIYQEPFKNLK	QIYQ	A11	http://hiv-web.lanl.gov
8	Pol	416-424	FVNTPPLVK	FVNT	A11	http://hiv-web.lanl.gov
9	Pol	179-188	AVFIHNFKRK	AVFI	A11	http://hiv-web.lanl.gov
10	Env	6-15	TQMNWPNLWK	TQMN	A11	http://hiv-web.lanl.gov
11	Env	36-46	VTVYYGVPVWR	VTVY	A11	http://hiv-web.lanl.gov
12	Env	109-117	ISLWDQSLK	ISLW	A11	http://hiv-web.lanl.gov
13	Env	309-318	ITVGPGQVFY	ITVG	A11	http://hiv-web.lanl.gov
14	Env	340-348	RVLKQVTEK	RVLK	A11	http://hiv-web.lanl.gov
15	Env	762-770	SLCLFSYHR	SLCL	A11	(15)
16	Nef	73-82	QVPLRPMTYK	QVPL	A11, A3, B27, B35	http://hiv-web.lanl.gov
17	Nef	83-92	GAFDLSFFLK	GAFD	A11	http://hiv-web.lanl.gov

Table 1 The HLA-A11 restricted HIV-specific CTL epitopes used in this study

4. Peripheral Blood Mononuclear Cells (PBMC) preparation

Heparinised blood was taken from donors every 4 mouth for 3 times. The heparinised blood was diluted with RPMI in a ratio of heparinised blood: RPMI (GIBCO BRL, U.S.A.) equals 1: 1. PBMC were separated using density gradient centrifugation method. Briefly, blood was added to centrifuge tube added with ficoll hypaque (Robbins Scientific, Norway) in a ratio of blood: ficoll hypaque equals 3: 1 and then centrifuged for 30 min at 1500 rpm. PBMC were separated from a part of ring and then washed twice with 10 ml of RPMI, and centrifuged for 10 min at 2000 rpm and 1800 rpm, respectively. The separated PBMC were resuspended with R10 (10% fetal bovine serum in RPMI-1640) supplemented with L-glutamine, penicillin 100 U/ml, streptomycin 100 µg/ml. Cell count was performed and adjusted to desired cell concentration and used in an ELISpot assay or stored in liquid nitrogen until use.

5. EBV-transformed B Lymphoblastoid cell line (BLCL) preparation

The $10x10^6$ cells/ml of PBMC were incubated with 1 ml of supernatant of B95-8 and incubated at 37^0C and 5%CO_2 for 1 h. After incubation, cells were washed twice with RPMI and then resuspened in R20 (20% fetal bovine serum in RPMI 1640) at $1x10^6$ cells/ml and added 100 µl of Cyclosporin A (1 µg/ml). After that, cells were added to the culture medium. R20 medium was changed every 1 week. After 3 week of culture, the cells from 24 well plate (IWAKI, Japan) were transferred to flask and changed medium twice weekly with R10 medium.

6. Generation of HIV-1-specific CTL lines by peptide stimulation

The $10x10^6$ cells/ml of PBMC were incubated with 100 µl of peptide (200 µg/ml) (Minotope, Australia) for 1 h. at 37^0C and 5%CO_2 and then washed twice with RPMI. PBMC were resuspended in R10 medium and transferred to 24 well plates in $5x10^6$ cells/well. 12 µl of IL-7 (330 units/ml) (R&B, UK.) is added to each well and then incubated at 37^0C and 5%CO_2. On day 3, in culture, the R10 with IL-2 (100 units/ml)

(R&B, UK.) was added to culture media in a ratio of IL-2: R10 equals 1 µl: 1ml. After that, the R10 with IL-2 was changed twice a week. After 10 to 14 day, cytotoxicity function of the cells were evaluated in a standard ^{51} Cr release assay.

7. HIV-specific T cell analysis

7.1 Cytotoxicity assay

The 100 µl of fetal bovine serum (FBS) was added to $5x10^5$ cells/ml of the pellet of BLCL and then labeled with ^{51}Cr for 1 h. at 37°C and 5 % CO_2. After incubation, cells were washed 3 times with 5 ml of RPMI. The labeled BLCL were pulsed with 25 µl of peptide (200 µg/ml) and incubated for 1 h. at 37°C and 5 % CO_2 and then washed with 5 ml of RPMI.

During the time, CTL were adjusted at the concentration $2.5x10^6$ cells/ml with R10. 5000 cells/well of the labeled BLCL (target cells) were incubated with CTL (effector cells) in a ratio of effector: target equals 50: 1, 25: 1, and 12.5: 1. After incubation at 37°C and 5 % CO_2 for 4 h, 100 µl of supernatants are harvested and then radioactivity is counted on gamma-counter.

Specific lysis is calculated with the formula:

$$\% \text{ specific lysis} = \frac{\text{experimental release (E)} - \text{spontaneous release (M)}}{\text{total release (T)} - \text{spontaneous release (M)}} \times 100$$

E = effector cell + ^{51}Cr target cell

T = ^{51}Cr -target cell + TritonX-100

M = ^{51}Cr -target cell in R10 alone

The criteria of positive CTL activity are as following

- Spontaneous release is always < 30%.
- Positive CTL response is defined as antigen-specific lysis of > 10 % at one or more E: T ratio.

7.2 ELISpot assay

Ninety-six well nitrocellulose plates (Milipore, U.S.A.) were coated with 50 µl of monoclonal antibody to IFN-γ (anti-IFN-γ) (Mabtech, Sweden) at the concentration 10 µg/ml for 3 h at 37^0C and 5%CO_2. Unbound antibody were washed 6 times with phosphate buffer saline (PBS) in 200 µl/well and blocked with R10 for 1 h at room temperature. Plates were again washed 6 times with PBS and then $2.5x10^5$ cells/well of PBMC were added the coated plated. 5 µl of peptide (200 µg/ml) were added at each well in duplicate. For negative control wells, 10 µl of R10 was added instead of peptide; for positive control wells, 2 µl of phytoheamagglutinin (PHA) (1µg/ml) (Sigma, U.S.A.) was added into the well instead of peptide. The ELISpot plate was incubated for 15 h. at 37^0C and 5%CO_2. Plates were then washed 6 times with PBS-Tween (PBS containing 0.05% Tween 20 [USB, U.S.B.]) and once with PBS before 50 µl of biotinylated anti IFN-γ (1 µg/ml) (Mabtech, Sweden) was added. After 3 h. of incubation, plates were then washed 6 times with PBS Tween and once with PBS. 50 µl of streptavidin-conjugated alkaline phosphatase (1µg/ml) (Mabtech, Sweden) was added and incubated at room temperature for 1 h before washed 6 times with PBS Tween and once time with PBS. Individual cytokine-producing units were detected as blue spots after 20-60 min reaction with 100 µl of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Bio-rad, U.S.A.). The wells were washed with tap water to terminate the colorimetric reaction and plates were then air-dried. The spots were counted under microscope and were expressed as spot-forming unit (SFU) per 10^6 PBMC.

Results of spots higher than 20 spots/well and 2.5 time more than negative controls were considered positive (Figure 3). Negative controls were < 20 spots/well.

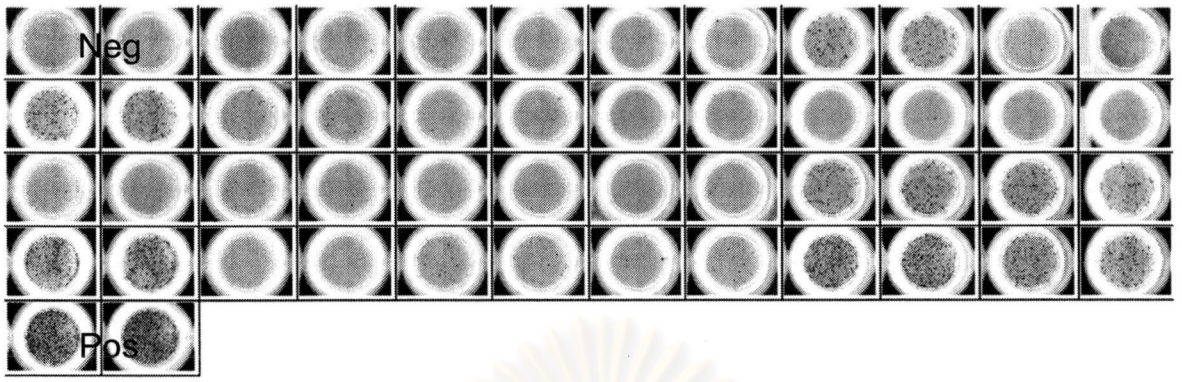

Figure 3 The representative example of ELISpot plate. This plate comprises negative control wells, tested wells and positive control wells.

7.3 CD8+ T cell depletion

The patients who HIV-specific T cell responses were positive by ELISpot assay were selected to test by CD8 depletion. This method was used to prove that these responses were mediated solely or mainly by CD8+ T cell. Briefly, 10x106 cells/ml of PBMC were incubated with 72 µl of anti-CD8-coated magnetic bead (Dynal beads CD8 T-cytotoxic /suppressor cells) at 4 ºC for 20 min, mixed during incubation every 5 min. After that, cells were washed twice with 5 ml of RPMI at 1,500 rpm for 5 min and then resuspened them with R10. The cell depleted CD8+ T cell were tested by ELISpot assay. The detected responses were considered being mediated by CD8+ T cell if they equal to or more than 50% of spots were decreased.

8. DNA sequencing

8.1 DNA preparation

Two million PBMC were cultured with 10 µl of 100 µg/ml of PHA to stimulate HIV replication for 48 h as previously described (125). DNA was extracted using the QIAamp DNA blood mini kit (Qiagen, Germany) according to the manufacturer's protocol. Briefly, each dried cells pellet in a cryotube was added with 200 µl of PBS. The 20 µl of Qiagen

mixed it with vortex machine. The cryotube were incubated for 10 min at 56°C to increase yield. The 200 µl of 96-100% ethanol was added and mixed again by vortexing for 15 s. The mixture from cryotube was transferred to the QIAamp spin column and then centrifuged at 14000 rpm for 1 min. After that, QIAamp spin column was removed to a clean 2 ml collection tube. The 500 µl of buffer AW1 was added into the spin column and then centrifuged at 8000 rpm for 1 min. After centrifugation, the 500 µl buffer AW2 was added to the QIAamp spin column and centrifuged 2 times at 14000 rpm for 3 min and 1 min. Finally, The QIAamp spin column was removed to a clean 1.5 ml microcentrifuge tube and added 80 µl of buffer TE to eluted extracted DNA. After incubation for 5 min, the QIAamp spin column was centrifuged at 8000 rpm for 1 min. The concentration of purified DNA was determined by optical density (OD) at OD260.

8.2 PCR amplificaiton

The nested polymerase chain reaction (PCR) was done to amplify a 703-bp fragment containing the entire *nef*. Primers were: 5′-GGTGGAACTTCTGGGACACAGC-3′ / 5′-GGGTTAGCTACTCCCCAACTCC-3′ for the primary reaction, and 5′-CCTAGAAGAATCAGACAGGGCTTAG-3′ / 5′-TCCCCTGGAAAGTCCCCAGC-3′ for the secondary reaction. The nested PCR carried out with a total volume of 50 µl containing a 0.2 mM concentration of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 2.5 U of *Taq* polymerase (Promega, U.S.A.), and 12.5 pmole of each primer. The two amplification rounds were identical, with an initial denaturation step at 92 °C for 5 min, followed by 27 cycles of 94 °C for 45 sec, 50°C for 30 sec, and 72 °C for 1 min 30 sec and a final extension of 5 min at 72 °C.

8.3 Gel electrophoresis and visualisation

The amplified products were analysed by gel electrophoresis in a 1.5% agarose gel and electrophoresed in 0.5X Tris borate EDTA buffer (TBE) running buffer. The electrophoresis was carried out at 100 constant voltages for 1 h. and then stained with

ethidium bromide (0.5 μg/ml of 1X TBE) for 15 min. After that, it was rinsed and destained with water for 15 min followed by photographing under UV illumination.

8.4 PCR purification

PCR products were purified by QIAquick® PCR purification kit (Qiagen, Germany) according to the manufacturer's protocol. Briefly, 5 volumes of buffer AL to1 volume of PCR products were added and transferred to the QIAquick column and then centrifuged at 13000 rpm for 1 min. After centrifugation, the 750 μl of buffer PE was added to QIAquick column and then centrifuged at 13000 rpm for 1 min. Finally, the QIAquick column was removed into a new collection tube and added 20 μl of buffer TE to elute DNA. After incubation for 5 min, the QIAquick column was centrifuged at 8000 rpm for 1 min.

8.5 Cloning

PCR products were cloned into pGem®-T Easy Vector systems (Promega, U.S.A.) as recommended by the manufacturers. Briefly, the reactions carried out with a total volume of 10 μl containing 5 μl of 2x Rapid Ligation Buffer, 1 μl of pGem®-T Easy Vector, 1 μl of T4 DNA ligase, PCR products, and deionised water. After incubation overnight at room temperature, 6 μl of each ligation reaction were added to a steriled 1.5 ml microcentrifuge tube on ice. 100 μl of the competent cells (*E. coli* strain DH5α) from -70°C were transferred into each tube. After that, the tube was gently flicked to mix and placed them on ice for 20 min. The cells were heat-shocked for 50 seconds in a water bath at exactly 42°C and immediately returned to ice for 2 min. 200 μl of the SOC medium were added to the tube containing cells transformed and then incubated for 1.5 h. at 37°C with shaking (~150rpm). After incubation, the transformation cultures were plated onto IPTG/X-Gal plates and incubated overnight (16-24 hours) at 37°C.

8.6 Purification of plasmid DNA

Plasmid DNAs were purified by QIAprep® Miniprep kit (Qiagen, Germany) according to the manufacturer's protocol. Briefly, the bacterial pellets were resuspended in 250 µl buffer P1 and transferred to a microcentrifuge tube. 250 µl buffer P2 was added and the tube was gently inverted 4-6 times to mix. After that, 350 µl buffer N3 was added and the tube was inverted immediately but gently 4-6 time and then centrifuge for 10 min at maximum speed with a tabletop microcentrifuge. The supernatants from step 4 were applied to the QIAprep columns by pipetting and then centrifuged for 30-60 s. After discarding of the flow-through, the QIAprep spin columns were washed by adding 0.75 ml buffer PE and centrifuged for 1 min. After that, the QIAprep spin columns were centrifuged for an additional 1 min to remove residual wash buffer. The QIAprep spin columns were placed in a clean 1.5 ml microcentrifuge tube and then 25 µl buffer EB was added to the center of each QIAprep column, let stand for 5 min, and centrifuged for 1 min.

8.7 Sequencing

Sequencing was carried out by BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, U.S.A.) according to the manufacturer's protocol. Briefly, the reactions carried out with a total volume of 10 µl containing 1 µl BigDye sequencing buffer, 3.2 pmole primer, 150-300ng template, and distilled water. The sequencing was performed with the inner primer set of nested PCR described above. The sequencing cycle performed for 25 cycles: 96°C for 30 sec, 55°c for 10 sec, and 60°c for 4 min. After that, the amplified products were transferred to a 1.5 microcentrifuge tube containing 1 µl of 0.25 M EDTA and 30 µl of 100 % ethanol and then incubated for 30 min at 4°C. The tube was centrifuged for 30 min at 4°C (13000 rpm) and the supernatant was removed from the tube. 60 µl of 70% ethanol was added to each tube and centrifuged for 10 min at 4°C. The supernatant was discarded from the tube and then tube was dried for 2 min at 95°C. The amplified product was sequenced using automated sequencer ABI Prism 310 Genetic Analyser (Applied Biosystems, U.S.A.).