CHAPTER IV
MATERIALS AND METHODS

Part I Subjects and sample collection

1. Healthy donors

Ten healthy adult volunteers were enrolled into this study. There were 3 male and 9 female donors with age ranging from 22 to 45 years with a mean of 27 years.

2. Patients

A total of 10 immunocompromised individuals were recruited into the study including 10 HIV infected patients attending the Anonymous Clinic, Thai Red Cross Society. They were 2 male and 8 female patients with age ranging from 20 to 38 years with a mean of 29 years. The patients were enrolled into the study when their serological test was positive in both anti-EBV and anti-HIV. All patients who were on antiretroviral therapy and on immunosuppressive drugs were excluded. Patients who had active current infection and/or opportunistic infectious were also ruled out from the study. All donors were informed the detail of this study and asked to sign the consent form.

3. Specimen collection

7 ml of EDTA blood and 20 ml of heparinised blood were obtained from each volunteer. After collecting, the blood sample was immediately processed.

3.1. Plasma collection

In order to collect plasma for EBV serological screening, the heparinised blood containing tube was centrifuged at 3,000 rpm for 10 minutes and 1 ml of plasma was collected and stored at -20°C until used.

3.2. Peripheral blood mononuclear cell (PBMC) preparation

PBMC were obtained from heparinised blood from 3.1 by density-gradient centrifugation. Brieftly the pellet was resuspended with 7 ml of RPMI 1640 (GIBCO BRL, U.S.A.) and overlay on ficoll-hypaque (density gradient 1.077 g/l), followed by centrifugation at 1,500 rpm for 30 minutes at room temperature. After centrifugation, PBMC were harvested and washed twice with RPMI 1640 and then resuspended in 10% Fetal Bovine Serum in RPMI 1640 (R10, Appendix II). The PBMC were collected twice from each volunteer, the first time was for establishing EBV-transformed B-

lymphoblastoid cell line (B-LCL) and the second was for analysis of EBV-specific T cell responses by Enzyme-Linked Immunospot (ELISpot) assay and for establishing EBV-specific CTL lines.

3.3. Dried pack cell preparation

PBMC were collected from density-gradient centrifugation of EDTA blood through a ficoll-hypaque gradient as previously described. The PBMC were counted and resuspended in PBS before aliquot into microtube. The cells were pelleted by centrifugation at 13,000 rpm for 2 minutes and stored at -70° C until DNA extraction was performed.

<u>Part II</u> Study of EBV serological response

- Detection of anti-VCA and anti-EBNA antibodies

To screen the volunteers enrolling into the study, anti-VCA IgG from their plasma were examined by ELISA commercial kit (Vironostika, Organon Teknika BV Boxtel, The Netherlands). The kit is based on the principle of indirect ELISA. Briefly, plasma was added to a VCA peptide-coated well and incubated at 37° C for 60 minutes. The antigen-antibody complexes would be formed if anti-VCA IgG was present in the sample. Conjugate (anti-human IgG conjugated with horseradish peroxidase) was added to bind to the antigen-antibody complexes. The enzyme-immune complexes detected after adding tetramethylbenzine (TMB) substrate, leading to colour development. The absorbance of solution measuring at 450 nm is related to the concentration of anti-VCA IgG in plasma.

The testing procedures were as followed; plasma sample was diluted to 1:1,000 with sample diluent. 100 μl of diluted plasma was then added to 96-well plate coated with synthetic VCA peptide. For control, 100 μl of each calibrator I, II, III and IV (concentration 10, 20,110 and 170 AU/ml respectively), supplied by the kit was added sequencially to first four wells. After incubation at 37°C for 60 minutes, the plate was washed 4 times with washing buffer and 100 μl of anti-human IgG conjugated with horseradish peroxidase was dispended into each well. The reaction was left at 37° C for 60 minutes and washed 4 times with washing buffer. Thereafter 200 μl of TMB and peroxide in citrate buffer as substrate was applied to each well and incubated at 37° C for 30 minutes in the dark. The reaction was then halted by 200 μl of stop solution (H_2SO_4). The absorbance was measured as optical density

(OD) at 450 nm. Positive result was reported when the absorbance of sample was equal to or more than the absorbance of calibrator II (20 AU/ml). The results could be reported if they reached the following criteria.

1. Absorbance of calibrator I was less than 0.250.
2. The remainder value from subtraction of calibrator IV absorbance with the calibrator II was more than 0.750.
3. The absorbance correlation value (R^2) of calibrator I, II, III and IV, which were automatically calculated by spectrophotometer software (Organon Teknika BV Boxtel, the Netherlands), was greater than 0.9.

Part III Development of EBV-DNA estimation technique by real-time PCR

1. Standard EBV-DNA

To quantified EBV-DNA in clinical sample, known concentration standard EBV-DNA should be prepared. EBV-DNA load in sample was then estimated comparing with the standard DNA. Therefore 3 types of standard EBV-DNA were conciderable prepared. These satndard were Namalwa EBV-DNA, B95-8 EBV-DNA and plasmid containing EBNA-1 DNA per cell.

1.1. Namalwa EBV-DNA

Namalwa DNA, at concentration 100 ng/µl, was generously provided by Associate Professor Dr. Apiwat Muthirangura, Department of Anatomy, Faculty of Medicine, Chulalongkorn University. The calculation in this study was based on the information that the Namalwa cells contained 2 copies of EBV-DNA.

1.2. B95-8 EBV-DNA

The DNA was obtained from B95-8 cells, EBV-infected marmoset lymphoblastoid cell line. Therefore EBV could infect, droved the viral life cycle and parallel replicated with cell cycle. So this cell was the main source of EBV.

1.2.1. Culture of B95-8 cells

B95-8 cells were cultured in R10 (see Appendix II). $5x10^6$ Cells were grown with 5 ml of R10 in 25 cm^2 tissue culture flask at 37°C under 5% CO_2 with 95% humidity. Cultures were subpassaged at a ratio 1:5 twice a week.

1.2.2. B95-8 DNA extraction

Briefly, $5X10^6$ of B95-8 cells were harvested by centrifugation at 1,500 rpm for 5 minutes. The DNA was then extracted from pellet using DNA blood mini kit (Qiagen, Germany) according to the manufacturer's instruction. Briefly, the 20µl of Qiagen protease as well as 200 µl of buffer AL was added into microtube and mixed thoroughly by vortex. After incubation at 56° C for 10 minutes, 200 µl of absolute ethanol was added into the suspension tube and mixed well. Total volume 700 µl of sample was carefully applied to a spin column which setting on a 2-ml collection tube. The column was centrifuged at 14,000 rpm for 1 minute before transferred to a new collection tube. 500 µl of washing buffer (AW1) was pipetted into a spin column, and the column was centrifuged at 8,000 rpm for 1 minute. The new collection tube was replaced and the spin column was again washed once with 500 µl of AW2 buffer at 14,000 rpm for 3 minutes. For drying the spin column membrane, centrifugation at 14,000 rpm for 1 minute was performed. Finally, to elute extracted DNA, the spin column was transferred into a new 1.5 ml microtube, and 50 µl of AE buffer was dropped directly on the column's membrane. After incubation at room temperature for 5 minutes, the column was centrifuged at 8,000 rpm for 1 minute to collect the extracted DNA. The eluted DNA was stored at -20° C until real-time PCR was performed. DNA concentration was determined by measurement of optical density (OD) at a wavelength of 260 nm.

1.3. Plasmid containing EBNA-1 DNA

A 294 bp fragment of *EBNA-1* was amplified from B95-8 EBV-DNA by real-time PCR and cloned into *Escherichia coli*, DH5α strain, using TOPOTA cloning kit (Invitrogen, USA). The recombinant plasmid was confirmed by melting curve analysis and DNA sequencing (BigDye Terminator V3.1 cycle Sequencing Kit, Applied Biosystems, U.S.A.).

1.3.1. Amplification of EBNA-1 DNA and purification method

A 294 bp fragment of *EBNA-1* was amplified from B95-8 EBV-DNA using real-time PCR as described below. The amplicon was confirmed by agarose gel electroporesis and purified using Qiaquick PCR purification kit (Qiagen, Germany) according to manufacturer's

instruction. One volume of PCR product was dispensed into microtube containing 5 volume of AL buffer. The mixture was mixed thoroughly by vortex and transferred to Qiaquick column standing on a 2-ml collection tube. The column was then centrifuged at 13,000 rpm for a minute before 750 μl of PE buffer was applied. After a minute of centrifugation at 13,000 rpm, the column was placed into a steriled microcentrifuge tube. 50 μl of AE buffer was directly added to the column. For collecting the purified product, the column was left at room temperature for 5 minutes and then centrifuged at 8,000 rpm for one minute. DNA concentration was determined by measurement of OD at a wavelength of 260 nm. The purity of DNA was determined by ratio of OD 260: OD 280 which should be equal to or higher than 1.8.

1.3.2. Preparation of competent cells

Escherichia coli (*E. coli*), DH5α strain, was used as competent cell source in this study. The cells were kindly provided by Assistant Professor Chintana Chirathaworn, Ph.D., Deparment of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Briefly, *E. coli* stored at -70° C was thawed in waterbath at 37° C and grown on LB plate (Appendix II) at 37° C overnight. Each single colony was then picked up and cultured at 37° C in 5 ml of LB broth (Appendix II) overnight. The 500 μl of cell suspension was transferred into conical tube containing 50 ml of LB broth and incubated with horizontal shaking at 37°C for 2 to 3 hours. The amount of cells was determined by measurement at OD 600 nm. When the OD was reached the optimum point ranging from 0.4 to 0.5, the suspension tube was placed on ice for 30 minutes and centrifuged at 5,000 rpm, 4°C for 8 minutes. After discarding of supernatant, the pellet was resuspended with 10 ml of 15% glyceral in 50 mM $CaCl_2$ (Ca/Glyceral solution, Appendix II), always keep cool and placed on ice for 30 minutes. After centrifugeation at 5,000 rpm at 4°C for 8 minutes, pellet was resuspended with 1 ml of cold Ca/Glyceral solution and was aliquoted in microcentrifuge tube and then stored at -70° C until used.

1.3.3. Cloning of *EBNA-1* fragment

The purified *EBNA-1* PCR product (from 1.3.1) was cloned by TOPOTA cloning kit (Invitrogen, USA) according to manufacturer's instruction. The kit was composed of linearised plasmid (pCR®2.1 TOPO®) containing ampicillin resistance gene, Topoisomerase I and single 3'-thymidine (T) overhang within the LacZα fragment. As the knowledge that Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. This allows A-tail of target PCR product complementary bounds to T-tail of vector. Topoisomerase I, covalently bond to the vector was then activated resulting in complete ligation between PCR product and the vector. The recombinant plasmids were transformed into competent cell (*E. coli* DH5α strain) by chemical reaction. For screening the colony acquired plasmid containing target DNA located within *LacZ*, X-gal/IPTG plate was needed. Based on the principle that *LacZ* was encoded for β-galactosidase that metabolised colourless substrate, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) into galactose and a blue insoluble product. This reaction was activated by IPTG (isopropyl-β-D-thiogalactopyranoside) which was an inducer of lac operon. The white colony was presented when β-galactosidase transcription was disrupted by insertion of target DNA within *LacZ*. So, each single white colony was selected.

The brief procedures were as followed, 0.5-4 μl of PCR product at concentration of approximately 50 ng was added to the reaction mixture that composed of 1 μl of salt solution (supplied by the kit), 1 μl of TOPO vector and water (add up to 5 μl). The reaction was incubated at room temperature for 5 minutes and placed on ice, while the vial containing competent cells was thawed on ice. 2 μl of TOPO cloning reaction was then added into the competent cell vial and mixed gently. After incubation on ice for 30 minutes, the cells were heated-shocked at 42° C for 30 seconds without shaking and immediately placed on ice. 250 μl of SOC medium, supplied by the kit was added to the culture and the tube was shaked horizontally (200 rpm) at 37°C for an hour. To select the clone,

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100 μl of cell suspension was spreaded on a selective LB plate containing X-gal/IPTG and 100 μg/ml amplicillin. The plate was then incubated at 37° C overnight. The white colony was picked up and cultured in LB broth containing 100 μg/ml amplicillin at 37° C overnight. The suspension was centrifuged at 1,500 rpm for 5 minutes before the plasmid extraction was performed.

1.3.4. Plasmid DNA extraction

The recombinant plasmid was extracted from bacterial cell using QIAprep® Miniprep (QIAGEN, germany) according to manufacturer's instruction. In brief, one white colony was selected and resuspended in 250 μl of P1 buffer and transferred to microcentrifuge tube. 250 μl of P2 buffer was added, and the solution was mixed gently. The tube was immediately mixed after dispensing of 300 μl N3 buffer. The mixture was then centrifuged at 14,000g for 10 minutes. The supernatant was transferred to QIAprep column standing on 2-ml collection tube and centrifuged at 4,00Cg for a minute. After centrifugation, the 750 μl of PE buffer was added into the column and centrifuged twice at 14000 rpm for 1 min. The QIAprep column was placed in a steriled microcentrifuge tube. 50 μl of EB buffer was then added to the column. To elute plasmid, the column was left at room temperature for 5 minutes and centrifuged at 14,000g for a minute. DNA concentration in elute was determined. After that, the purified DNA plasmid was stored at -20° C until used.

1.3.5. Sequencing of plasmid containing *EBNA-1*

To determine whether the inserted *EBNA-1* in the plasmid was similar to that of the *EBNA-1* from B95-8 cells, the nucleotide sequence of purified plasmid was further analysed. The sequencing principle was based on Sanger method modification. The reaction composed of 3 steps, denaturation, annealing and extension similar to PCR method except it was combined deoxynucleotide triphosphate (dNTP) and dideoxynucleotide triphophate (ddNTP) together in the mixture containing single primer. During annealing phase, primer binded complementarily to the DNA target, leading to synthesis of DNA product at the end of primer in 3' direction. As the fact that the extension step required a free –OH group at

the 3' position of sugar (ribose). Chain termination was occurred when ddNTP was incorporated instead of dNTP at each nucleotide position in product fragment. These resulted in a various size of the products which were separated by gel electrophoresis. In automatic sequencing cycle, ddNTPs were labeled with 4 different fluorescence dyes. When it was excited by laser light, fluorescence light was emitted and could be detected in automatic sequencing instrument.

In this study, the purified plasmid containing *EBNA-1* was sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, U.S.A.) according to manufacturer's instruction. Briefly, *EBNA-1* recombinant plasmid at concentration 150-300 ng was added into a PCR tube containing sequencing mixture. The mixture was composed of 3.2 ρmole primer (5'-TGTAAAACGACGGCCAGT-3'), 3 μl of BigDye solution and 1 μl of sequencing buffer. Total volume of the reaction was filled up to 10 μl with water. The reaction tube was placed in thermol cycler. Then 25 cycles PCR reaction was performed as followed: 10 seconds of denaturation at 96°C, 5 seconds of annealing at 50°C and 4 minutes of extension at 72° C. Thereafter the DNA was precipitated by 3M sodium acetate, cold absolute ethanol, and incubated at 4°C for 30 minutes. The tube was centrifuged at 14,000 rpm, 4°C for 30 minutes and washed once with 1 ml of cold 70% ethanol. After centrifugation at 14,000 rpm for 10 minutes, the supernatant was removed, and pellet was dried at 92° C for 2 minutes. To determine the nucleotide sequences, pellet was resuspended with injection buffer and placed in ABI Prism 3100 Genetic analyzer (Applied Biosystem, U.S.A.).

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2. Real-time PCR

In establishment of real-time-based PCR, PCR condition should be optimised. In optimisation, *EBNA-1* from B95-8 cells which routinely cultured in laboratory was prepared. When PCR condition had optimized, as protocol below, the assay was standardised by known concentration Namalwa EBV-DNA. Standard curve which was established from plasmid containing *EBNA-1* was then performed and the experiment was started.

Standard EBV-DNA was detected by real-time PCR with fluorogenic probe based on the principle of Fluorescence Resonance Energy Transfer (FRET). The 297-bp segment of *EBNA-1* was used as target DNA in this study. The forward and reverse primers were 5'-GAG GGT GGT TTG GAA AGC-3' (EB-30) and 5'- AAC AGA CAA TGG ACT CCC TTA G-3' (EB-40). The fluorescence probes were 5' -AGT CGT CTC CCC TTT GGA ATG GC-FITC-3' (3-FL) and 5'-LC-Red 640-CTG GAC CCG GCC CAC AAC CTG-3' (5-LC). All primers and probes were purchased from TIB MOLBIOL (Berlin, Germany). Briefly, 5 μl of DNA sample was loaded into a capillary tube which contained 12 μl of PCR mixture. The mixture was composed of 0.5 μM of each primer, 0.3 μM of each probe, 2 μl of LightCyCler FastStart DNA Master Hybridization Probes (Roche Diagnostics) and 4 mM $MgCl_2$. All of capillary tubes were centrifuged before they were placed in the LightCyCler. Then PCR reaction was performed sequentially as followed: 2 seconds of Uracil-N-glycosylase (UNG) inactivation at 95°C, 10 minutes of Taq DNA polymerase activation at 95°C, 60 cycles of 0 second of denaturation at 95°C, 20 seconds of annealing at 57°C and 10 seconds of extension at 72°C. Fluorescence signal was measured at the end of annealing phase in a single mode. After data summarisation by LightCycler software version 3.5, amplification curves were plotted against the fluorescence value versus the cycle number in 2 fit pint-arithmetic mode.

For melting point analysis, a single cycle of 95° C for 0 second, 40° C for 20 seconds and 95° C for 0 second was immediately performed after amplification with continuous fluorescence acquisition, permitted a construction of melting curve from fluorescence value and temperature.

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<u>Part IV</u> Quantitation of EBV-DNA in clinical specimens

1. Sample for EBV viral load

DNA was extracted from PBMC of 10 healthy volunteers and 10 patients was isolated using QIAamp DNA blood mini kits (Qiagen, U.S.A.) according to the manufacturer's instruction already mentioned in part III. In this study, PBMC pellets approximately $4x10^6$ to $10x10^6$ cells were used. All these DNA samples were further quantitated for the amount of EBV-DNA in copies/10^6 cells or copies/μg DNA by real-time PCR which have previously been described.

Part V Study of EBV-CTL responses

Viral antigen from virus-infected cells could be processed into short peptide (8-12 amino acid), loaded to MHC I molecule and presented to the complex to $CD8^+$ specific T cell which could recognise and destroy the infected cell. When PBMC were infected with recombinant vaccinia containing EBV gene, in the similar way, infected cell would be eliminated by the specific T cells. Within the process, IFN-γ was trap and enumerous by ELISpot, as described below. The recombinant vaccinia were propagated and titrated in TK^-143 cells as following procedure.

1. Preparation of TK-143 cells and recombinant vaccinia containing EBV genes

   1.1. Culture of TK^-143 cells

   TK^-143 cells, osteosarcoma cell lacking thymidine kinase, were cultured in Dubecco Modified Essential Minimal Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 units/ml penicillin G, 100 ug/ml streptomycin and 0.01 M HEPES (N-2-hydroxyethyl-piperaine-N'-2-ethan sulfonic acid) (all were from GIBCO BRL, U.S.A). The cells were grown in 75-cm^2 tissue culture flask at 37°C under 5% CO_2 with 95% humidity. After culturing for 3 days, cells were split at ratio 1:3 into a new flask. These cells were continuously grown and subpassaged until used. TK^-143 cells were kindly provided by Professor Pilaipan Puthawalanon, Ph.D, Department of Microbiology, Faculty of Medicine, Siriraj hospital, Mahidol University, Bangkok, Thailand.

   1.2. Propagation of recombinant vaccinia containing EBV genes.

   A total of 8 recombinant vaccinias containing EBV genes (*LMP-1, LMP-2A, EBNA-1, EBNA-3A, EBNA-3B, EBNA-3C, EBNA-LP* and *BHRF-1*) and one wild type vaccinia were generously provided by Professor Alan B. Rickinson, University of Birmingham, United Kingdom. In briefly, supernatant of each recombinant vaccinia containing EBV genes (r-vaccinia) was inoculated in 1-2 day-old TK^-143 cell monolayer at multiplicity of infection (MOI) of 1. After 2 hours adsorption, the cells were washed twice with PBS, and 5 ml of DMEM with 2% FBS was then added. The infected cells were incubated at 37° C under 5% CO_2 with 95% humidity for 2 days before supernatant and cells were harvested into 15 ml conical tube. The suspension was immediately freeze-thawed at -70°C and 37°C for 3 times. The

supernatant was collected by centrifugation at 5,000 rpm, 4°C for 30 minutes, aliquoted to 1.5 ml microcentrifuge tube and stored at -70° C.

1.3. Titration of recombinant vaccinia containing EBV genes.

The amount of viruses was determined by plaque titration assay. A number of 4.0 x 10^5 of TK^-143 cells was cultured as monolayer in each well of 6-well tissue culture plate for 1-2 days. After washing once with PBS, 1 ml of serial 10-fold dilution of r-vaccinia was immediately added into the culture. Each dilution was done in duplicated wells. After incubation at 37°C under 5% CO_2 with 95% humidity for 2 hours, the infected cells were washed once with PBS. Then 3 ml of DMEM with 2%-FBS was added into each well. The cultures were incubated at 37° C under 5% CO_2 with 95% humidity for another 2 days. The number of plaque was counted after staining with 1% crystal violet containing 20% ethanol solution (Appendix II). One ml of staining solution was added into each well after removing the medium. After 20 minutes, the staining solution was discarded, and the plate was air-dried. Plaques, transparent unstained area of death cells, were enumerated. The titre of r-vaccinia was calculated using the following formula.

$$\textbf{Titre}\ (\text{PFU/ml}) = \frac{P_1 + P_2 + \ldots P_n}{N} \times \textbf{Dilution} \times \frac{1}{V}$$

P = number of plaque counted in each well at selected dilution

N = number of wells

V = volume inoculated in each well (in millilitres)

2. Estimation of EBV-specific T cell responses by IFN-γ producing by ELISpot assay

MultiScreen Immobilon-P (IP) 96 well filtration plate (Milipore Corporation, U.S.A.) was coated with 50 µl of 10 µg/ml of IFN-γ monoclonal antibody (MABTECH AB) and incubated at 37°C for 3 hours. Unbound antibodies were removed by 6 times washing with steriled PBS. Then the coated plate was blocked by R10 AT 37°C for an hour. After 6 times washing with PBS, 100 µl of PBMC at a concentration 2.5x10^6cell/ml were added into each well. These cells were infected with 50 µl of r-vaccinia at MOI of 4 in duplicate. Whilst the positive control were 50

μl of recombinant vaccinia containing HIV genes (*Gag, Nef* and *Env*) at MOI of 4 and 4 μl of PHA (20 μg/ml) in 100 μl of RPMI containing 10 μl of PBMC at a concentration 2.5×10^6cell/ml, the negative controls were 50 μl of wild type vaccinia at MOI of and 100 μl of PBMC at a concentration 2.5×10^6cell/ml in 50 μl of RPMI. After incubation at 37° C under 5% CO_2 with 95% humidity for 16 hours, the plate was washed 6 times with 0.05% tween20 in PBS (PBS-tween) and once with PBS. 50 μl of 1 μg/ml biotinylated anti-IFN-γ monoclonal antibodies (MABTECH AB) was dispended into each well, and the reaction was incubated at room temperature for 3 hours. The plate was then washed 6 times with PBS-tween and once with PBS before 50 μl of streptavidin-conjugated alkaline phosphatase (MABTECH AB) at concentration of 1 μg/ml was added into each well. For removing unbound conjugate, the plate was washed 6 times with PBS-tween and once with PBS. Then 100 μl of 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium substrate (AP conjugate substrate kit, BIO-RAD) was added, and the reaction was incubated at room temperature for 15 to 20 minutes in the dark. When spots were completely developed, the reaction was stopped by washing with distilled water. After drying, the number of spot was enumerated under dissecting microscope. The results were reported as mean of number of spot per one million cells PBMC.

$$\text{Number of spot (spot/}10^6\text{ cells)} = \left[\left(\frac{S_1+S_2}{2}\right) - \left(\frac{N_1+N_2}{2}\right)\right] \times 4$$

S = number of spot counted in each wells of each reaction

N = number of spot counted in each wells of negative control

In order to prove whether the responses were mediated through $CD8^+$ T cell, the $CD8^+$ depletion experiments were performed. Briefly, CD8 antibody conjugated with magnetic bead (Dynabeads M-450; Dynal Biotech ASA, Norway) was pre-washed by transferred the suspension-bead into 1.5 ml microcentrifuge. The beads were then washed twice with 1 ml of RPMI, mixed through by vortex and placed the tube into the magnetic holder for 2 minutes. This step, the immuno-magnetic beads were trapped by magnetic bond with the holder. Thereafter the medium was removed, and 1 ml of R10 containing 1×10^7 PBMC was added to the tube. The cell suspension was mixed thoroughly by vortex and gentle continuously shacked on ice for 20 minutes with 5 minutes mixing interval. To harvest $CD8^-$ cells, the tube was placed in magnetic holder for 2 minutes. Then the suspension containing

unbound cell was transferred to 15 ml conical tube and filled up with 3 ml of R10. The cells were used in ELISpot assay as described previously.

3. Preparation of EBV-contained supernatant of B95-8 culture

For preparing EBV stock virus, B95-8 cells were grown in 75 cm^2 tissue culture flask for 3 days, and then supernatant was harvested into a 15 ml conical tube. After centrifugation at 2000 rpm, 4°C for 10 minutes, the supernatant was filtered pass through a Minisart 0.45 μ filter (sartorius, Germany). One ml of supernatant was aliquot out in microcentrifuge tube and stored at -70° C until used.

4. Establishment of EBV-transformed B-Lymphoblastiod Cell Line (BLCL)

Approximately $1x10^7$ PBMC were transferred to 15 ml conical tube and washed once with RPMI by centrifugation at 1500 rpm for 5 minutes. The pellet was inoculated with 1 ml of B95-8 supernatant for 1 hour at 37°C under 5% CO_2 with 95% humidity. The infected cells were then washed once with RPMI and resuspended with 10 ml of 20% fetal bovine serum in RPMI 1640 (R20) (see appendix II). After that, cyclosporin A at final concentration 1 μg/ml was added to the suspension. Two ml of cells suspension was transferred to each well of 24-well tissue culture plate. The plate was incubated at 37° C under 5% CO_2 with 95% humidity for 7 days. Thereafter 1 ml of supernatant was replaced with R20 in the same volume. The cells were left in the grown until characteristic clumping pattern of BLCL was seen in more than 50% of cell population. The culture was then transferred to 25 cm^2 tissue culture flask and added up to 5 ml with R20. The cells were incubated at 37° C under 5% CO_2 with 95% humidity until they were completely transformed. The cultures were then split at ratio 1:3 to a new flask and continually maintained until used.

5. Establishment of EBV specific cytotoxic T lymphocyte lines (EBV-CTL)

EBV-CTL were generated from fresh PBMC collected from donors on the day of ELISpot testing. The CTL were prepared by stimulating donor lymphocytes with autologous EBV-transformed Lymphoblastoid cell lines in flat-bottomed 24-well tissue culture plate. Briefly, $2x10^6$ PBMC per well were co-cultivated with $5x10^4$gamma-irradiated (3,000 rads) autologous BLCL in 2 ml of R10 supplemented with IL-7 at final concentration of 25 ng/ml. After incubation at 37°C under 5% CO_2 with 95% humidity for 10 days, viable cells were isolated by ficoll hypaque density-gradient centrifugation. The cells were subcultured at $5x10^5$ cells per well and restimulated with 1.25 $x10^5$ gamma-irradiated autologous BLCL (T lymphocyte:B Lymphoblastoid cell ratio of 4:1). The reaction was obtained in 2 ml of R10

containing IL-7 (25 ng/ml) and incubated at 37°C under 5% CO_2 with 95% humidity. Four d ays a fter l ater, s upplement w ith IL-2 at f inal c oncentration o f 5 0 U /ml. T he culture was continuously expanded and supplemented with IL-2 three times a week, each time with 50 U/ml IL-2, and the third time with irradiated autologous BLCL at a T lymphocyte:B Lymphoblastoid cell ratio of 4:1 until chromium release assay was performed.

6. Analysis of EBV-specific cytotoxic T lymphocyte responses by chromium release assay

To label target cell with chromium, $5x10^5$ cell/ml of autologous BLCL were transferred to 15 ml conical tube. Cells were washed twice with RPMI and resuspended with 100 μl of FBS. After that 80 μCi of chromium-51 was added to the suspension and incubated at 37°C under 5% CO_2 with 95% humidity for 1 hour. In the mean time, effector cells at concentration $5x10^6$ cell/ml were prepared and kept at 37° C under 5% CO_2 with 95% humidity. After incubation, target cells were washed twice with RPMI and resuspended with R10 to adjust final concentration of target cell to $5x10^4$ cell/ml. Effector and target cells were co-cultivated in U-bottom 96 well tissue culture plate at effector:target ratio of 50:1, 25:1, 12.5:1 and 6.25:1, respectively. For the total release (TR) or maximum release, 5% triton-X 100 (TX100) was added to the target cells. Minimum release or spontaneous release (SR) only R10 was cultured with target cells. Plate were centrifuged at 500 rpm for 5 minutes and incubated at 37°C, 5% CO_2 under 95%-humidity for 4 hours. Thereafter plate was centrifuged at 500 rpm for 5 minutes and the supernatant, 100 μl each, was transfer to microtube. Then gamma ray was counted with a gamma counter. % specific lysis was calculated as follow:

$$\%\ \text{Specific lysis} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Total release} - \text{Spontaneous release}} \times 100$$

$$\%\ \text{Non specific lysis} = \frac{\text{Spontaneous release}}{\text{Total release}} \times 100$$

The test criteria was reached when the percentage of non specific lysis was less than or equal to 30.