CHAPTER IV

MATERIALS AND METHODS

4.1 Cell culture

The human B cell line, Raji (kindly gift from Prof Winyu Mitarnun, Prince of Sonkla University), was cultured in RPMI-1640 medium containing 10 % fetal bovine serum, non essential amino acid, and 50 µg/ml gentamycin. Cells were splitted and cultured medium was changed every 3-5 days, depending on the cell density, in order to keep the cell in a log phase. For experiments, Raji cell culture was centrifuged at 1,000 rpm for 5 minutes at 4° C. Cells were then washed 3 times with cold PBS and resuspended in RPMI complete medium and counted by staining with 0.4% Trypan blue.

Character of Raji cell line

Isotype: IgM; lambda light chain

Organism; Homo sapiens

Source; Disease: Burkitt's lymphoma

Cell type: B lymphocyte

Cellular products: immunoglobulin

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4.2 B cell stimulation

The 1.5 x10^6 cells/ml Raji cells were stimulated with 2.5 μg/ml PWM (Pokeweed mitogen) in combination with human recombinant IL-2 20 unit/ml in a 24 well culture plate and incubated at 37° C 5%CO_2 in humidified atmosphere for 9 days. Supernatants were harvested on day 0, 3, 6, and 9 for the detection of antibody production. For RNA isolation, cells were harvested at the same time interval and cell lysis buffer for RNA were added, and the cell lysates were kept at -20°C until used.

4.3 Isolation of RNA

Raji cell RNA was extracted by using an RNeasy mini spin column (QIAGEN, Germany). The number of cell up to 5x10^6 was disrupted by the addition of RLT buffer at 350 μl. The cells were mixed vigorously and the cell lysate was passed through a 20-G needle fitted to a syringe 20 times. An equal volume of 70% ethanol was added to the cell lysate and mixed well by pipetting. A sample was applied to the RNeasy mini spin column sitting in a 2 ml collection tube and centrifugation for 15 seconds at 13,000 rpm and the flow-through was discarded. 500 μl RPE buffer was added to the column and centrifuged for 15 seconds at 13,000 rpm. The flow-through was discarded again and another PRE buffer at 500 μl was added to the column. The column was centrifuged for 2 minutes at 13,000 rpm and transferred into a new 1.5 ml collection tube. RNase free water at 30 μl was applied to the RNeasy membrane and centrifuged for 1 minute at 13,000 rpm to elute. The elution step was repeated twice. The RNA was divided into 3 aliquots and kept at -70° C.

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4.4 RT-PCR

4.4.1 Synthesis of first strand cDNA for PCR amplification

The Revert^{TM} Aid First Strand cDNA Synthesis Kit (fermentus) synthesis first strand cDNA. The reaction condition was performed according to the manufacture's protocol

For a final volume of 20 µl.

RNA template 0.1-0.5 µg of total RNA		
Random hexamer primer 0.2 µg	1	µl
Water was added to	12	µl

Reaction was mixed gently and spun down for 3-5 sec before incubating at 70°C for 5 minutes. It was then chilled on ice and the drops were collected by brief centrifugation. Further reagents were added as follows:

5x reaction buffer	4	µl
RiboLock^{TM} Ribonuclease inhibitor 20 unit	0.5	µl
20 mM dNTP mix	1	µl
The reaction was incubated at 25°C for 5 minutes		
The Revert^{TM} M-MulV Reverse Transcriptase 200 unit	1	µl

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The mixture was incubated at 25°C for 10 minutes and then 37°C for 60 minutes. The reaction was stopped by heating at 70°C for 10 minutes, and chilled on ice before being kept at -20°C until used.

4.4.2 PCR reaction

The PCR reaction was used to amplify the first strand cDNA.

The condition was performed in 25 µl of the PCR reaction as follows:

2x PCR Mastermix	12.5	µl
20 µM sense primers	1	µl
20 µM antisense primers	1	µl
cDNA template	1	µl

Sterile deionized water to 25 µl.

The thermal cycle condition for amplification was:

initial denature	94° C	30 minutes
denature	94° C	30 seconds
annealing	50° C	30 seconds
extension	72° C	30 seconds
final extension	72° C	10 minutes

For 45 cycles

4.4.3 One step RT-PCR

One step RT-PCR was used to reverse transcribe and amplify the full length and Δ7 Blimp-1 isoform genes.

The condition was performed in 25 µl of the RT-PCR reaction as follows:

RNA template	1	µl
5x QIAGEN OneStep RT-PCR buffer	5	µl
dNTP mix (400 µM of each dNTP)	1	µl
20 M sense primers	1	µl

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20 M antisense primers	1	µl
QIAGEN OneStep RT-PCR Enzyme mix	1	µl
10 RNase inhibitor (20 unit/µl)	0.5	µl

The thermal cycle conditions used for amplification were:

Reverse transcription	50° C	30 minutes
Initial PCR activation step	95° C	15 minutes
Denature	94° C	30 seconds
Annealing	50° C	30 seconds
Extension	72° C	30 seconds
Final extension	72° C	10 minutes

For 45 cycles.

The sequences of the primers used to amplify Blimp-1 and GAPDH were as follows:

1. Blimp-1 isoform primers

Forward	5' GACGAAGCCATGAATCTCA 3'		
Backward	5' TGAGGCTACAGAGATGGAT 3'		
PCR product	full length isoform	477	bp
	Δ 7 isoform	348	bp

2. GAPDH primers

Forward	5' GGTCATCCCTGAGCTGAACG 3'
Backward	5' TCGTTGTCATACCAGGAAAT 3'
PCR product	295 bp

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4.5 Agarose gel electrophoresis

The preparation of 1.5% (w/v) agarose gel (sigma) was performed by melting agarose in 0.5x TBE buffer in a microwave oven. Ethidium bromide was added in to the melted agarose gel to a final concentration of 0.5 μg/ml and poured into the i-Mupid mini gel imgration chamber (Cosmo Bio, Japan). The comb was then placed on the gel and withdrawn when the gel was placed in an electrophoresis tank and 0.5x TBE buffer was added to the electrophoresis tank to cover the gel. Two microlitres of PCR product was prepared by mixing with of 6x loading buffer. Three microlitres of samples and 100 bp ladder DNA marker (Fermentas^{R}) were applied into the wells. The gel was run at 80 volts for about 50 minutes. The DNA was visualized on a UV light source and photographed (SynGene).

4.6 Analysis of the band intensity

The intensity of PCR products was measured using the GeneTools from SynGene program. The value of the band intensity was determined as:

Volume = the integrated volume of the object

Mean = average O.D. of the pixels within the object

Where O.D. = Volume/Area

Adjusted volume count = the integrated volume of the object adjusted for background removal.

The intensity of bands between the full length and Δ 7 Blimp-1 isoforms was compared and presented in an intensity ratio.

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4.7 Staining of CD138 and Blimp-1 protein on Raji and LP-1 cells

4.7.1 Titration of anti CD138 and conjugated antibody for staining

The mouse anti CD138 antibody (Santa Cruz Biotechnology[R]) dilution of 1:50, 1:100 and 1:200, were used to stain the LP-1 and Raji cell lines as a positive and negative cell control for Blimp-1, respectively. The 5×10^5 cells were stained initially with 20 µl of mouse anti CD138 for 30 minutes and then with 20 µl of rabbit anti mouse IgG_1 FITC (Jackson ImmunoReseach[R]) dilution of 1:100, 1:200 or 1:400 for 30 minutes on ice. They were washed 3 times with cold 1%BSA in PBS in every step by centrifugation at 1,000 rpm for 5 minutes at 4°C. Cells were resuspended in 500 µl of sheath fluid and then analysed by a flow cytometer.

4.7.2 Determination of the CD138 expression in PWM+IL-2 stimulated Raji cells

Raji cells, stimulated at different time intervals; day 0, 3, 6 and 9 were stained for a plasma cell surface marker, CD138. The optimal condition and dilution of antibody was selected from the titration above. Cells 5×10^5 were stained initially with 20 µl of mouse anti CD138 dilution of 1:200 for 30 minutes and then with rabbit anti mouse IgG_1 FITC dilution of 1:200 for 30 minutes and washed in every step, the cells were then analysed by a flow cytometer.

4.7.3 Determination of different permeabilization reagents for intracellular Blimp-1 protein staining.

Cold methanol and 0.1% Triton-X were compared for their capability of permiabilizing cells. For intracellular Blimp-1 protein staining, 5×10^5 cells were washed twice with 2 ml of cold PBS by centrifuged at 1,000 rpm, for 5 minutes at

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4°C, The cells were fixed with 200 µl of 1.5% formaldehyde for 10 minutes at room temperature and then washed once. The cells were then permeabilized for 10 minutes with 500 µl ice cold methanol or 0.1% Triton-X in PBS and then washed twice with cold 1%BSA in PBS. They were stained firstly of goat anti Blimp-1 antibody (C-21: Santa Cruz Biotechology[R]: recognized a peptide mapping near the C-terminus of human Blimp-1) dilution 1:50 for 1 hour, secondly stained with 20 µl of rabbit anti goat IgG conjugated FITC dilution to 1:50 or 1:100, They were washed with 2 ml of cold 1% BSA-PBS and resuspended in 500 µl of sheath fluid before analysis by a flow cytometer.

4.7.4 Titration of Blimp-1 antibody for staining

Three dilution of goat anti Blimp-1 antibody: 1:50, 1:100, 1:200, were used to stain LP-1, a human plasma cell line, which should be positive for Blimp-1, the Raji B cell line, a negative cell control for Blimp-1 was stained in parallel. The staining procedure was performed in same was as above, but with cold methanol used as the permeability reagent. Cells were analyzed by a flow cytometer.

4.7.5 Determination of Blimp-1 expression in PWM+IL-2 stimulated Raji cells

Raji cells, stimulated at different time interval; day 0, 3, 6, 9 were stained for Blimp-1 expression. The optimal condition and dilution of antibody was selected from the titration above. Cells $5x10^5$ were fixed with cold methanol and then permabilizing with 0.5% formaldehyde. They were stained firstly with goat polyclonal anti Blimp-1 antibody at dilution 1:50 for 1 hour and secondly with rabbit anti goat IgG conjugated FITC dilution 1:200. The staining process was performed in the same way as above.

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An LP-1 plasma cell line was stained in paralled as a control for Blimp-1 expression. The cells were then analysed by a flow cytometer and using the program FACDiva Version 6.1 and merge by program WinMDI version 2.9

4.8 Detection of antibody production by ELISA

4.8.1 Titration of goat anti IgG + IgM (H+L) concentration coating to the plate

Serial dilution of Goat anti human IgG+IgM (Jackson ImmunoResearch[R]) between 10-0.2 μg/ml in coating buffer were coated on each well of an ELISA plate and incubated at 4 °C overnight. The plate was washed in 3 times with 0.05% PBS-Tween and then blocked with 5% BSA 50 μl for 1 hour. The cell culture supernatant and standard IgM at concentrations between 0.1-100 μg/ml were added to each well (triplicate), the plate were incubated for 1 hour at room temperature. Fifty microlitters of anti human IgG conjugate HRP (Jackson ImmunoResearch[R]) in concentration of 1:10,000 was added in each well 50 μl, incubated for 1 hour at room temperature. The plate was washed 3 times with 0.05% PBS-Tween. The TMB substrate (SureBlue Reserve TMB (KPL[R])) was added in each well of 50 μl and incubated for 30 minutes. The reaction was stopped by adding the 1 N sulfuric acid (H_2SO_4). The intensity of colour development was measured at wave length 450 nm by an ELISA reader.

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4.8.2 Detection of antibody production in cell culture supernatants by ELISA

To verify that the Raji cells were activated and differentiated into antibody producing plasma cells, supernatants harvested from PWM stimulated Raji B cells were tested for antibody secretion in supernatants from day 3 to day 9.

Goat anti human IgG+IgM 0.2 μg/ml in coating buffer were coated on each well of an ELISA plate and incubated at 4°C overnight. The plate was washed in 3 times with 0.05% PBS-Tween and then blocked with 5% BSA 50 μl for 1 hour. The cell culture supernatant and standard IgM were added to each well (triplicate), the plate were incubated for 1 hour at room temperature. Fifty microlitters of anti human IgG conjugate HRP at dilution of 1:20,000 was added in each well and incubated for 1 hour at room temperature. The plate was washed 3 times with 0.05% PBS-Tween. The TMB substrate was added in each well 50 μl and incubated for 30 minutes. The reaction was stopped by adding 1 N sulfuric acid (H_2SO_4) and the colour intensity was measured at the wave length 450 nm by an ELISA reader.

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