CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals, antibodies, cell lines and instruments used in this study are shown in Appendix.

2.2 Antigen Preparation

2.2.1 Hemoglobin Bart's (Hb Bart's)

2.2.1.1 Preparation of hemolysates

Blood, using EDTA as anticoagulant, was centrifuged at 400 g for 5 minutes at room temperature. The plasma was discarded and red blood cells (RBCs) were washed with 0.9% NaCl isotonic solution for 3 times. Then, packed RBCs were lysed by adding an equal volume of distilled water and mixed vigorously to release hemoglobins (Hbs). After that, the organic carbon tetrachloride (CCl_4) was added to a half volume of the mixture and mixed for 5 minutes. The mixture was centrifuged at 2,000 g for 10 minutes at room temperature. Then, the upper layer containing hemolysate was collected and stored at -20°C until used.

2.2.1.2 Purification of Hb Bart's

Hb Bart's was separated from the hemolysate of Hb Bart's hydrop fetalis by cellulose acetate electrophoresis with an alkaline buffer solution. The cellulose acetate membrane was soaked in Tris-Borate-EDTA (TBE) buffer pH 8.6 for 3-5 minutes. Then, the sample applicator was used to apply the hemoglobin Bart's hydrop fetalis

hemolysate on the membrane and placing on the electrophoresis chamber by presenting of the hemolysate on the cathode side. The electrophoresis was performed until the band of Hb Bart's was clearly separated from Hb Portland. After electrophoresis, the band of Hb Bart's was cut and eluted from the cellulose acetate membrane by soaked in phosphate buffer saline (PBS) pH 7.2 at 4°C overnight.

2.2.2 Hemoglobin E (Hb E) and Hemoglobin F (Hb F)

Hb E and Hb F were purified from adult heterozygous beta-thalassemia with Hb E and hemolysates of normal umbilical cord blood by DEAE Sepharose chromatography, respectively. Briefly, the hemolysate was dialyzed against the binding buffer (Tris-HCl–KCN (THK) pH 9.0) for overnight. The C10/10 column containing DEAE Sepharose beads was equilibrated with THK buffer pH 9.0. Afterward the hemolysates were applied to the column. The pH gradient separation for Hb was performed by increasing the concentration of eluting buffer (THK buffer pH 6.5). The Hb fractions were collected by AKTA prime fraction collector.

2.2.3 Recombinant Ag85B-BCCP protein

The Antigen 85 (Ag85) complex is a major secretion product of *M. tuberculosis*. The Ag85 complex comprises three closely related proteins, 85A, 85B, and 85C. The Ag85B in the form of biotin carboxyl carrier protein (BCCP) fusion protein, named Ag85-BCCP protein, was produced according to the method described elsewhere (Tayapiwatana *et al.* 2006). Briefly, the digested DNA encoding Ag85B was ligated with digested pAK400CB plasmid vector by T4 DNA ligase. The constructed recombinant plasmid vector was named pAK400CB-Ag85B. The pAK400CB-Ag85B plasmid was transformed into *E. coli* Origami B. The clone of *E. coli* Origami B containing the pAK400CB-Ag85B plasmid was cultured in SB

medium containing antibiotic and supplemented with 0.05% glucose and 4 μM biotin. The Ag85B was biotinylated at the BCCP motif in *E. coli* Origami B strain. The Ag85B-BCCP (biotinylated Ag85B) was extracted from bacterial pellet using B-PER II extracting reagent. Finally, Ag85B-BCCP was purified from bacterial extract containing Ag85B-BCCP by Streptactin Sepharose as was described in manufacturer's instruction.

2.3 The development of conditioned medium for hybridoma production

2.3.1 Preparation of PMA-induced BW and BW conditioned media

$1x10^7$ cells of BW5147 mouse thymoma cells were cultured in 10 ml of Iscove's Modified Dulbeco's Medium (IMDM) containing 10% fetal bovine serum (FBS), gentamycin 40 mg/l and fungizone 5 mg/l (10% FBS-IMDM) with or without 20 ng/ml phorbol myristate acetate (PMA) at 37°C in a 5% CO_2 incubator for various incubation times. After cultivation, the culture supernatants were harvested by centrifugation at 550 g for 5 minutes and filtrated by 0.1 μm membrane filter. The culture supernatants were aliquot and stored at -20°C until used.

2.3.2 Study the utilization of conditioned media for hybridoma single cell cloning

2.3.2.1 Single cell cloning

Hybridoma were counted and cell concentration were adjusted to 4, 2 cells and 1 cell per 150 μl in 10% FBS-IMDM, 10% FBS-IMDM supplemented with 10, 20 or 50% of the produced conditioned media or 10% FBS-IMDM supplemented with 10% BM-Condimed H1 commercially conditioned medium. Then, 150 μl of each cell suspension were added in to 96-well tissue culture plate. The cells were cultured at

37°C in a 5% CO_2 incubator. After 7, 10, 13, 15, and 18 days of cultivation, number and size of hybridomas clones were determined under an inverted microscope**.** The clone size was scored into four levels, i.e. very small (VS), small (S), medium (M) and large (L) as described below (Figure 2.1).

Very small	clone size less than 1 in 8 of well
Small	clone size approximately 1 in 8 to less than 1 in 4 of well
Medium	clone size approximately 1 in 4 to less than 1 in 2 of well
Large	clone size approximately 1 in 2 of well

Comparison of the produced and commercial conditioned media for supporting of hybridoma single cell cloning was compared.

2.3.3 Study the utilization of conditioned media for generation of hybridomas by hybridoma technique

2.3.3.1 Mouse immunization

BALB/c mice were used in this study. Blood was collected by tail-bleeding for using as pre-immunized serum. The mice were intraperitoneally (IP) immunized with 100 µg purified Ag85B-BCCP recombinant protein or purified Hb E in 300 µl sterile PBS mixing with 200 µl complete Freund's adjuvant at 2-weeks interval. At the second immunization, incomplete Freund's adjuvant was used instead of complete Freund's adjuvant. At the third immunization, the antigen was mixed with sterile PBS and immunized without adjuvant. Two weeks after the third immunization, immunized serum was prepared from blood collection and stored at -20°C for antibody detection.

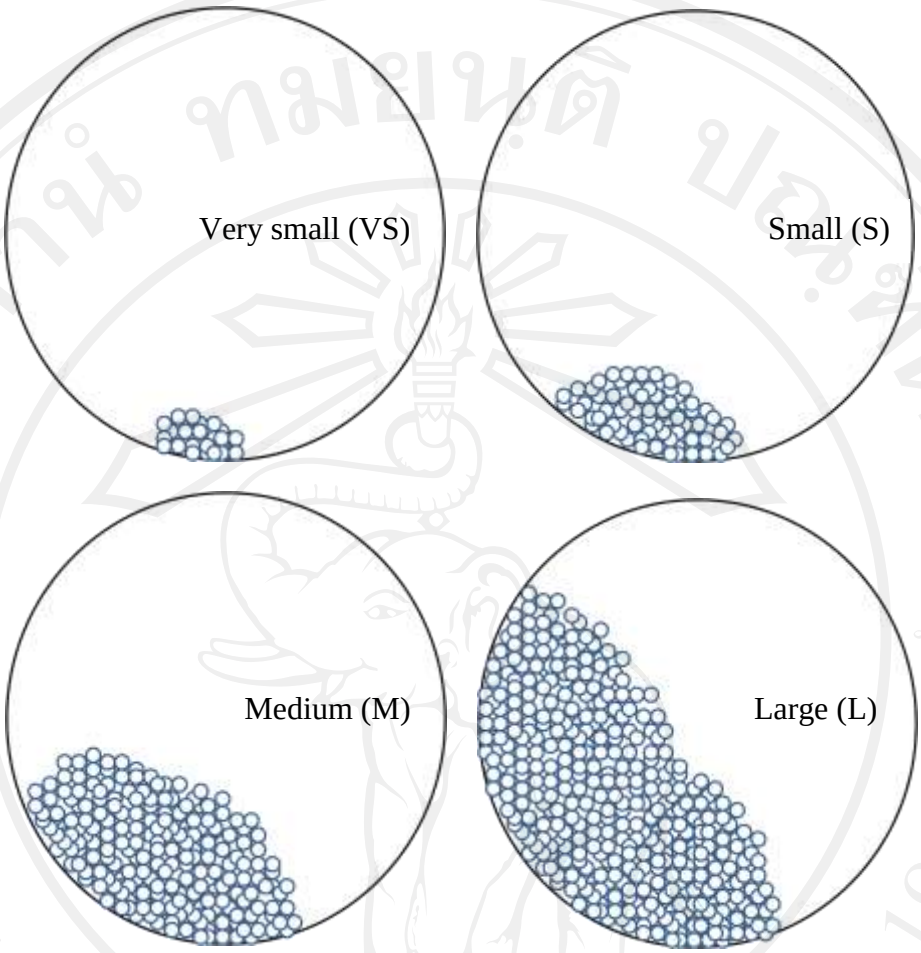

Figure 2.1 Size of single hybridoma clone. Hybridomas single cell cloning were determined under an inverted microscope**.** The clone size was scored into four levels, i.e. very small (VS), small (S), medium (M) and large (L).

2.3.3.2 Hybridoma technique

The BALB/c mice that produced the specific antibody response were sacrificed. The spleen was aseptically removed from the immunized mice. Splenocytes were isolated from the spleen and fused with myeloma cells (P3-X63Ag8.653) by a standard hybridoma technique (Kohler and Milstein 1975). Briefly, the splenocytes were fused with myeloma cells at the cell ratio of 2:1 using 50% PEG. After cell fusion, cells were resuspended in HAT selective medium with 20% of produced conditioned media or 10% of BM-Condimed H1 to obtain spleen cell concentration of $3x10^5$ cells/ml and 100 µl of the cell suspension was seeded into each well of 96-well plates. The plates were then cultured at 37°C in a 5% CO_2 incubator. After 5 days of cultivation, 150 µl of HT medium supplemented with 20% of produced conditioned media or 10% of BM-Condimed H1 were added into each well. The plates were incubated at 37°C in a 5% CO_2 incubator for hybridoma generation. The generated hybridomas were monitored by an inverted light microscope. Culture supernatants were collected from the hybridoma containing wells and tested for specific antibody by ELISA using the corresponding antigen. The numbers of hybridoma clones obtained from the produced and commercial conditioned medium were compared.

2.3.4 Determination of antibody response in the immunized mice and culture supernatant by ELISA

2.3.4.1 ELISA for anti-Ag85B-BCCP

Briefly, 10 µg/ml of avidin were coated into 96-well ELISA plate using carbonate/bicarbonate coating buffer pH 9.6 at 50 µl/well and incubated at 4°C for overnight. The plates were then washed 3 times with PBS containing 0.05% Tween

20 (PBS-Tween). After that the plate were blocked with 80 µl of 2% skim milk-PBS at 37°C for 1 hour. Then, plates were washed with PBS-Tween, 50 µl of 10 µg/ml of purified Ag85B-BCCP and purified CD147-BCCP (as control) were added to each well and incubated at 37°C for 1 hour. Plates were washed with PBS-Tween, then 50 µl diluted sera or culture supernatants were added to each well and incubated at 37°C for 1 hour. After 3 washing, horseradish peroxidase conjugated rabbit anti-mouse immunoglobulins antibody at a dilution of 1:2,000 were added to each well and incubated at 37°C for 1 hour. Thereafter, the plate was washed and 50 µl tetramethylbenzidine (TMB) substrate was added. The reaction was stopped with 1M HCl and the absorbance was measured at 450 nm.

2.3.4.2 ELISA for anti-Hb E antibody

Briefly, 10 µg/ml of purified Hb E was coated into 96-well ELISA plate using carbonate/bicarbonate coating buffer pH 9.6 at 50 µl/well and incubated at 4°C for overnight. The plates were washed with PBS-Tween. After that the plate was blocked with 80 µl of 2% BSA-PBS at 37°C for 1 hour. After washing, 50 µl diluted sera or culture supernatants were added to each well and incubated at 37°C for 1 hour. The plates were then washed, then horseradish peroxidase conjugated rabbit anti-mouse immunoglobulins antibody at a dilution of 1:2,000 were added to each well and incubated at 37°C for 1 hour. Thereafter, the plate was washed and 50 µl TMB substrate was added. The reaction were stopped with 1M HCl and the absorbance were measured at 450 nm

2.3.5 Determination of antibody response in the immunized mice and culture supernatant by immunofluorescence staining

2.3.5.1 Lysed whole blood immunofluorescence staining

50 µl of K_3EDTA blood were incubated at 4°C with 50 µl of tested hybridoma culture supernatants. After 30 minutes incubation, cells were then washed twice with PBS containing with 1% bovine serum albumin (BSA) and 0.02% sodium azide (1% BSA-PBS-0.02% NaN_3). The FITC labeled anti-mouse immunoglobulin conjugate was added and incubated for another 30 minutes at 4°C. Then, 1 ml of BD FACS™ lysing solution was added and let stand at room temperature in the dark for 10 minutes. Cells were washed three times with 1% BSA-PBS-0.02%NaN_3 and fixed with PBS containing 1% paraformaldehyde. The stained cells were then analyzed by a flow cytometer.

2.3.6 Analysis of proteins in the produced conditioned medium by sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The produced conditioned medium, BM condimed H1, and 10% FBS-IMDM were diluted to 1:8 and separated by SDS-PAGE using 10% separating gel and 4% stacking gel. After electrophoresis, the gels were stained with Coomassie Blue and de-stained by filtrated water. The amounts of protein band from the produced and commercial conditioned medium were compared.

2.4 The develop high efficiency hybridoma technology for production of monoclonal antibody

2.4.1 Pre-isolation of B cell strategy

The BALB/c mouse was IP immunized with 100 µg purified hemoglobin Bart's as was described in 2.3.3.1. Two weeks after the third immunization, mouse serum was collected and detected for antibodies presentation by ELISA using purified Hb Bart's coated plate as was described in 2.3.4.2.

After having high antibody titer, spleen cells were isolated from spleen of the immunized mice. B cells were then isolated from the spleen cells by using a Magnetic Cell Sorting System (MACS). Briefly, spleen cells were washed twice with PBS containing 2 mM EDTA and 0.5% BSA (2 mM EDTA-0.5% BSA-PBS) by centrifugation. The washed cells were mixed with rat anti-mouse IgM MicroBeads and goat anti-mouse IgG MicroBeads as described in manufacturer's instruction ($1x10^7$ spleen cells: 20 µl of each MicroBeads) and incubated by rotating at 4°C for 30 minutes. Cells were washed twice with PBS containing 2 mM EDTA-0.5% BSA-PBS by centrifugation. The cell pellet was resuspended in 500 µl 2 mM EDTA-0.5% BSA-PBS. The cell suspension was then applied onto the LD column and separated by MidiMACS Separator according to the manufacturer's instruction. The B cells, contained in the positive fraction, were isolated. The negative fraction of spleen cells was also collected as non B-cells fraction.

B cells (positive cell fraction), non B-cells (negative cell fraction) and total spleen cells were fused with myeloma cells at the cell ratio of 1:1 by a standard hybridoma technique as was described in 2.3.3.2. The generated hybridomas were monitored by an inverted light microscope. Culture supernatants were collected from

the hybridoma containing wells and tested for specific antibody by ELISA using purified Hb Bart's coated plate as was described in 2.3.4.2. The total spleen cells were used as standard hybridoma technique control. The numbers of hybridoma clones obtained from positive and negative cell fractions were determined and compared the standard hybridoma technique.

2.4.2 Pre-isolation of antigen specific B cell strategy

Biotin-streptavidin system was used for this strategy. Production of mAb against Ag85B-BCCP was used as a model.

2.4.2.1 Isolation of Ag85B-BCCP specific B cell for hybridomas production

The mice were intraperitoneally (IP) immunized with 100 µg purified Ag85B-BCCP as was describe in 2.3.3.1. Two weeks after the third immunization, mice serum were collected and determined for antibody responses. Antibody responses to Ag85B-BCCP in the immunized mice were determined by ELISA as was described in 2.3.4.1.

B cells that produced specific antibody to Ag85B-BCCP were isolated from spleen cells of the immunized mice by using MACS. Briefly, spleen cells were incubated with purified Ag85B-BCCP (200 µg purified Ag85B-BCCP: 1-5x10^7 spleen cells) in 5 ml 10% FBS-IMDM and rotated at 4°C for 2 hours. Cells were washed twice with 2 mM EDTA-0.5% BSA-PBS by centrifugation. These cells were mixed with Streptavidin MicroBeads as described in manufacturer's instruction (1x10^7 spleen cells: 10 µl Streptavidin MicroBeads) and incubated by rotating at 4°C for 45 minutes. The labeled cells were washed twice with 2 mM EDTA-0.5% BSA-PBS by centrifugation. The cell pellet was resuspended in 500 µl of 2 mM EDTA-

0.5% BSA-PBS. The cell suspension was then applied onto the LD column and separated by MidiMACS Separator according to the manufacturer's instruction. The Ag85B-BCCP specific B cells, contained in the positive fraction, were isolated. The negative fraction of spleen cells was also collected.

Ag85B-BCCP specific B cells (positive fraction), negative fraction and total spleen cells were fused with myeloma cells at the cell ratio of 1:1 by a standard hybridoma technique as was described in 2.3.3.2. The generated hybridomas were monitored by an inverted light microscope. Culture supernatants were collected from the hybridoma containing wells and tested for specific antibody by ELISA as was describe in 2.3.4.1. The total spleen cells from immunized mouse were used as standard hybridoma technique control. The numbers of hybridoma clones obtained from positive cell fraction, negative cell fraction, and the standard hybridoma technique were determined and compared.

2.5 Development of hybridoma techniques for production of monoclonal antibody having a desired isotype

2.5.1 Isolation of IgM and IgG expressing cells for hybridomas production

BALB/c mice were IP immunized with 100 µg purified Hb F as was described in 2.3.3.1. Two weeks after the third immunization, mice serum were collected and detected for antibodies presentation by ELISA using purified Hb F as was described in 2.3.4.2.

IgM and IgG surface expressing cells were isolated from the immunized mice spleen cells by using MACS. Briefly, spleen cells were mixed with rat anti-mouse IgM MicroBeads as described in manufacturer's instruction ($1x10^7$ spleen cells: 20 µl

anti-mouse IgM MicroBeads) and incubated for 30 minutes on ice. Cells were washed twice with PBS containing 2 mM EDTA-0.5% BSA-PBS by centrifugation. The cell pellet was resuspended in 500 µl 2 mM EDTA-0.5% BSA-PBS. The cell suspension was then applied onto the LD column and separated by MidiMACS Separator according to the manufacturer's instruction. The IgM surface expressing cells (IgM^+ cells) were magnetically isolated and contained in the positive fraction. The negative fraction of spleen cells was collected and labeled with goat anti-mouse IgG MicroBeads ($1x10^7$ spleen cells: 20 µl anti-mouse IgG MicroBeads) and the isolation performed as described above. The IgG surface expressing cells (IgG^+ cells) were contained in positive fraction. The final negative fraction, the non-IgM and IgG surface expressing cells (IgM^-/IgG^- cells), was also collected.

The isolated IgM^+ cell, IgG^+ cell and IgM^-/IgG^- cell fractions were fused with myeloma cells at the cell ratio of 1:1 by a standard hybridoma technique as was described in 2.3.3.2. The generated hybridomas were monitored by an inverted light microscope. Culture supernatants were collected from the hybridoma containing wells and tested for specific antibody against Hb F by ELISA as was described in 2.3.4.2.

2.5.2 Determination of antibody isotyping by ELISA

The isotypes of antibodies produced by the generated hybridomas were determined by indirect ELISA. Briefly, 50 µl rabbit anti-mouse immunoglobulins (10 µg/ml) were coated on a 96-well ELISA plate using carbonate/bicarbonate coating buffer pH 9.6. The plate was blocked with 2% BSA-PBS. Hybridoma culture supernatants were added to each well and incubated at 37°C for 1 hour. The plate was washed 4 times with PBS containing 0.05% Tween 20 and 50 µl horseradish peroxidase conjugated goat anti-mouse IgM or IgG antibody (dilution 1:10,000) were

added to each well and incubated at 37°C for 1 hour. Thereafter, 50 µl of TMB substrate were added. The reaction was stopped by adding 50 µl of 1 M HCl and the absorbance was measured at 450 nm.

2.6 *In vitro* immunization for antibody production

2.6.1 To optimize of conditions for *in vitro* immunization

Spleen was harvested from a non-immunized BALB/c mouse. The spleen cells were isolated from the harvested spleen; the contained red blood cells were lysed with hypotonic solution. Then the obtained spleen cells were washed with RPMI 1640 medium. spleen cells ($5x10^6$) were then suspended in 0.5 ml RPMI 1640 medium containing 50µM β-mercaptoethanol and added with 40 µg/ml muramyldipeptide (MDP) as an adjuvant. Spleen cells were then activated with 10, 20, or 40 µg/ml Ag85B-BCCP. After 30 minutes, 0.5 ml of complete RPMI 1640 medium containing 40% FBS (40% FBS-RPMI) were then added to each suspension of spleen cells followed by incubation at 37°C in a humidified 5% CO_2 for various incubation days. Regular (non-refed) and re-fed conditions of culture were performed. For regular (non-refed) condition, 200 µl culture supernatant from each wells were collected for determining antibody activity and the wells were replaced by adding with 200 µl of 20% FBS-RPMI medium. For re-fed condition, 800 µl culture supernatant from each wells were collected for determined antibody activity and the wells were added with 800 µl of 20% FBS-RPMI medium. The antibody activity in the collected culture supernatants were determining by an appropriate ELISA as was described in 2.3.4.2.

2.6.2 Production of monoclonal antibody by *in vitro* immunization

Spleen cells were induced for antibody responses by *in vitro* immunization using the optimal conditions obtained from the previous section. After *in vitro* stimulation, the culture supernatants from *in vitro* immunization well were collected and detected antibody response on day 3, day 5, and day 7 by ELISA using Ag85B-BCCP as antigen (as was described in 2.3.4.2). The activated spleen cells were also determined for antibody against Ag85B-BCCP on cell surface by immunofluorescence staining on day 3, 5 and 7.

After *in vitro* immunization, the spleen cells were fused with myeloma cells (P3-X63Ag8.653) at the cell ratio of 1:1 by a standard hybridoma technique as was described in 2.3.3.2. The generated hybridomas were monitored by an inverted light microscope. Culture supernatants from hybridoma containing wells were collected from the hybridoma containing wells and tested for specific antibody by ELISA.

2.6.3 Determination of anti-Ag85B-BCCP antibody expressing cells by immunofluorescence staining

The $2x10^5$ spleen cells were resuspended in 50 µl of 1% BSA-PBS-NaN_3 and incubated with 50µl of 20µg/ml purified Ag85B-BCCP at 4°C for 30 minutes. The cells were washed twice with 1% BSA-PBS-NaN_3 and incubated with 10% FBS at 4°C for 30 minutes to block nonspecific Fc-receptor-mediated binding of mAbs. Fifty microliters of the cell suspension were incubated with 50 µl of 20 µg/ml rabbit anti-Ag85B-BCCP polyclonal antibody at 4°C for 30 minutes. The cells were washed twice with 1% BSA-PBS-NaN_3 and then incubated with FITC-conjugated swine anti-rabbit immunoglobulins antibody at 4°C for 30 minutes. The stained cells were then

washed for twice with 1% BSA-PBS-NaN_3 and resuspended in 350 µl PBS containing 1% paraformaldehyde. The stained cells were analyzed by flow cytometry.