CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and equipments

Chemicals and equipments used in this study were shown in Appendix A.

2.2 Preparation of plasmid vector encoding CD147Ex-BCCP

2.2.1 CD147Ex gene amplification by PCR

A pair of specific primers, CD147*NdeI* (5' GAG GAG GAG GTC ATA TGG CTG CCG GCA CAG TCT TC 3') with underlined *NdeI* restriction site and CD147*EcoRI* (5' GAG GAG GAG CTG AAT TCG TGG CTG CGC ACG CGG AG 3') with underlined *EcoRI* restriction site, was synthesized in order to amplify CD147 extracellular domain coding sequence from the phagemid vector pComb8-CD147Ex (kindly provided by Dr. Nutjeera Intasai, Chiang Mai University, Chiang Mai, Thailand) (Intasai et al., 2003). Fifty nanograms of pComb8-CD147Ex vector was annealed with 250 ng of each described primer in the 100 µl of the PCR mixture containing 5U of ProofStart DNA polymerase (QIAGEN). The amplification condition included a jump start at 95°C for 5 min and followed by the three steps of PCR amplification: denaturation at 94°C for 50 s, annealing at 50°C for 50 s and extension at 72°C for 1 min. After 35 amplification cycles, the mixture was incubated at 72°C for 10 min. The amplified product was then analyzed by 1% agarose gel electrophoresis for expected molecular size (552 bp).

2.2.2 Restriction enzyme digestion of amplified CD147Ex

The PCR product was purified using QIAquick PCR purification kit (QIAGEN), in accordance with the manufacturer's instructions, prior to be treated with restriction enzymes. Approximately 6 μg of purified PCR product was digested with 30 U of both *NdeI* and *EcoRI* (Fermentas) at 37°C for 18 hours. The buffer for specific enzymes was followed the commercial recommendation. Then, the digested DNA was checked by agarose gel electrophoresis. The digested CD147Ex fragment was further purified by QIAquick PCR purification kit (QIAGEN) before subcloning into plasmid vector.

2.2.3 Preparation of pAK400CB plasmid cloning sites

Approximately 5 μg of plasmid vector pAK400CB (Santala and Lamminmaki, 2004) was digested with 25 U of both *NdeI* and *EcoRI* (Fermentas) at 37°C for 18 hours. The buffer for specific enzymes was followed the commercial recommendation. To purify the digested pAK400CB vector, the plasmid was firstly separated by agarose gel electrophoesis and the desired fragment of approximately 4 kbp was excised from the gel after ethidium bromide staining and UV light exposure. The digested pAK400CB plasmid was retrieved using QIAquick Gel extraction kit (QIAGEN). The purified plasmid vector was further check by agarose gel electrophoresis.

2.2.4 Construction of plasmid containing CD147Ex-BCCP gene

The pretreated pAK400CB plasmid vector was ligated with digested CD147Ex fragment using T4 DNA ligase (Roche Molecular Biochemicals). Five

units of T4 ligase was introduced to the ligation mixture containing 50 ng of digested CD147Ex and 100 ng of digested vector. The ligation mixture was subsequently incubated at 4°C for 18 hours. The newly constructed plasmid was named pAK400CB-CD147Ex.

2.3 Introduction of plasmid DNA to the bacterial cells

2.3.1 Bacterial cell transformation

For plasmid amplification, the ligated product was firstly transformed into *E. coli* XL-1 Blue [*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*F' [*proAB*$^{+}$, *lac*I^{q} *lac*ZΔM15 Tn10 (tet^{r})]] (Stratagene). The ligated DNA was co-incubated with 200 μl of cold-thawed $CaCl_2$-treated XL-1 Blue competent cells on ice for 1 hour. The mixture was transferred into cooled screw cap tube and subsequently shocked at 42°C for 1.5 minute, then abruptly chilled on ice for 1 min. Three milliliters of non-antibiotic LB broth was added and further cultured with shaking (120 rpm) at 37°C for 3 hours. The transformed cell was centrifuged (3,000 rpm) at RT for 10 minutes and plated on LB agar containing 25 μg/ml chloramphenicol and 10 μg/ml tetracycline. The plates were then incubated at 37°C overnight.

2.3.2 Purification of plasmid DNA by alkaline lysis method

The antibiotic-resistant isolated colonies were randomly picked and grown in 3 ml of LB broth (containing 25 μg/ml chloramphenicol) with shaking (180 rpm) at 37°C for 18 hours. The 1.5 ml of culture was centrifuged 10,000*xg* at 4°C for

5 minutes. The supernatant was discarded and 100 μl of 1x glucomix-lysozyme was added to the cell pellet, followed by vigorous shaking. 200 μl of freshly prepared NaOH/SDS was added and mixed by inverting. Then, 150 μl of potassium acetate was added and gently mixed by vortex. The solution was centrifuged 10,000 g at 4°C for 5 minutes and the supernatant was collected. 900 μl of analytical grade absolute ethanol was added and the solution was kept on ice for 2 minutes. The DNA was spun down at 10,000 g at 4°C for 5 minutes and the supernatant was discarded. The DNA pellet was reconstituted by adding 100 μl of sterile DW, followed by adding 50 μl of 7.5M ammonium acetate, and incubated at –70°C for 10 minutes. The supernatant was collected by centrifugation at 10,000 g at 4°C for 5 minutes. 300 μl of absolute ethanol was added to the supernatant and incubated at –70°C for 10 minutes. The solution was spun down and the pellet was cleaned up with 1 ml of 70% ethanol by centrifugation at 10,000 g at 4°C for 5 minutes. The DNA pellet was dried at 37°C, reconstituted with 30 μl of sterile DW and stored at –20°C.

2.3.3 Characterization of recombinant clones

The purified plasmids were firstly checked by fractionating in 1% agarose gel electrophoresis. To verify the correct *E. coli* clones, the purified plasmid from the individual clone was characterized by digesting with *NdeI*, *EcoRI*, or both, to identify the band of correct size, and by PCR re-amplification of CD147Ex fragment using primer pair as above. After the correct clone was identified, the purified plasmid retrieved from that clone was used to transform *E. coli* strain Origami B [F^- *ompT* *hsdS*$_B$ (r_B^- m_B^-) *gal dcm lacY1 ahpC gor522*::Tn10 *trxB* (Kan^R, Tet^R)] (Novagen)

according to the described protocol, except that 15 μg/ml of kanamycin was included in the LB agar. The antibiotic-resistant colonies were picked and the plasmids were purified by alkaline lysis method. The *E. coli* clone harboring pAK400CB-CD147Ex was identified by restriction analysis and PCR re-amplification, as described above.

2.4 Expression of biotinylated CD147Ex-BCCP fusion protein

The selected clone of *E. coli* Origami B harboring pAK400CB-CD147Ex was grown in 10 ml of SB medium (30 g/l tryptone, 15 g/l yeast extract, and 10 g/l MOPS, pH 7.0) containing tetracycline (10 μg/ml), chloramphenicol (25 μg/ml), kanamycin (15 μg/ml) and supplemented with glucose (0.05%) and 4 μM biotin. The culture was shaken 300 rpm at 37°C until an optical density (OD) at 600 nm of 0.5 was reached. The cultured bacteria were subsequently transferred to 20 ml of the same media and further shaken at 37°C until the OD at 600 nm reached 0.8. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the cultured at a final concentration of 100 μM. The culture was further cultivated at 24°C with 200 rpm shaking for 22 hours.

2.5 Bacterial total protein extraction

The bacterial cells were harvested by centrifugation (3000x*g*, 4°C, 10 min), resuspended in 2.5 ml of B-PER^{TM}II bacterial protein extraction reagent (PIERCE) and mixed by pipetting until the cell suspension was homogeneous. The mixture was gently shaken at room temperature for 20 minutes. The soluble proteins were separated from insoluble debris by centrifugation at 27,000x*g*, 4°C for 15

minutes. The supernatant that contain the fusion protein was kept and dialyzed against 5 change of ammonium buffer, pH 9. Then the solution was subjected to lyophilization and the dried extract was kept in -20°C freezer.

2.6 Detection of biotinylated CD147Ex-BCCP fusion protein by immunological techniques

2.6.1 Indirect ELISA

Microtiter plate (NUNC) was coated with 0.5 μg of egg white avidin (Sigma) in 50 μl of carbonate/bicarbonate buffer, pH 9.6 at 4°C for 18 hours. The coated plate was blocked by adding 2% skim milk in PBS, pH 7.2, and incubated for 1 hour at room temperature. After washing 3 times with washing buffer (0.05% Tween 20 in PBS pH 7.2), 5 μg of bacterial extract from Origami B-pAK400CB-CD147Ex, as well as from Origami B-pAK400CB-survivin and Origami B without any plasmid, in 50 μl of 2% skim milk in PBS were added into the wells. After incubation at room temperature for 1 hour, the plate was washed 3 times with washing buffer. The bound CD147Ex-BCCP was traced by adding 50 μl of 10 μg/ml mouse anti-CD147 mAb M6-1D4, M6-1B9, M6-1E9, M6-1F3, M6-2B1 and M6-2F9 (kindly provided by Dr. Watchara Kasinrerk, Chiang Mai University, Chiang Mai, Thailand) (Kasinrerk et al., 1999). Mouse anti-survivin mAb (Santa Cruz Biotechnology, Inc) was used as positive control for biotinylated survivin-BCCP fusion protein. After washing, the same volume of HRP-conjugated rabbit-anti-mouse immunoglobulins (Zymed) was added. After 1 hour incubation and 3 times washing, 100 μl TMB (3', 3', 5', 5'-tetramethylbenzidine) color substrate was applied to each well. The plate

was allowed to stand at room temperature for a short duration for signal development. The enzymatic reaction was stopped by adding 100 µl of 1 N HCL and the absorbance at 450 nm was measured.

2.6.2 SDS-PAGE and Western immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was employed for analysis of biotinylated CD147Ex-BCCP fusion proteins. The protein components of bacterial extracts were separated in 12% polyacrylamide gel under reducing condition. For Western immunoblotting, the separated proteins were electroblotted onto polyvinylidene fluoride (PVDF) membrane. Blotted membrane was blocked at 4°C for 18 hours in 5% skim milk in PBS, pH 7.2, and then incubated with the pooled CD147 mAbs (M6-1E9, M6-1B9 and M6-1D4 at 5 µg/ml each) or HRP-conjugated streptavidin (Zymed) for 1 hour at room temperature on a shaking platform. Following washing four times with 0.05% Tween 20 in PBS, pH 7.2, HRP-conjugated rabbit-anti-mouse immunoglobulins antibody (Zymed) diluted in 5% skim milk in PBS, pH 7.2, was added to the CD147 mAbs-stained membrane and incubated at room temperature for 1 hour. After washing step, the immuno-reactive bands were then visualized by chemiluminescent substrate detection system (Amersham–Pharmacia Biotech).

2.7 Purification of biotinylated CD147Ex-BCCP fusion protein by streptavidin-coated magnetic beads

Biotinylated CD147Ex-BCCP fusion protein was concentrated and separated from the bacterial proteins by being trapped on the streptavidin-coated

magnetic beads (MagnaBind^{TM} Streptavidin Beads; PIERCE). 100 μl of streptavidin beads (equal to $5x10^7$ beads) was washed 3 times with 1 ml of PBS followed by magnetic field separation. 200 μg of bacterial extract was added to the washed beads, and the beads were gently agitated and incubated for 30 minutes at room temperature. Then the beads were washed 3 times with PBS by using magnetic field and resuspended with 100 μl of PBS. The beads that carry CD147Ex-BCCP on their surface were termed CD147Ex-BCCP beads.

2.8 Detection of CD147Ex-BCCP on streptavidin magnetic beads by indirect immunofluorescence

The existence of CD147Ex-BCCP on streptavidin beads was determined by immunofluorescence and flow cytometry. $2.5x10^6$ CD147Ex-BCCP beads, or uncoated beads, were incubated in 50 μl of 1% BSA in PBS/NaN_3 containing 5 μg/ml mouse anti-CD147 mAb M6-1D4, with gently agitation for 30 minutes at room temperature. The beads were washed 3 times with 1 ml of 1% BSA in PBS/NaN_3 by applying the magnetic field and resuspended in 25 μl of the same buffer. FITC-conjugated sheep $F(ab')_2$ anti-mouse immunoglobulins antibodies (Dako) was then added and incubated with mild agitation at room temperature for a further 30 minutes. The beads were washed 3 times with the same buffer and resuspended in 400 μl of 1% paraformaldehyde/PBS, then analyzed by FACSCalibur flow cytometer.

2.9 Animal and immunization

Three 6 week-old female BALB/c mice were intraperitoneally immunized three times with $5x10^7$ CD147Ex-BCCP beads in 500 µl sterile PBS at two-week intervals. The immunizations were done without any adjuvant. One day before the first immunization, blood from each mouse was collected by tail bleeding. Each mouse was further bled before every injection, and additional 3 times at 8, 14, and 20 weeks after the last immunization. The obtained mouse sera were screened for anti-CD147 antibodies by indirect ELISA, indirect immunofluorescence and Western immunoblotting.

2.10 Determination of activity and specificity of polyclonal antibody in mice sera

2.10.1 Optimization of CD147-hIgG and CD2-hIgG concentration

In order to analyze the mouse anti-CD147 antibodies raised against CD147Ex-BCCP fusion protein by ELISA, the coated antigens must not contain the BCCP part. Therefore, the different form of recombinant CD147 molecule was encountered. The culture supernatants containing the soluble fusion protein of CD147 and the Fc part of human IgG (CD147-hIgG), or CD2 and Fc part of human IgG (CD2-hIgG) (kindly provided by Dr. Watchara Kasinrerk) were titrated to achieve the quantity that gave the best reactivity when reacted with corresponding antibodies. The microtiter plate was coated at 4°C for 18 hours with various dilution of CD147-hIgG, or CD2-hIgG supernatant, in carbonate/bicarbonate buffer, pH 9.6. The plate was blocked by adding 2% skim milk in PBS and incubated at room temperature for 1 hour. After 3 times washing with 0.05% tween-PBS, serial dilution of CD147 mAb

M6-1D4, or CD2 mAb (kindly provided by Dr. Watchara Kasinrerk) was added and the plate was further incubated at room temperature for 1 hour. Then the plate was washed, followed by being added with the HRP-conjugated rabbit anti-mouse immunoglobulins in 2% skim milk/PBS. After 1 hour incubation and 3 time washing, TMB substrate was added to each well followed by terminating the reaction with 1 N HCl. The absorbance of the reaction was measured at 450 nm by ELISA reader.

2.10.2 Indirect ELISA

The kinetic of anti-CD147 polyclonal antibodies induced by immunizing with CD147Ex-BCCP beads was evaluated using indirect ELISA. The microtiter plate was coated overnight at 4°C with the optimal dilution of CD147-hIgG, or CD2-hIgG, in carbonate/bicarbonate buffer, pH 9.6. The plate was blocked by adding 2% skim milk in PBS and incubated at room temperature for 1 hour. After 3 times washing with 0.05% tween-PBS, the mice sera were diluted in two-fold series and added into the wells. Then the plate was incubated for further 1 hour and washed 3 times with the same reagent. To trace the antibody binding, HRP-conjugated rabbit anti-mouse immunoglobulins was added into each well. After incubation and washing step, TMB color substrate was added. After a period of time, the reactions were stopped by adding with 1 N HCl and the colorimetric signal was measured at 450 nm by ELISA reader.

2.10.3 Indirect immunofluorescence

The reactivity of mice antibodies against CD147 was determined by indirect immunofluorescence and flow cytometry. CD147-expressing and non-

expressing BW5147 cell lines, or CD298-expressing BW5147 cell line (kindly provided by Dr. Sawitree Chiampanichayakul, Chiang Mai University, Chiang Mai, Thailand), were cultured in RPMI1640 medium supplemented with 10% FCS and maintained at 37°C in 5% CO_2 incubator. The cells were counted and adjusted to $1x10^7$ cells/ml in 1% BSA-PBS/NaN_3. After that, the cells were incubated for 30 minutes at 4°C with 10% human AB serum for blocking the non-specific Fc receptor binding. Then aliquot of 50 μl of the cell suspension was incubated on ice with 50 μl of diluted mice sera (1:50) for 30 minutes. After 3 times washing with cold 1% BSA-PBS with NaN_3, the cells were resuspended in 20 μl of the same reagent and 25 μl of FITC-conjugated sheep anti-mouse immunoglobulins (Dako) was added to the cells suspension. After 30 minutes incubation on ice, the cells were washed 3 times with the same reagent and fixed in 1% paraformaldehyde in PBS. Then the stained cells were analyzed by FACSCalibur flow cytometer.

2.10.4 SDS-PAGE and Western immunoblotting

To ensure the specificity of CD147 polyclonal antibodies, mice sera were subjected to react against protein extract of CD147-expressing BW5147 cell line, which was separated by SDS-PAGE. CD147-expressing and non-expressing BW5147 cell lines, or CD298-expressing BW5147 cell line, were washed twice with PBS and adjusted to $5x10^7$ cells/ml. The packed cells ($5x10^7$ cells) were solubilized in 1 ml lysis buffer (1% NP-40, 1 mM PMSF, 5 mM iodoacetamide, 10 μg/ml aprotinin, Tris-HCl pH 8.2) at 4°C for 30 minutes. The lysate was clarified by centrifugation at 18,000x*g*, 4°C for 20 minutes. The protein quantity of the cell lysate was measured by biurette method (Olympus AU 400 Chemistry Analyzer). Then the cell lysate was

separated in 12% polyacrylamide gel under reducing condition. For Western immunoblotting, the separated proteins were electroblotted onto polyvinylidene fluoride (PVDF) membrane. The membrane was blocked at 4°C for 18 hours in 5% skim milk in PBS, pH 7.2, and then incubated with the diluted mice sera (1:5,000) for 1 hour at room temperature on a shaking platform. Pooled CD147 mAbs (M6-1E9, M6-1B9 and M6-1D4 at 5 µg/ml each) were used as positive control. Following washing four times with 0.05% Tween 20 in PBS, pH 7.2, HRP-conjugated rabbit-anti-mouse immunoglobulins antibody (Zymed) diluted in 5% skim milk in PBS, pH 7.2, was added to the membrane and incubated at room temperature for 1 hour. After washing step, the immuno-reactive bands were then visualized by chemiluminescent substrate detection system (Amersham–Pharmacia Biotech).