CHAPTER III

RESULTS

3.1 Construction of plasmid expression vector encoding CD147Ex-BCCP fusion protein

The extracellular domain of CD147 coding sequence was amplified from pComb8-CD147Ex plasmid vector using a set of primer that was designed to have the short restriction sequences for *NdeI* and *EcoRI* at the upstream and downstream of CD147Ex gene, respectively. Successful amplification of the CD147Ex gene was clearly indicated by the presence of a band at approximately 552 bp (Figure 3.1, lane 1). The amplified CD147Ex fragment was purified and digested with *NdeI* and *EcoRI*, followed by another purification step (Figure 3.1, lane 2). The digested CD147Ex was inserted and ligated into the pAK400CB plasmid vector, which was pre-treated with the same enzymes (Figure 3.1, lane 4). The ligated product was transformed into the *E. coli* XL-1 Blue and the chloramphenicol-resistant colonies were selected for purification of the plasmid vector. The plasmid containing the inserted CD147Ex gene was identified and used to transform *E. coli* Origami B. The molecular size at approximately 4.6 kb of plasmid isolated from chloramphenicol-resistant colonies was revealed by digesting with either *NdeI* or *EcoRI* (Figure 3.2, lane 2 and 3). The inserted fragment of CD147Ex at 552 bp was retrieved from the isolated plasmid by digestion with *NdeI* and *EcoRI* (Figure 3.2,

lane 4) or re-amplification by PCR (Figure 3.2, lane 5). The newly synthesized recombinant plasmid vector was named pAK400CB-CD147Ex (Figure 3.3).

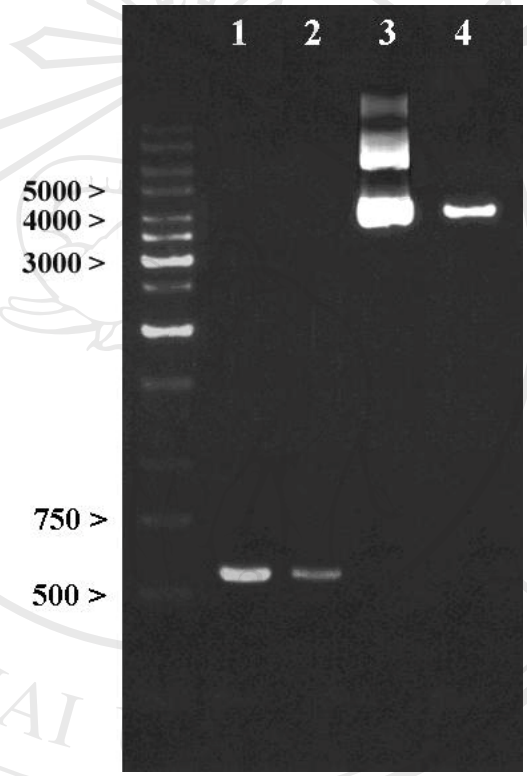

Figure 3.1 Agarose gel electrophoresis revealed PCR product of CD147Ex (552 bp) which was amplified from pComb8-CD147Ex (lane 1), the purified CD147Ex fragment after *NdeI* and *EcoRI* treatment (lane 2), the undigested and purified product of *NdeI* and *EcoRI*-treated pAK400CB plasmid vector (lane 3 and 4, respectively).

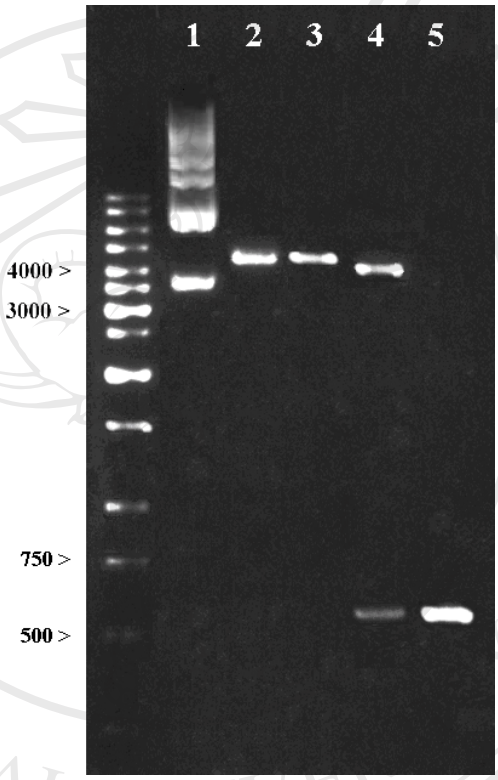

Figure 3.2 Analysis of plasmid vector purified from *E. coli* Origami B harboring pAK400CB-CD147Ex. Lane 1 demonstrates the uncut vector. The plasmid vector was digested with either *NdeI* (lane 2), or *EcoRI* (lane 3). CD147Ex DNA insert (552 bp) was retrieved by double-digestion with *NdeI* and *EcoRI* (lane 4), or re-amplification of CD147Ex gene (lane 5).

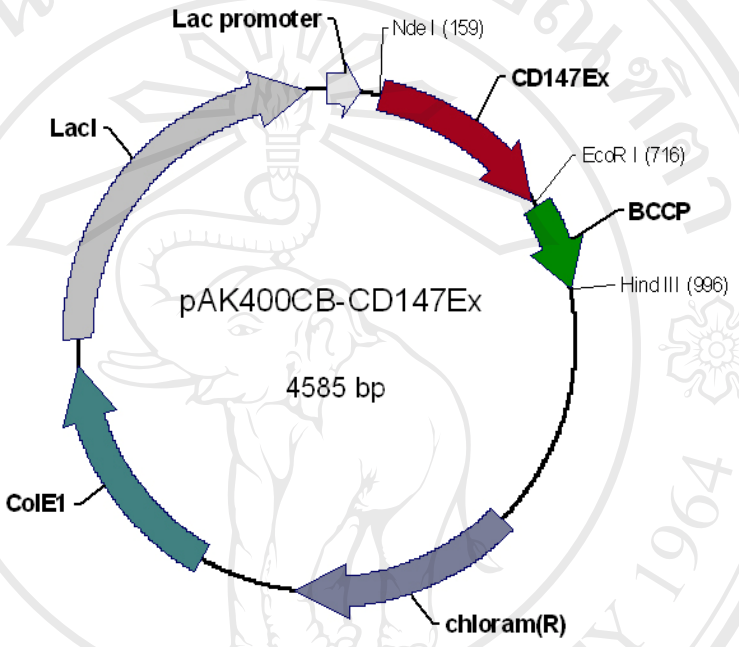

Figure 3.3 Schematic illustration represents the pAK400CB-CD147Ex vector. The CD147Ex was inserted between the *NdeI* and *EcoRI* restriction sites; origin of replication (ColE1), lac promoter, lac repressor (lacI), chloramphenicol resistant gene (chloram(R)) and BCCP are shown.

3.2 Production of biotinylated CD147Ex-BCCP fusion protein

The selected clone of *E. coli* Origami B was grown in shaking flask to allow protein expression. The pellet of cultured bacteria was solubilized in protein extraction reagent and the supernatant that contained the recombinant protein was obtained. To verify the presence of biotinylated CD147Ex-BCCP fusion protein in bacterial extract, indirect ELISA and Western immunoblotting were carried out.

3.2.1 Detection of biotinylated CD147Ex-BCCP fusion protein by indirect ELISA

Indirect ELISA was employed to determine whether the recombinant protein was produced and biotinylated *in vivo*. The ELISA wells were coated with avidin for capturing the biotinylated CD147Ex-BCCP, followed by addition of CD147 mAbs and HRP-conjugated rabbit-anti-mouse immunoglobulins to detect the bound fusion proteins. By this system, only CD147Ex-BCCP that was biotinylated by the *E. coli* biotin ligase showed the positive signal. As shown in Figure 3.4, CD147Ex-BCCP was strongly reacted with all of the CD147 mAbs, but not for anti-survivin control antibody. The non-significant reactivity of CD147 mAbs against the captured survivin-BCCP, or the well that applied with extract from mock-transformed Origami B (data not shown), assured that the other elements, such as BCCP and avidin, did not encounter to the positive signal observed in CD147Ex-BCCP-captured wells. In addition, the biotinylation of CD147Ex-BCCP was assured since the uncoated wells could not capture the CD147Ex-BCCP, leading to undetectable signal (data not shown).

3.2.2 Analysis of CD147Ex-BCCP fusion protein by Western immunoblotting

The protein components of bacterial extracts containing CD147Ex-BCCP fusion protein were separated in 12% polyacrylamide gel under reducing condition, electroblotted onto PVDF membrane, and probed with pooled CD147 mAbs (M6-1B9, M6-1D4, and M6-1E9). By tracing with HRP-conjugated rabbit anti-mouse Igs, an immuno-reactive band with molecular weight of approximately 30 kDa (CD147Ex, 20 kDa plus BCCP, 9 kDa) was visualized (Figure 3.5, lane 1A). The same size of protein band was obtained when probed with HRP-conjugated streptavidin (Figure 3.5, lane 1C). HRP-conjugated streptavidin also recognized the biotinylated F_2-BCCP fusion protein (20 kDa), while CD147 mAbs did not detect any cross-reactive protein in Origami B-pAK400CB-F_2, or untransformed Origami B extracts (Figure 3.5, lane 2C, 2A and 3A, respectively). The Origami B-pAK400CB-CD147Ex extract did not show any positive band when CD147 mAbs were excluded (Figure 3.5, lane 1B).

As demonstrated by both indirect ELISA and Western immunoblotting, the CD147Ex-BCCP fusion protein was efficiently expressed in *E. coli* Origami B with *in vivo* biotin attachment. The biotinylated fusion protein was further purified and immobilized on streptavidin magnetic beads prior to immunization.

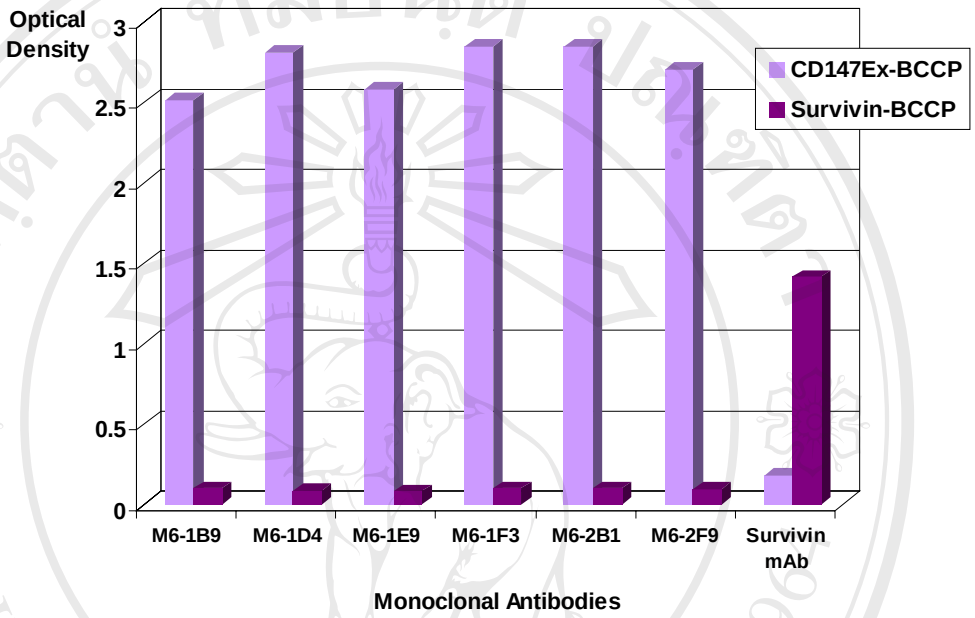

Figure 3.4 Detection of biotinylated CD147Ex-BCCP fusion protein in bacterial extracts by indirect ELISA using six CD147 mAbs (M6-1B9, M6-1D4, M6-1E9, M6-1F3, M6-2B1, and M6-2F9) and HRP-conjugated rabbit anti-mouse immunoglobulins as a tracer. The biotinylated fusion proteins were captured by egg white avidin, which was coated in the wells, followed by the addition of specific mAbs. The bacterial extract containing survivin-BCCP fusion protein was used as a control.

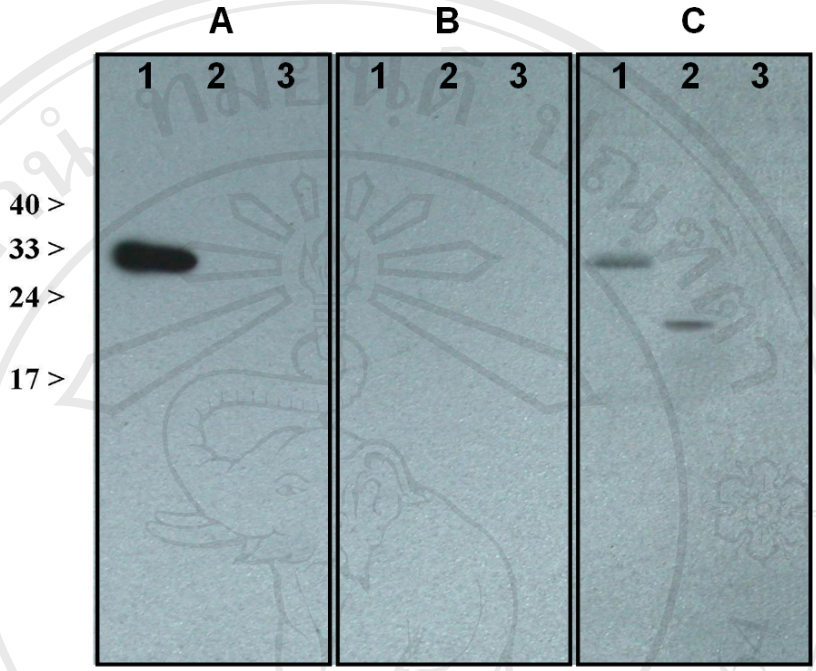

Figure 3.5 Analysis of biotinylated CD147Ex-BCCP fusion protein by Western immunoblotting technique. The protein extracts from Origami B harboring pAK400CB-CD147Ex (lane 1), pAK400CB-F_2 (lane 2), and untransformed Origami B (lane 3) were separated by reducing SDS-PAGE and subsequently blotted onto PVDF membrane. The fractionated proteins were probed with pooled CD147 mAbs (M6-1E9, M6-1B9 and M6-1D4) followed by HRP-conjugated rabbit-anti-mouse immunoglobulins antibody (A), without CD147 mAbs (B), or probed with HRP-conjugated streptavidin (C). The immuno-reactive bands were visualized by chemiluminescent substrate detection system. Molecular weight markers in kDa are indicated.

3.3 Purification of biotinylated 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**.** The CD147Ex-BCCP-immobilized magnetic beads were further used as immunogen to induce the antibody responses against CD147 in mice. To verify the existence of CD147Ex-BCCP on the beads, indirect immunofluorescence was performed using CD147 mAb M6-1D4 and FITC-conjugated sheep $F(ab')_2$ anti-mouse Igs antibodies. The fluorescent profiles of stained beads, which analyzed by flow cytometer, were shown in Figure 3.6. The strong fluorescent intensity of CD147Ex-BCCP beads stained with M6-1D4 indicated the immobilization of the fusion protein on the beads (Figure 3.6, A). The uncaptured streptavidin beads did not react with the CD147 mAb, ensuring the specificity of the test (Figure 3.6, B). Thus, the CD147Ex-BCCP beads were ready to be injected into the mice.

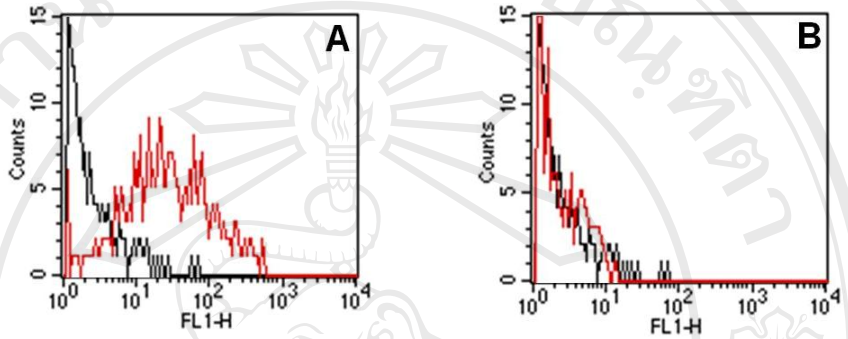

Figure 3.6 Flow cytometric analysis of CD147Ex-BCCP-immobilized streptavidin magnetic beads. CD147Ex-BCCP beads (A) or uncaptured beads (B) were stained with CD147 mAb M6-1D4 and traced with FITC-conjugated sheep anti-mouse Igs (red line), black line represents conjugate control.

3.4 Production of anti-CD147 polyclonal antibodies in mice

Three female BALB/c mice were intraperitoneally immunized three times with CD147Ex-BCCP beads at two-week intervals. Blood from each mouse was collected at 2, 4, 6, 12, 18, and 24 weeks after the first immunization. The obtained mouse sera were screened for anti-CD147 antibodies by indirect ELISA, indirect immunofluorescence and Western immunoblotting.

3.4.1 Optimization of CD147-hIgG and CD2-hIgG concentration

In order to determine the presence of CD147 antibodies in mice sera by using indirect ELISA, the coating antigens that contained BCCP part (i.e. CD147Ex-BCCP) should be avoided. Hence, another recombinant CD147, CD147-hIgG, was utilized. The culture supernatant containing CD147-hIgG, or an irrelevant molecule CD2-hIgG, was titrated to achieve the dilution that gave the highest reactivity against the lowest concentration of corresponding mAbs. By plotting the O.D. of each dilution of the supernatant against various concentrations of mAbs, the dilution that showed the highest sensitivity of CD147-hIgG (Figure 3.7 A, 1:64), or CD2-hIgG (Figure 3.7 B, 1:2) in carbonate/bicarbonate buffer was obtained. The specificity of the assay was assured since the mAbs did not react to the uncoated wells.

3.4.2 Determination of anti-CD147 antibody responses in mice by indirect ELISA

In order to analyze antibody response to CD147 in the immunized mice, indirect ELISA using recombinant CD147-hIgG fusion protein was carried out. The CD147-hIgG, or CD2-hIgG, was coated in ELISA wells at their optimal dilution

followed by addition of serially two-fold diluted mice sera, and the bound antibodies were traced by HRP-conjugated rabbit anti-mouse Igs antibodies. By setting the reactivity of pre-immunized sera as cut-off titer, the significant titer (about $5x10^5$) of polyclonal anti-CD147 antibodies was observed in all immunized mice (Figure 3.8). In all mice, the antibody response could be clearly detected right after the first immunization and extremely increased after the third immunization. Moreover, the antibody response was still detected in the sera even at 5 months after the last immunization. Since the reactivity against CD2-hIgG of all immunized sera was comparable to the pre-immunized sera (cut-off titer), the binding of mice antibodies to IgG part or irrelevant components was excluded and the specificity of mice antibodies to CD147 was ensured.

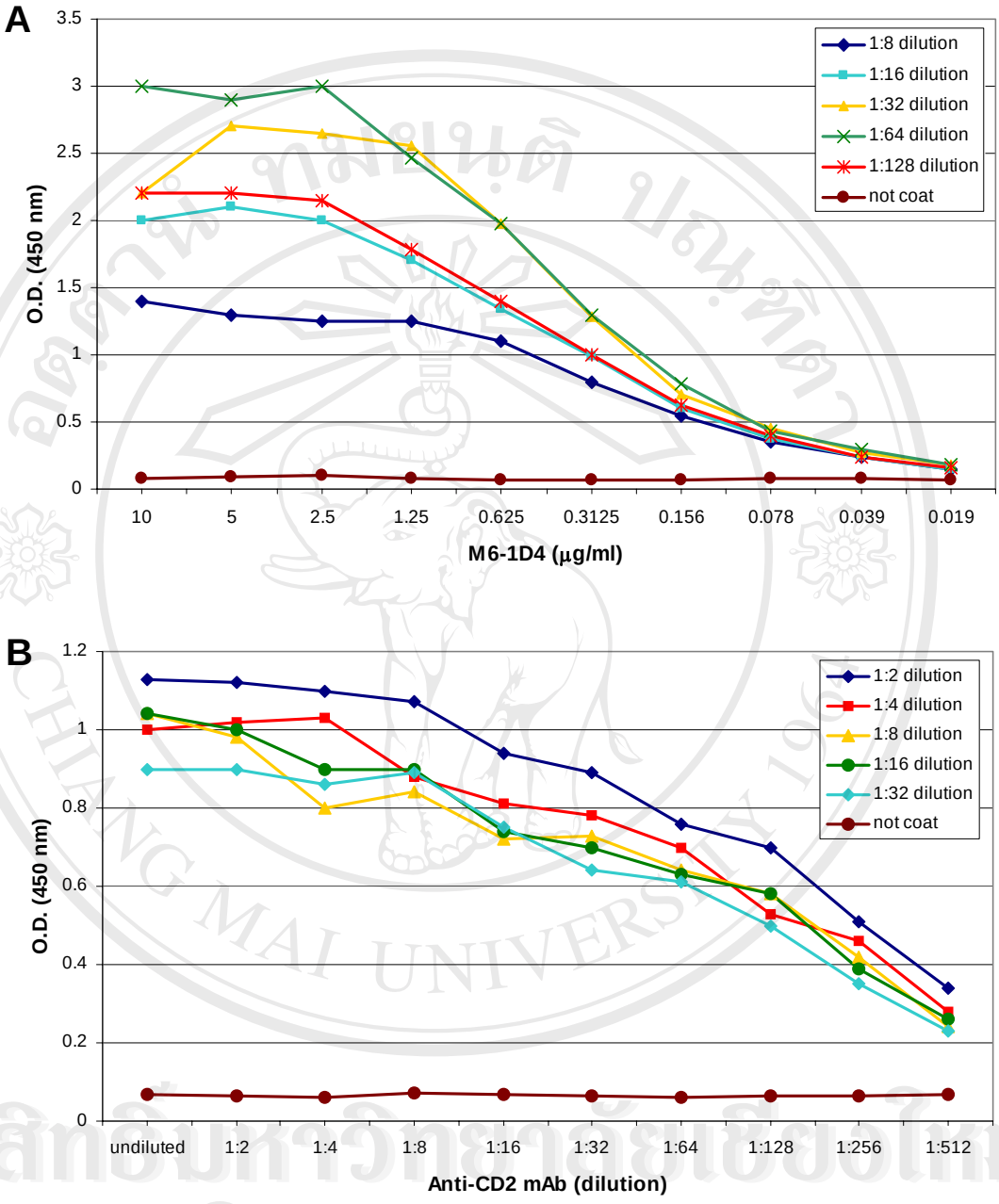

Figure 3.7 Titration of culture supernatant containing CD147-hIgG (A) or CD2-hIgG (B) by indirect ELISA. The O.D of each dilution was plotted against various concentrations of the corresponding mAbs.

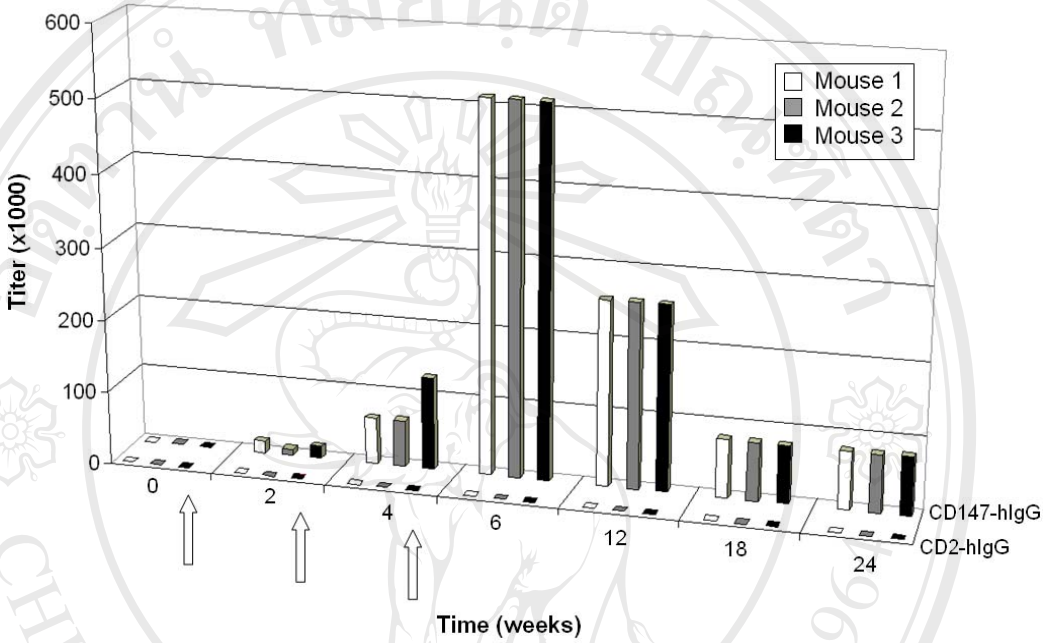

Figure 3.8 Anti-CD147 antibody responses of mice after immunizing with CD147Ex-BCCP beads were determined by indirect ELISA. The titer of each serum against CD147-hIgG, or control antigen CD2-hIgG, was plotted to compare the individual antibody reactivity between three mice. The pre-immunized serum of each mouse was used as a cut-off titer. Arrows indicate the first, second and third immunizations.

3.4.3 Determination of the reactivity of mouse anti-CD147 antibodies by indirect immunofluorescence

The activity and specificity of mouse polyclonal antibodies against CD147 was determined by indirect immunofluorescence on CD147-expressing BW5147 cell line, or CD298-expressing BW5147 cell line. Both BW5147 cell lines, which derived from mouse thymoma, were stably transfected with cDNA of human CD147, or CD298, respectively (Chiampanichayakul et al., 2002). Serum at 6^{th} week from mouse number 3, the representative mouse in this experiment, strongly reacted with CD147-expressing BW5147 cell, but not with the CD298-expressing and untransfected BW5147 cells (Figure 3.9, B, E, and G) while pre-immunized serum did not reacted to any cell type (Figure 3.9, A, D, and F). The positive control CD147 mAb, M6-1D4, also strongly reacted with CD147-expressing BW5147 cells (Figure 3.9, C).

3.4.4 Determination of the reactivity of mouse anti-CD147 antibodies by Western immunoblotting

To assure that the positive fluorescent signal was derived from the binding of mouse antibodies to the cell surface CD147, proteins extracted from CD147-expressing BW5147 cells were separated on reducing SDS-PAGE and blotted onto PVDF membrane. The blotted membrane was probed with sixth week-serum of mouse number 3, the representative mouse of the experiment. Mouse antibodies recognized the broad protein band at molecular weight approximately 50 kDa of the lysate from CD147-expressing BW5147 cells (Figure 3.10, lane 3B). The same position of reactive band was observed in the lane of CD147-expressing BW5147 cell

lysate after probing with pooled CD147 mAbs (Figure 3.10, lane 3C), indicating the mature form of CD147. In control experiments, both mouse hyperimmune serum and CD147 mAbs did not reacted to any components of the lysates from CD298-expressing and untransfected BW5147 cells (Figure 3.10, lane 1-2B and 1-2C). Moreover, pre-immunized serum, as well as HRP-conjugated secondary antibody alone, did not recognize any reactive band in any of the three cell lysates (Figure 3.10, A and D). The protein quantities of the three cell lysates were comparable, as shown in Figure 3.10 lane 1-3E. Thus, anti-CD147 antibodies in mouse serum had high reactivity and specificity to CD147, both in native and denatured forms.

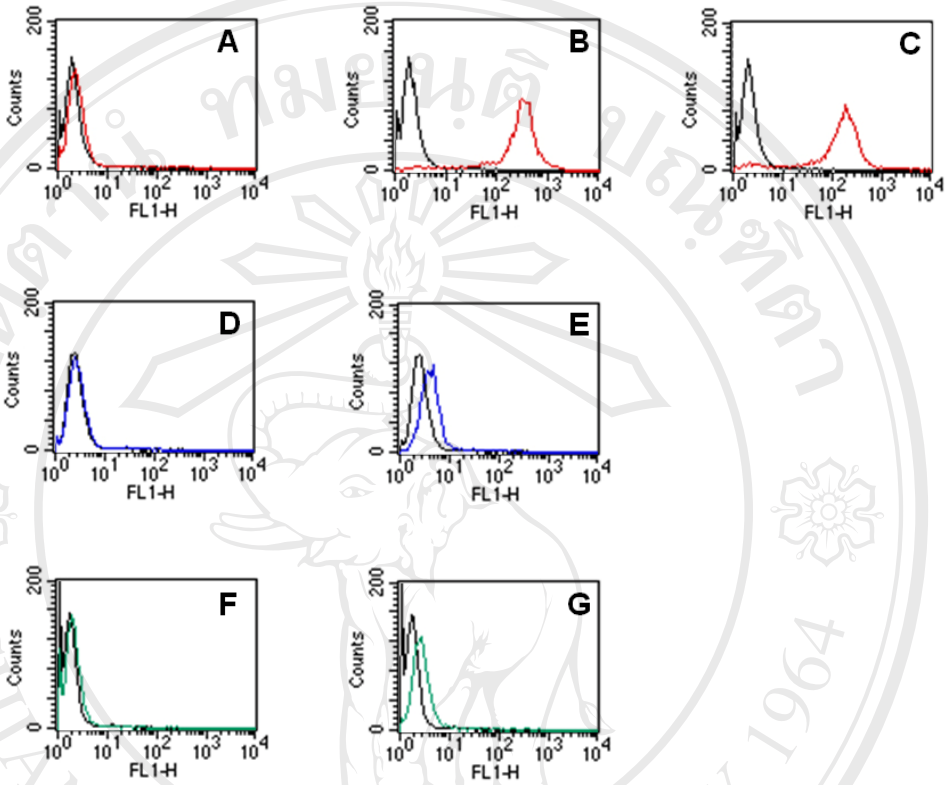

Figure 3.9 Immunofluorescence profiles of the reactivity of anti-CD147 polyclonal antibodies in mouse sera. CD147-expressing BW5147 cell line were stained with pre-immunized serum (A) or serum at 6th week (B) of mouse number 3 after having immunized with CD147Ex-BCCP beads. The pre-immunized serum and serum at 6th week of mouse number 3 were also used to stain CD298-expressing BW5147 cell line (D and E) or BW5147 cell line (F and G, respectively). The fluorescent signal of CD147-expressing BW5147 cell line against positive control antibody (M6-1D4) was indicated (C). Black lines represent the background fluorescence of negative control, and colored lines represent the cells stained with mouse sera or positive control antibody.

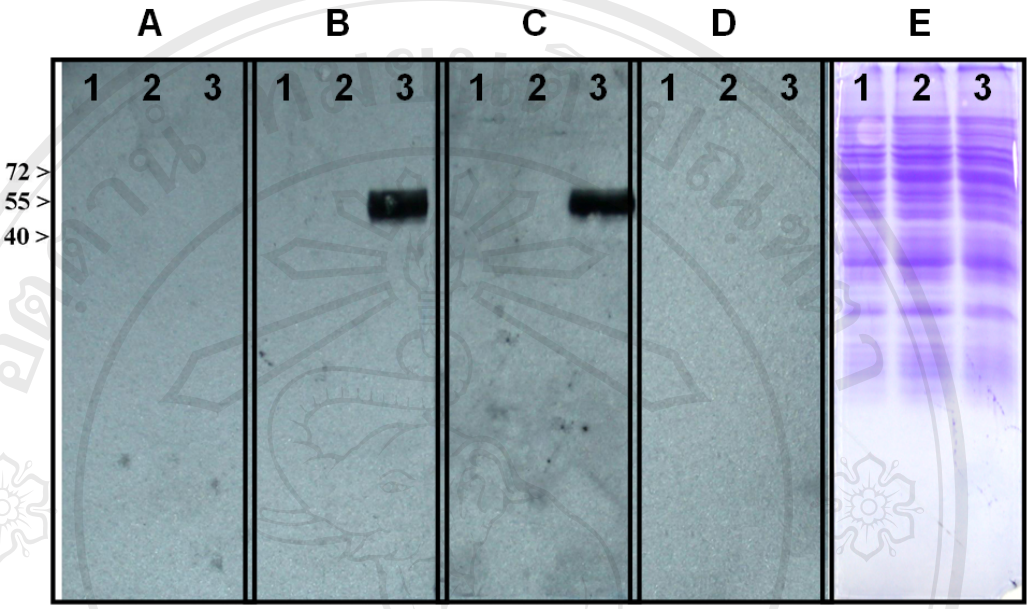

Figure 3.10 Analysis of mouse anti-CD147 polyclonal antibodies by Western immunoblotting technique. The protein components in lysates of CD298-expressing BW5147 cell (lane 1), untransfected and CD147-expressing BW5147 cells (lane 2 and lane 3, respectively) were separated by reducing SDS-PAGE and subsequently blotted onto PVDF membrane. The membranes were probed with either pre-immunized serum (A) or serum at 6th week (B) of mouse number 3 after being immunized with CD147Ex-BCCP beads. Combination of CD147 mAbs (M6-1E9, M6-1B9 and M6-1D4) was used as positive control (C), while membrane in D represented negative control (secondary antibody only). Polyacrylamide gel stained with coomassie blue demonstrates the presence of comparable amount of proteins in these cell lysates (E). Molecular weight markers in kDa are indicated.