CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and instruments used in this study are shown in Appendix A-C

2.2 Production of supernatant containing CD147–hIgG

2.2.1 Preparation of COS cells

COS cells were grown in MEM medium supplemented with 10% FCS in a tissue culture flask. On the day before COS cell transfection, the culture medium was discarded and 0.5 mM EDTA-PBS was added into the tissue culture flask. The solution was incubated for 7 minutes at room temperature. Then, COS cells were removed and collected into 15 ml tube. They were washed twice with MEM medium and counted. The cell concentration was adjusted to $1x10^6$ cells in 4 ml of 10% FCS-MEM, plated into 6 cm tissue culture dish and incubated overnight at 37°C in 5% CO_2 incubator.

2.2.2 COS cell transfection

Transfection mediated by DEAE-Dextran is one of many methods used for introducing plasmid DNA into cultured mammalian cells such as COS cells. The procedures for transfection of plasmid DNA encoding soluble CD147-human IgG fusion protein (CD147-Rg-DNA) (kindly provide by Dr. Hannes Stockinger,

University of Vienna, Vienna, Austria) into COS cells by using DEAE-Dextran transfection were as follows. The medium was removed from the COS cell dishes and the cells were washed with 4 ml of MEM medium. Subsequently, COS cells were transfected with 2 ml of MEM containing 250 μg/ml DEAE-Dextran, 400 μM chloroquine diphosphate and 2 μg of CD147-Rg-DNA and incubated for 3 hours at 37°C in 5% CO_2 incubator. The transfection solution was aspirated and 2 ml of 10% DMSO-PBS was added into the COS cell dish, left to stand for exactly 2 minutes at room temperature and then removed rapidly. The cells were washed with 3 ml of MEM medium, after that 4 ml of 10% FCS-MEM were added into the dish. Cells were cultured in a CO_2 incubator at 37°C. After overnight incubation, the medium was discarded and 4 ml of 10% FCS-MEM was replaced. The transfected COS cells were cultured for another 2 days to allow expression of the corresponding proteins. The supernatant containing CD147-human IgG fusion protein was collected and stored at -20°C.

2.2.3 Purification of CD147-hIgG from culture supernatant

To purify the CD147-hIgG from culture supernatant, affinity chromatography was used. Protein A coated Sepharose column was used to purify CD147-hIgG fusion protein.

Protein A coated Sepharose column was equilibrated with 50 ml of 20 mM sodium phosphate and adjusted the solution to the bed volume. Then, 80 ml of the diluted supernatant were added to the column, incubated for 5 minutes. The unbound proteins were removed by washing with 50 ml of 20 mM sodium phosphate. To elute antibodies from the column, 2 ml of elution buffer (0.1 M citric acid, pH 3.0) were

added and the solution was drained out by re-adding the elution buffer into the column. The eluate was collected and adjusted to pH 7.0 with neutralization buffer (2M Tris-HCL, pH 8.0). The column was then washed with 50 ml of 20 mM sodium phosphate and retained in storage buffer (see appendix) and stored at 4°C.

The obtained eluates were dialyzed against PBS overnight by microdialyzer system. The absorbance of antibody preparation was determined at 280 nm and protein concentration was calculated.

2.3 Determination of activity and specificity of the purified CD147-hIgG

To confirm that the purified CD147-IgG fusion protein can bind to anti-CD147 antibodies, sandwich ELISA was employed. The microtiter plate was coated overnight with 25 μg/ml CD147 mAb, M6-1D4, in 0.1 M carbonate bicarbonate buffer, then washed four times with 0.05% Tween/PBS. The wells were blocked with 5% bovine serum albumin/PBS and washed one time with 0.05% Tween/PBS. Serial dilutions of purified CD147-hIgG were added to wells and incubated for 1 hour at 37°C. After washing four times, rabbit anti-human immunoglobulins peroxidase conjugate was added and incubated for 1 hour at 37°C. Plates were washed four times with 0.05% Tween/PBS. Next, 50 μl of ortho-phenylenediamine (OPD) substrate was added to each well and incubated at room temperature for 15 minutes followed by the addition of 50 μl of 4 N of H_2SO_4. The OD of each well was measured by using Microplate Reader at 490 nm.

2.4 Production of plasmid DNA encoding of CD147 molecule

2.4.1 Transformation of competent *E. coli* by plasmid DNA

Competent *E. coli* (MC1061/P3) were removed from –70°C and thawed on ice. Then, 100 µl of each competent bacteria was aliquoted into a pre-cooled 10 ml tube. Ten microliter of pCDM8-CD147, plasmid DNA encoding CD147 molecule, concentration of 200 µg/ml was then added into the tube, mixed gently and incubated on ice for 40 minutes. The tube was put into a 42°C water bath for exactly 45 seconds, then, rapidly placed on ice for 2 minutes. Luria-Bertani broth (0.9 ml) was added into the tube and a shaking incubator at 37°C at 200 rpm for 1 hour. Ten microliters of bacteria suspension was then spread on Luria-Bertani plate containing 15 µg/ml of ampicillin and 10 µg/ml of tetracycline, and incubated at 37°C overnight. The resulting transformed bacterial colonies were screened for their pCDM8-CD147 containing.

2.4.2 Screening of transformed bacterial colonies

2.4.2.1 Isolation of plasmid DNA from transformed bacteria

Each bacterial colony was inoculated and incubated separately in a 50 ml tube containing 5 ml LB-antibiotic broth and shaken at 200 rpm in 37°C, overnight. On the next day, 1.5 ml of bacterial suspension was added into the microtube and centrifuged with a microcentrifuge at 13,000 g for 1 minute. The medium was then discarded. Then, the bacterial pellets were resuspended in 250 µl of A1 buffer (NucleoSpin® Plasmid Mini kit). Two hundred and fifty microliters of A2 buffer (NucleoSpin® Plasmid Mini kit) was added into each microtube and samples were mixed by gently

inverting the tube 4-6 times. The tube was left at room temperature for 5 minutes. Three hundred microliters of A3 (NucleoSpin® Plasmid Mini kit) were then added into each tube, mixed by gently inverting the tube 4-6 times, and centrifuged with a microcentrifuge at 13,000 g for 10 minutes. The supernatant containing plasmid DNA was collected. Then, supernatant was applied into the NucleoSpin column and centrifuged with a microcentrifuge at 13,000 g for 1 minute. The column was washed with 600 µl of A4 buffer (NucleoSpin® Plasmid Mini kit) and centrifuged with a microcentrifuge at 13,000 g for 1 minute. The plasmid DNA was eluted with 50 µl of AE buffer (NucleoSpin® Plasmid Mini kit) and left at room temperature for 1 minute. It was then centrifuged for 1 minute at 13,000 g and the eluated solution was collected. The absorbance at 260 nm and 280 nm was measured. The concentration of the plasmid DNA was determined by the following equation:

The concentration of plasmid DNA (µg/ml) = OD at 260 x 50

2.4.2.2 Restriction fragment analysis of plasmid DNA

Two microliters of 10x restriction buffer, were added to 7 µl of distilled water and 1 µl of *Xba* I restriction enzyme in a sterile microtube. The mixture was then mixed by tapping the tube and centrifuged at 13,000 g for 2-3 seconds, then 10 µl of plasmid DNA was added and mixed by tapping the tube. The tube was centrifuged at 13,000 g for 2-3 seconds and the mixture was incubated at 37°C for 2 hours. Afterwards, 4 µl of 6x loading buffer was added to the digested plasmid DNA. The tube was incubated at 65°C for 5 minutes and plunged into an ice bath immediately,

and left on ice for 10 minutes. Ten microliters of each sample were subsequently loaded into 1% agarose gel containing EtBr. The lid of the electrophoresis chamber was closed and the electrical leads were attached so that the DNA was migrated toward the anode by applying 120 volts. The sample ran until the bromphenol blue migrated to the appropriate distance through the gel. Later on, the electric current was turned off and the gel was removed from the gel tank. The gel was destained with distilled water for 10 minutes. It was then visualized for fragments of DNA with a UV transiluminator, photographed. The DNA bands were compared with the standard DNA markers.

2.4.3 Large-scale production of plasmid DNA

The transformed *E. coli* colonies were selected from cultured plates and grown in LB broth supplemented with ampicillin and tetracycline by shaking at 200 rpm for 8 hours at 37°C. Then, 1 ml of cultured broth was added to 250 ml of LB broth and the mixture was shaken at 200 rpm at 37°C, overnight. The bacterial pellets were harvested by centrifugation at 2,400g, 4°C, for 30 minutes. Fifty milliliters of P1 buffer (QIAGEN plasmid mega kit) was then added into a bacterial pellet and resuspended by vortex mixer. Fifty milliliters of P2 buffer (QIAGEN plasmid mega kit) was added to the suspended bacteria, and samples were mixed by gently inverting the tube 4-6 times. The tube was left at room temperature for 5 minutes. Fifty milliliters of P3 buffer (QIAGEN plasmid mega kit) were then added into each tube, mixed by gently inverting the tube 4-6 times. The tube was left on ice for 30 minutes and centrifuged at 2,400g, 4°C for 30 minutes. The supernatant containing plasmid DNA was collected to 50 ml tube by filtration with autoclaved gauze.

Before applying the supernatant, QIAGEN-tip 2500 column (QIAGEN plasmid mega kit) was equilibrated with 35 ml of QBT buffer and allowed to empty by gravity flow. Then, supernatant containing plasmid DNA was applied into the QIAGEN-tip 2500 column and allowed to empty by gravity flow. The column was washed with 150 ml of QC buffer and allowed to empty by gravity flow. The plasmid DNA was eluted with 35 ml of QF buffer and collected into a new tube. After that, 0.7 volumes of isopropanol were added to the supernatant and centrifugation at 15,000 g at 4°C for 30 minutes. The solution was removed from DNA pellet. Fourteen milliliters of 70% ethanol was added to the DNA pellet for washing. After centrifugation at 15,000 g at 4°C for 30 minutes, the supernatant was removed from the DNA pellet. The DNA pellet was allowed to dry for 10 minutes. Finally, DNA pellet was resuspended with 250 µl of sterile water. The concentration of plasmid DNA was measured as described in 2.4.2.1.

2.5 Determination of activity and specificity of the large-scale production of plasmid DNA

To confirm that the production of plasmid DNA could express CD147 molecule, the obtained pCDM8-CD147 were analyzed by using restriction fragment analysis and COS cell transfection experiments.

2.5.1 Restriction analysis of plasmid DNA

The restriction analysis of plasmid DNA was performed as described in 2.4.2.2.

2.5.2 Expression of CD147 protein by COS cell transfection expression system

2.5.2.1 COS cell transfection

The preparation of COS cells for transfection was performed as described in 2.2.1. COS cells were then transfected with plasmid DNA encoding intact CD147 molecule (pCDM8-CD147), or plasmid DNA encoding CD8 molecule (CD8-DNA) by DEAE-Dextran transfection as described in 2.2.2.

2.5.2.2 Staining of transfected COS cells by indirect immunofluorescent method

Three days after COS cell transfection, the culture medium was removed and 0.5 mM EDTA-PBS was added into the culture dishes. The solution was incubated for 7 minutes at room temperature. Then, COS cells were removed and collected into a 15 ml tube. Cells were washed 3 times with PBS and resuspended in cold 1% BSA-PBS-NaN_3. Cells were counted and adjusted to$1x10^7$ cells/ml with the same reagent. To block non-specific Fc receptor binding, cells were incubated for 30 minutes at 4°C with 10% human AB serum before staining. Aliquot of 50 µl of cell suspension was incubated with 20 µg/ml purified antibodies on ice for 30 minutes. After that, cells were washed twice with cold 1% BSA-PBS-NaN_3 and resuspended with 20 µl of the same reagent. Twenty five microliters of FITC-conjugated $F(ab')_2$ fragment of sheep anti-mouse immunoglobulins antibody were subsequently added and incubate on ice for a further 30 minutes. Finally, the stained cells were washed three times with 1%

BSA-PBS-NaN_3 and fixed with 1% paraformaldehyde-PBS and then analyzed for fluorescent staining by a fluorescent microscope.

2.6 Construction of the phagemid expressing CD147 molecule

2.6.1 Construction of phagemid and preparation of phage expressing CD147

Four microgams of purified PCR product, which were estimated by comparing to the known concentration band from DNA marker, were treated with 60 U of *Sfi* I at 50°C for 18 hours, whereas 20 μg of phagemid vector, pComb3HSS (kindly provided by Dr. Carlos F. Barbas, Scripps Institute, USA) was treated with 100 U of the same enzyme. Both *Sfi* I-treated DNA fragments were further purified by the PCR Purification Kit (QIAGEN). After that, five units of T4 ligase was introduced to the ligation mixture containing 50 ng of purified *Sfi* I-digested CD147Ex and 100 ng of purified *Sfi* I-digested pComb3HSS (M.W. 3,300 bp). The volume of the ligation reaction was 15 μl. Then, the ligation mixture was subsequently incubated at 4°C for 18 hours.

2.6.2 Phage display technique for the expression of recombinant CD147 molecule

2.6.2.1 Bacterial cell transformation

Fifteen microliters of the ligated DNA were co-incubated with 200 μl of cold-thawed $CaCl_2$ competent *E. coli* cells on ice for 1 hour. The transforming mixture was transferred into cooled screw cap tube and subsequently shocked at 42°C for 1.5 minutes, then, rapidly placed on ice for 1 minute. After that, three milliliters of LB

broth were added and further incubated at 37°C at 120 rpm while shaking for 3 hours. The transformed cell was centrifuged 1,900 g at RT for 10 minutes and plated on ampicillin-containing LB agar (100 μg/ml). Then, several ampicillin resistance colonies were selected and grown for plasmid miniprep using the alkaline lysis protocol as was described in 2.6.2.2. The purified phagemid were firstly checked by fractionating in 1 % agarose gel electrophoresis. In order to verify the correct *E. coli* clones, the purified phagemid from the individual clone was further digested with *Sfi* I and identified the band of correct insert (approximately 552 bp). To confirm that the CD147 DNA was inserted in pComb3HSS phagemid vector, the PCR reamplification was used to determine the correct size of the PCR product. The newly synthesized phagemid was named pComb3H-CD147.

2.6.2.2 Purification of phagemid by using alkaline lysis method

An ampicillin resistant colony was picked and grown in 3 ml of ampicillin containing broth (100 μg/ml) with vigorous shaking 180 rpm at 37°C for 8 hours. The 1.5 ml of cultured were centrifuged 10,000 g at 4°C for 5 minutes. The supernatant was discarded and the cell wall of bacterial pellet was lysed by 100 μl of 1x glucomix-lysozyme and vortex vigorously. Two hundred microliters of freshly prepared NaOH/SDS were added and mixed by inverted. Then, 150 μl of potassium acetate were added and gently mixed by vortex. The solution was centrifuged at 10,000 g 4°C for 5 minutes for collecting the clear supernatant. After that, the nine hundred microliters of analytical grade absolute ethanol were added and kept on ice for 2 minutes. The DNA was spun down at 10,000 g at 4°C for 5 minutes and supernatant was removed. The DNA pellet was reconstituted by 100 μl of sterile

distill water and followed by adding 50 µl of 7.5 M 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. Then, three hundred microliters of absolute ethanol were added to the supernatant and incubated at -70°C for 10 minutes. The solution was spun down to harvest the pellet. Finally, the pellet was washed with 1 ml of 70% ethanol by centrifugation 10,000 g at 4°C for 5 minutes. The DNA pellet was dried at 37°C about 30 minutes and reconstituted with 30 µl of sterile distilled water and stored at -20°C until use.

2.6.2.3 Preparation of phage-displayed CD147 using the *E. coli* TG-1

The selected clone of transformed *E. coli* TG-1 harboring pComb3H-CD147 was cultured in antibiotic-containing 2xTY broth (100 µg/ml of ampicillin) with shaking 180 rpm at 37°C. When the OD at 600 nm of 0.8 was reached, the precultured bacteria were transferred into 100 ml of the same medium, which contained 2 ml of glucose and further cultured in the same condition until 2 hours or the OD at wavelength 600 nm was reached 0.5. Thirty milliters of the culture were divided to infect with 3 ml of 10^{11} t.u.of VCSM13 helper phage and incubated without shaking in water bath at 37°C for 30 minutes. Virus-infected bacterial cells were centrifuged 2,500 g at 4°C for 10 minutes. The supernatant was discarded and the pellet was resuspended in 2xTY broth containing antibiotic (100 µg/ml of ampicillin and 70 µg/ml of kanamycin). Finally, Fifteen milliliters of resuspended cells were transferred into 250 ml of the same media and further cultured with shaking (180 rpm) at 37°C for 18 hours.

2.6.2.4 Harvesting of phages by PEG precipitation

The VCSM13-infected *E. coli* from section 2.6.2.3 were centrifuged 2,500 g 4°C for 30 minutes. The culture supernatant was collected and phages in the culture supernatant were precipitated by adding 4 % w/v of PEG 8,000 and 3 % w/v of NaCl were completely dissolved. The supernatant was kept on ice for 30 minutes and centrifuged 8,000 g 4°C for 30 minutes. The pellet was air dried for 30 minutes and reconstituted in 2.5 ml of 1 mM PBS pH 7.2. The solution was centrifuged 9,000 g at 4°C for 10 minutes and the supernatant was preserved in 30 % glycerol. The precipitated phages were stored at -70°C.

2.6.2.5. Detection of the phage-displayed CD147 by sandwich ELISA

Multi-well plate was separately coated with 50 µl/well of 10 µg/ml anti-CD147 monoclonal antibody, M6-1D4, which diluted in carbonate/bicarbonate buffer pH 9.6, at 4°C for 18 hours. The antibody coated wells were blocked with 2 % skimmed milk diluted in 1 mM PBS pH 7.2 at RT for 1 hour. The wells were washed with 0.05 % tween-20 diluted in 1 mM PBS. Then, precipitated phages were added into each well and incubated at RT for 1 hour. After washing, the peroxidase-conjugated sheep anti-M13 Ab was added and incubated at RT for 1 hour. The reaction wells were washed and TMB (3', 3', 5', 5'-tetramethylbenzidine) substrate was applied to each well. The enzymatic reaction was stopped using 1 N HCL and measured the absorption at OD at wavelength 450 nm.

2.7 Animals and immunizations

The immunization of mice with phage expressing CD147 molecules and pCDM8-CD147 was performed by using the immunization schedule as follows:

For phage expressing CD147 molecules were immunized into two 6 week-old female BALB/c mice using the immunization schedule as follows: On day 0, blood from each mouse was collected by tail bleeding. On day 1, mice were injected intraperitoneally (i.p) with 500 μl of phage expressing CD147 molecule (concentration $4x10^{11}$ t.u./700μl) solution emulsified in 700 μl of Complete Freund's Adjuvant. On day 14, blood from each immunized mouse was collected by tail bleeding. On day 15, the mice were boosted i.p. with the same amount of phage expressing CD147 molecules solution emulsified in 700 μl of Incomplete Freund's Adjuvant. On day 28, blood from each immunized mouse was collected by tail bleeding. On day 29, the mice were finally boosted i.p. with the same amount of phage expressing CD147 molecules as done on day 15. After that, blood from the immunized mice were collected every 14 days after 3rd immunizations by tail bleeding and screened for anti-CD147 antibodies by using indirect ELISA, indirect immunofluorescence staining and flow cytometric analysis.

For DNA based immunization, pCDM8-CD147 was immunized into two 6 week-old female BALB/c mice using the immunization schedule as follows. On day 0, blood from each mouse was collected by tail bleeding. On day 1, mice were injected intramuscularly (i.m) with 50 μl of pCDM8-CD147 in 50 μl PBS (1 mg/ml). On day 14, blood from each immunized mouse was collected by tail bleeding. On

day 15, the mice were boosted i.m. with the same amount of pCDM8-CD147 as done on day 1. On day 28, blood from each immunized mouse was collected by tail bleeding. On day 29, the mice were finally boosted i.m. with the same amount of pCDM8-CD147 as done on day 1. After that, blood from each immunized mouse was collected every 14 days after 3rd immunizations by tail bleeding and screened for anti-CD147 antibodies by using indirect ELISA, indirect immunofluorescence staining and flow cytometric analysis.

2.8 Cultivation of BW5147 mouse thymoma cell line

CD147 expressing BW5147 cell line and CD147 non-expressing cell line were cultured in RPMI1640 medium supplemented with 10% FCS in a tissue culture flask at 37°C in 5% CO_2 incubator.

2.9 Determination of activity and specificity of polyclonal antibodies in mouse sera

To study the antibody response after pCDM8-CD147 and phage expressing CD147 molecules immunization, indirect ELISA, indirect immunofluorescence assay was carried out.

2.9.1 Indirect ELISA

To determine the anti CD147 protein response, microtiter plate wells were coated at 4°C overnight with either 10 μg/ml CD147-hIgG fusion protein in 1 ml of 0.1 M carbonate bicarbonate buffer washed four times with 0.05% Tween/PBS. The wells were blocked with 5% bovine serum albumin/PBS and washed one time with

0.05% Tween/PBS. Serial dilutions of mouse sera in 0.05% Tween/PBS were added to wells and incubated for 1 hour at 37°C. After washing four times, antibody binding was detected using rabbit anti-mouse peroxidase conjugates and incubated for 1 hour at 37°C. Plates were washed four times with 0.05% Tween/PBS. Next, 50 µl of ortho-phenylenediamine (OPD) solution reagent was added to each well and incubated at room temperature. The reaction was stopped with 50 µl of 4 N of H_2SO_4 and the absorbance was measured by ELISA reader at 490 nm.

2.9.2 Immunofluorescence technique

The indirect immunofluorescence staining and flow cytometry were used to monitor the antibody responses. CD147 expressing BW5147 cell line (Chiampanichayakul *et al.*, 2002) were counted and adjusted to $1x10^7$ cells/ml. To block non-specific Fc receptor binding, cells were incubated for 30 minutes at 4°C with 10% human AB serum before staining. Aliquot of 50 µl of cell suspension was incubated with 50 µl of dilution of mouse sera (dilution 1:50) on ice for 30 minutes. After that, cells were washed twice with cold 1% BSA-PBS-NaN_3 and resuspended with 20 µl of the same reagent. Twenty five microliters of FITC-conjugated sheep $F(ab')_2$ anti-mouse immunoglobulins antibodies were then added and incubate on ice for a further 30 minutes. After, washing three times with 1% BSA-PBS-NaN_3 and fixed with 1% paraformaldehyde-PBS, stained cells were analyzed by FACSCalibur flow cytometer using cellQuest software.