CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals, antibodies, cell lines and instruments used in this study are shown in Appendix A-D

2.2 Production of ascitic fluids containing anti-CD147 mAbs

To produce ascitic fluids containing mAbs of interest, hybridromas were grown in IMDM medium supplemented with 10% fetal calf serum (FCS). At one week before injection of hybridromas, 500 µl of pristane was injected into intraperitoneal cavity of Balb/c mice. Then, 0.5-1x10^7 hybridomas were injected into the pristane-primed mice. After 2-3 weeks, ascitic fluids were collected from the peritoneal cavity of Balb/c mice and centrifuged at 8,500 g for 20 minutes at 4°C. Clear ascitic fluids were collected and stored at -20°C.

2.3 Purification of anti-CD147 mAbs from ascitic fluids

To purify the anti-CD147 mAbs from ascitic fluids, affinity chromatography was used. Anti-mouse IgM coated Sepharose column was used to purify mAbs of IgM isotype and Protein A coated Sepharose column was used to purify mAbs of IgG isotype.

Four hundred microliters of ascitic fluid were clarified by centrifugation at 12,000 g 4°C for 20 minutes. The clarified ascitic fluid was then diluted with filtrated phosphate buffered saline (PBS) at a ratio of 1:5. For purification of IgM isotype, anti-mouse IgM coated Sepharose column was equilibrated with 50 ml PBS and the solution was then adjusted to bed volume. Then, 2 ml of the diluted ascitic fluid were added to the column and incubated for 5 minutes. The unbound proteins were removed by washing with 50 ml PBS. To elute antibodies from the column, 2 ml of elution buffer (0.2M glycine, pH 2.8) 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 (2 M Tris-HCl, pH 8.0). The column was then washed with 50 ml PBS, retained in 0.1% azide-PBS and stored at 4°C.

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For purification of IgG isotype, Protein A coated Sepharose column was equilibrated with 50 ml of 20 mM sodium phosphate and adjusted the solution to the bed volume. Then, 2 ml of the diluted ascitic fluid 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 (2 M 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 $4^{\circ}C$.

The obtained eluates were concentrated by speed vacuum system and dialyzed against PBS overnight by microdialyzer system. The absorbance of antibody preparation was determined at 280 nm and protein concentration was calculated using the following equations:

For IgM:

Concentration of antibody (mg/ml) = (O.D at 280 / 1.2)

For IgG:

Concentration of antibody (mg/ml) = (O.D at 280 / 1.35)

2.4 Measurement of activity and specificity of the purified anti-CD147 mAbs

To confirm that the purified antibodies still have specific activity in binding to CD147 molecule, the purified antibodies were used to stain U937 cell line and CD147 expressing COS cells by indirect immunofluorescent method.

2.4.1 Measurement of activity of the purified mAbs using U937 cell line

U937 cells were grown in RPMI-1640 medium supplemented with 10% FCS. To prove the activity of purified antibodies, cells were removed from tissue culture flask and washed 3 times with PBS. Then, cells were counted and adjusted to 1×10^7 cells/ml with 1% BSA-PBS-NaN_3. To block Fc receptor, human AB serum was added to the cell suspension at the ratio of 1:10 and the cells were incubated on ice for 30 minutes. Fifty microliters of 20 μg/ml purified antibodies in 1% BSA-PBS-NaN_3 were added into the 50 μl of blocked cells and incubated on ice for 30 minutes. Cells were washed twice with 1% BSA-PBS-NaN_3 and resuspended with 20 μl

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1% BSA-PBS-NaN_3. Subsequently, twenty five microliters of FITC-conjugated $F(ab')_2$ fragment of sheep anti-mouse immunoglobulins antibody were added and incubated on ice for 30 minutes. Finally, cells were washed 3 times with 1% BSA-PBS-NaN_3 and fixed with 1% paraformaldehyde-PBS. The fluorescent reactivity of the stained cells was analyzed by a flow cytometer (FACSCalibur).

2.4.2 Measurement of the specificity of the purified mAbs by using CD147 expressing COS cells

2.4.2.1 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 removed 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. Cells were washed twice with MEM medium and counted. Cells were then resuspended and transferred into 6 cm tissue culture dishes with the concentration of $1x10^6$ cells in 4 ml of 10% FCS-MEM. Dishes were incubated overnight at 37°C in 5% CO_2 incubator.

2.4.2.2 COS cell transfection

The medium was removed from the COS cell dishes and the cells were washed with 4 ml of MEM medium. Then, COS cells were transfected with 2 ml of MEM containing 250 µg/ml DEAE-Dextran, 400 µM chloroquine diphosphate and 2 µg cDNA encoding CD147 protein (M6-DNA) or cDNA encoding CD8 protein (CD8-DNA). Cells were incubated for 3 hours at 37°C in 5% CO_2 incubator. Then, the transfection solution was removed and cells were treated with 2 ml of 10% DMSO-PBS for 2 minutes at room temperature. After that, the solution was removed and washed with 3 ml of MEM medium. Finally, Cells were grown with 10% FCS-MEM for overnight at 37°C in 5% CO_2 incubator. The medium was replaced in next day. The transfected COS cells were cultured for another 2 days to allow expression of the corresponding proteins.

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2.4.2.3 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 1% BSA-PBS-NaN_3. Cells were counted and adjusted to $1x10^7$ cells/ml with 1% BSA-PBS-NaN_3. Fifty microliters of 20 µg/ml purified antibodies in 1% BSA-PBS-NaN_3 were added into the 50 µl of $1x10^7$ cells/ml transfected COS cells and incubated on ice for 30 minutes. Then, cells were washed twice with 1% BSA-PBS-NaN_3 and resuspended with 20 µl of 1% BSA-PBS-NaN_3. Twenty five microliters of FITC-conjugated $F(ab')_2$ fragment of sheep anti-mouse immunoglobulins antibody were subsequently added and incubated on ice for 30 minutes. Finally, cells were washed 3 times with 1% BSA-PBS-NaN_3 and fixed with 1% paraformaldehyde-PBS. The fluorescence reactivity on the cell surface was analyzed by a fluorescent microscope (Olympus; Tokyo, Japan).

2.5 Conjugation of purified anti-CD147 mAbs with fluorescein isothiocyanate (FITC)

To study the epitope mapping of anti-CD147 mAbs, purified anti-CD147 mAbs were conjugated with FITC and used in the cross-blocking experiments.

For IgG isotype, the purified anti-CD147 mAbs were adjusted to the concentration of 2 mg/ml in 0.1M $NaHCO_3$-PBS. For IgM isotype, the purified anti-CD147 mAbs were adjusted to the concentration of 1 mg/ml in PBS. Then, a volume of 10 mg/ml FITC in DMSO was added to the purified antibodies. The added volume of FITC solution was calculated by the following equations:

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ml of 10 mg/ml FITC in DMSO =

[(mg antibody x 0.1) / MW of antibody] x (R x MW of FITC)

R = molar incubation ratio of FITC : antibody.

R = 10 for IgM isotype and R = 20 for IgG isotype, were used in this study.

The FITC solution was slowly added into antibody with continuously mixed. The mixture was incubated for 90 minutes at room temperature. After that, the solution was dialyzed

with PBS by microdialyzer system. Finally, FITC-conjugated antibodies were measured at O.D 495 and 280 nm. The concentrations of FITC-conjugated antibodies were determined as described in 2.3.

2.6 Titration of FITC-conjugated anti-CD147 mAbs for epitope mapping

To obtain the optimal concentration of FITC-conjugated anti-CD147 mAbs, U937 cells were stained with various concentrations of FITC-conjugated anti-CD147 mAbs and was analyzed by indirect immunofluorescence method.

U937 cell line was grown in RPMI-1640 medium supplemented with 10% FCS. Cells were then removed from the tissue culture flask and washed 3 times with PBS. Then, the cell number was counted and adjusted to $1x10^7$ cells/ml with 1% BSA-PBS-NaN_3. To block Fc receptor, human AB serum was added to the suspension at the ratio of 1:10 and the cells were incubated on ice for 30 minutes. Twenty five microliters of 1% BSA-PBS-NaN_3 were added into the 50 µl of blocked cells followed by incubation on ice for 30 minutes. After that, twenty-five microliters of various concentrations of FITC-conjugated anti-CD147 mAbs in 1% BSA-PBS-NaN_3 were added and incubated on ice for 30 minutes. Finally, cells were washed 3 times with 1% BSA-PBS-NaN_3 and fixed with 1% paraformaldehyde-PBS. The stained cells were analyzed by a flow cytometer. The obtained fluorescent intensity of each sample was used to determine the optimal concentration of FITC labeled antibodies for further epitope mapping experiments.

2.7 Production of plasmid DNA encoding domain 1 or domain 2 of CD147 molecule

To produce plasmid DNA encoding domain 1 or domain 2 of CD147 molecule, the plasmid DNA encoding domain 1 or domain 2 of CD147 were transformed into *E. coli*. The plasmid DNA was then isolated using QIAGEN plasmid midi kit.

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2.7.1 Transformation of plasmid DNA into *E. coli*

Two microliters of cDNA encoding domain 1 or domain 2 of CD147 molecule were added to 100 µl of competent *E.coli* (MC1061/P3) in transformation tubes and incubated for 40 minutes on ice. Then, *E. coli* were shocked at 42°C for 40 seconds and further incubated on ice for 2 minutes. After that, 900 µl of LB broth were added into the transformation tubes and the

tubes were shaken at 200 rpm for 1 hour at 37°C. Transformed *E.coli* were spread on LB agar containing 15 μg/ml of ampicillin and 10 μg/ml of tetracycline. Plates were incubated overnight at 37°C.

2.7.2 Isolation of plasmid DNA

The transformed *E.coli* colonies were selected from cultured plates and grown in 5 ml of LB broth containing 15 μg/ml of ampicillin and 10 μg/ml of tetracycline. The bacterial cells were incubated in a 37°C incubator-shaker at 200 rpm overnight. Then, bacterial cells were harvested from 3 ml of cultured medium by centrifugation at 13,000 g for 1 minutes at room temperature. The bacterial pellets were resuspended in 300 μl of P1 buffer (QIAGEN plasmid mini kit). Three hundred microliters of P2 buffer (QIAGEN plasmid mini kit) were added, mixed and incubated for 5 minutes at room temperature. Then, 300 μl of P3 buffer (QIAGEN plasmid mini kit) were added to the suspended bacteria, mixed and incubated on ice for 5 minutes. The suspended bacteria were centrifuged at 13,000 g for 10 minutes at room temperature. Supernatant containing plasmid DNA was collected.

Before applying the supernatant, QIAGEN–tip 20 column (QIAGEN plasmid mini kit) was equilibrated with 1 ml of QBT buffer and allowed to empty by gravity flow. Then, supernatant-containing plasmid DNA was applied into the QIAGEN–tip 20 column and allowed to empty by gravity flow. The column was washed twice with 2 ml of QC buffer and allowed to empty by gravity flow. The plasmid DNA was eluted with 800 μl of QF buffer (QIAGEN plasmid mini kit), and collected into a new microtube. Five hundred and sixty microliters of isopropanol was added to the eluate and the mixture was centrifuged at 7,500 g for 30 minutes at room temperature. Then, the solution was removed from DNA pellet. One milliliter of 70% ethanol was added to the DNA pellet and centrifuged at 7,5000 g for 10 minutes at room temperature. After that, the solution was removed from DNA pellet and the DNA pellet was allowed to dry by air-dry. Finally, DNA pellet was resuspended with 100 μl of autoclaved water. 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 nm x 50

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2.7.3 Characterization of isolated plasmid DNA

Ten microliters of *Xba* I enzyme solution or buffer solution was added to 10 μl of 100 μg/ml of plasmid DNA and incubated for 2 hours at 37°C in a water bath. Then, 4 μl of 6X loading buffer was added to the digested plasmid DNA. The solution was incubated for 5 minutes at 65°C in water bath and incubated on ice for more than 10 minutes. Subsequently, the solution was loaded in 1% agarose gel containing EtBr and separated at 120 volt. Gel was destained with distilled water and the DNA bands were observed by UV transilluminator.

2.7.4 Large-scale production of plasmid DNA

The transformed *E.coli* were grown in 5 ml of 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 500 ml of LB broth and the mixture was shaken at 200 rpm overnight at 37°C. The bacterial cells were harvested by centrifugation at 2,400 g for 30 minutes at 4°C. The bacterial pellets were then resuspended in 20 ml of P1 buffer. Twenty milliliters of P2 buffer was added to the suspended bacteria, mixed and incubated for 5 minutes at room temperature. Then, 20 ml of P3 buffer was added, mixed and incubated on ice for 30 minutes. The solution was mixed several times during incubation. The bacteria suspension was centrifuged at 2,400 g for 30 minutes at 4°C. Supernatant containing plasmid DNA was collected to 50 ml tube by filtration with filter paper. Then, 0.7 volumes of isopropanol was added to the supernatant and centrifugation at 15,000 g for 30 minutes at 4°C. The supernatant was removed and the DNA pellet was allowed to dry.

The DNA pellet was re-dissolved with 500 μl of TE buffer and adjusted to the final volume of 5 ml with QBT buffer. Before applying of plasmid DNA, QIAGEN–tip 100 column (QIAGEN plasmid midi kit) was equilibrated with 4 ml of QBT buffer and allowed to empty by gravity flow. Then, plasmid DNA was applied to the column and allowed to empty by gravity flow. The column was washed 3 times with 10 ml of QC buffer and allowed to empty by gravity flow. The plasmid DNA was eluted with 5 ml of QF buffer and collected in a 15 ml tube.

For precipitation of DNA, 3.5 ml of isopropanol was added to the eluate followed by centrifugation at 15,000 g for 30 minutes at 4°C. Then, the solution was removed from DNA pellet. Ten milliliters of 70% ethanol was added to the DNA pellet for washing. After

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centrifugation at 15,000 g for 30 minutes at 4°C, the supernatant was removed from the DNA pellet. The DNA pellet was allowed to dry for 10 minutes. Finally, DNA pellet was resuspended with 100 µl of autoclaved water. The concentration of plasmid DNA was measured as described in 2.7.2.

2.8 Epitope mapping

To determine the epitope on CD147 molecule, cross-blocking analysis was carried out.

2.8.1 Epitope mapping of anti-CD147 mAbs by cross-blocking analysis using U937 cell line

U937 cells were grown in RPMI-1640 medium supplemented with 10% FCS. Cells were removed from tissue culture flask and washed 3 times with PBS. Then, cells were counted and adjusted to $1x10^7$ cells/ml with 1% BSA-PBS-NaN_3. To block Fc receptor, human AB serum was added to the cell suspension at the ratio of 1:10 and incubated on ice for 30 minutes. Twenty five microliters of 160 µg/ml un-labeled anti-CD147 mAbs in 1% BSA-PBS-NaN_3 were added to the 50 µl of blocked cells and incubated on ice for 30 minutes. After that, twenty five of 160 µg/ml FITC-conjugated anti-CD147 mAbs in 1% BSA-PBS-NaN_3 were added and the cells were incubated on ice for another 30 minutes. Finally, cells were washed 3 times with 1% BSA-PBS-NaN_3 and fixed with 1% paraformaldehyde-PBS. The stained cells were analyzed by a FACSCalibur flow cytometer. Fluorescent intensity of each tube was compared and the percentage of inhibition was calculated.

2.8.2 Epitope mapping of anti-CD147 mAbs using domain 1 or domain 2 of CD147 expressing COS cells

2.8.2.1 COS cell transfection

Preparation of COS cells for transfection was performed as described in 2.4.2.1. COS cells were then transfected with plasmid DNA encoding domain 1 or 2 of CD147 molecule or plasmid DNA encoding intact CD147 molecule (CD147-DNA), or plasmid DNA encoding CD8 molecule (CD8-DNA) by DEAE-dextran transfection as described in 2.4.2.2.

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2.8.2.2 Intracellular staining of transfected COS cells by indirect immunofluorescent method

After 3 days of COS cell transfection, the culture medium was removed and 0.5 mM EDTA-PBS was added into the culture dishes. Cells were incubated for 10 minutes at room temperature. Then, the cells were removed and collected into a 15 ml tube. Cells were washed 3 times with PBS, counted and adjusted to $5x10^6$ cells/ml with PBS. Then, 10 µl of COS cells were smeared on glass slides and dried at room temperature. Slides were fixed with cold acetone for 20 minutes and kept at room temperature until dry. Fixed slides were washed 3 times with PBS. Then, 50 µl of 20 µg/ml purified antibodies in 1% BSA-PBS-NaN_3 were added to the smear and incubated for 1 hour at 37°C in moist chamber. Slides were then washed 3 times with PBS. Fifty microliters of FITC-conjugated sheep anti-mouse immunoglobulins antibody were added onto the smear and incubated for 1 hour at 37°C in moist chamber. Slides were washed 3 times with PBS. Finally, 7 µl of PBS was added and the smear was covered with cover slip. The stained cells were analyzed by a fluorescent microscope.

2.9 Characterization of anti-CD147 mAbs by western blotting

2.9.1 Preparation of cell lysate

The U937 cell line was grown in RPMI-1640 medium supplemented with 10% FCS. Cells were removed from tissue culture flask and washed 3 times with PBS. To prepare cell lysate, cells were counted and adjusted to $5x10^7$ cells in 1 ml of lysis buffer (see Appendix), and incubated on ice for 30 minutes. After that, cells were transferred to pre-cooled microcentrifuge tubes and centrifuged at 12,000 g 4°C for 30 minutes. Finally, the cell lysates were collected and stored on ice.

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2.9.2 Protein separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotting

Non-reducing buffer (see Appendix) or reducing buffer (see Appendix) was added to the cell lysates. The samples were boiled for 5 minutes and then kept on ice. The protein components were separated by SDS-PAGE using 12.5% separating gel and 4% stacking gel at

120 volts (constant volt) and the proteins were transferred onto a nitrocellulose membrane by semi-dry electrophoretic blotting system at 40 mA (constant ampere) for 2 hours.

2.9.3 Immunodetection

The membrane was blocked with 5% skimmed milk in PBS overnight at 4°C and subsequently for an hour at room temperature. The blocked membrane was rinsed twice with PBS. Anti-CD147 mAbs or control antibodies (10 μg/ml in 1% skimmed milk-0.1% PBS-Tween 20) were added and incubated for 1 hour at room temperature. Then, the membrane was washed 5 times with 0.1% PBS-Tween 20 and a solution of peroxidase conjugated rabbit anti-mouse immunoglobulins antibodies at the dilution of 1:5,000 in 1% skimmed milk-0.1% PBS-Tween 20 was added. The membrane was incubated for 1 hour at room temperature. After that, the membrane was washed 3 times with 0.1% PBS-Tween 20 and twice with PBS. The reaction was then visualized by the chemiluminescence detection system. Briefly, the membrane was incubated with peroxide-luminol/enhancer solution for 5 minutes at room temperature. Then, the membrane was wrapped with enwrap and exposed with a light sensitive clear blue X-ray film (CL-X Posure™ Film). Finally, the film was developed with Kodak GBX solution.

2.10 Characterization of anti-CD147 mAbs by immunoprecipitation

2.10.1 Biotinylation and preparation of cell lysate

The U937 cell line was grown in RPMI-1640 medium supplemented with 10% FCS. Cells were removed from tissue culture flask and washed 3 times with PBS. Then, the cells were counted and adjusted to $5x10^7$ cells in 1 ml of 5 mM Sulfo-NHS-LC-biotin in PBS and incubated for 1 hour on ice. To stop the reaction, 1 mM glycine-PBS 50 ml were added and centrifuged at 600 g 4°C for 5 min. After that, cells were washed twice with cold-PBS and adjusted to $5x10^7$ cells in 1 ml of lysis buffer and incubated on ice for another 30 min. Finally, the cells were transferred to pre-cooled microcentrifuge tubes and subjected to centrifugation at 12,000 g 4°C for 30 minutes. Cell lysates were collected and stored on ice.

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2.10.2 Preclearing lysates

Rabbit-anti-mouse immunoglobulin antibodies were diluted with coating buffer at dilution 1:30 and added to 96 wells flat-bottom microplate at 100 μl/well. Plate was incubated for 1 hour at 37°C in moist chamber. Then, the wells were washed 3 times with PBS and 100 μl of 1:10 diluted normal mouse serum in PBS were added into the coated wells. The plate was incubated for 1 hour at 37°C in moist chamber. After that, the solution was removed and 120 μl of 2.5% bovine serum albumin (BSA) in PBS were added and the plate was incubated for another 1 hour at 37°C in moist chamber. Finally, the wells were washed 3 times with cold TRIS-lysis buffer. Then 100 μl of Biotinylated cell lysate were added to each well and the plate was incubated overnight at 4°C in moist chamber. After incubation, the precleared lysates were collected and stored on ice.

2.10.3 Immunoprecipitation

Rabbit anti-mouse immunoglobulins antibodies were diluted with coating buffer at the dilution of 1:25 and added to 96 wells of flat-bottom plate at 50 μl/well. The plate was incubated for 1 hour at 37°C in moist chamber. Then, the wells were washed 3 times with PBS and 50 μl of 20 μg/ml anti-CD147 mAbs or control antibodies were added. After an overnight incubation at 4°C in moist chamber, the wells were washed 3 times with cold-PBS and 60 μl of 2.5% BSA-PBS were added. The plate was incubated for 1 hour at 37°C in moist chamber. Subsequently, three washes were performed using TRIS-lysis buffer and 50 μl of percleared lysate were added followed by incubation for 3 hours at 4°C in moist chamber. The precipitated wells were washed 10 times with cold TRIS-lysis buffer. To elute out the precipitated protein, 50 μl of non-reducing or reducing buffer was added into the precipitated wells, mixed and the solution was collected. The solution was boiled for 5 minutes and kept on ice. The biotinylated-precipitated proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane by semi-dry electrophoretic blotting system as described in 2.9.2.

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2.10.4 Chemiluminescence detection

The membrane was blocked with 5% skimmed milk in PBS for 4°C overnight and for 1 hour at room temperature. Then, the blocked membrane was rinsed twice with PBS and

incubated with avidin-peroxidase conjugate at the dilution of 1:5,000 in PBS for 1 hour at room temperature. After that, the membrane was washed 3 times with 0.1% PBS-Tween 20 and twice with PBS. Finally, the biotinylated proteins were visualized by the chemiluminescence detection system as described in 2.9.3.

2.11 Functional study of CD147 molecule

2.11.1 Induction of apoptosis by anti-CD147 mAbs

The U937, KG1a and Sup-T1 cell lines were grown in RPMI-1640 medium supplemented with 10% FCS. Cells were removed from tissue culture flasks and washed twice with RPMI-1640 medium. Then, the washed cells were counted and adjusted to $5x10^6$ cells/ml with 10% FCS-RPMI-1640 medium. One hundred microliters of the washed cells were added to 96 well flat-bottom microplate. One hundred microliter of 20 μg/ml of anti-CD147 mAb or 10% FCS- RPMI-1640 medium were added to each well. The cultured plate was incubated for 4 hours at 37°C in 5% CO_2 incubator. After that, the activated cells were collected from the cultured plate. The number of apoptotic cells were measured by Annexin V-FITC Apoptosis detection kit. Briefly, cells were washed twice with cold-PBS and resuspended with 300 μl of Annexin V binding buffer (Apoptosis detection kit). Then, 100 μl of resuspended cells were transferred to 12x17 mm tube. Five microliter of Annexin V-FITC and 5 μl of PI were added and incubated for 15 minuets at room temperature in dark place. Then, 400 μl of Annexin V binding buffer were added and the apoptotic cells were analyzed by a FACSCalibur flow cytometer.

2.11.2 Study the effect of anti-CD147 mAbs on cell proliferation

2.11.2.1 Titration of anti-CD3 mAb for induction of T cell proliferation

2.11.2.1.1 Immobilization of anti-CD3 mAb

On the day before T cell proliferation assay, a 96 well flat-bottom microplate was pre-coated with sterile anti-CD3 mAb (OKT3 mAb) in PBS at concentration of 3.9 ng/ml to 4 μg/ml in 100 μl of sterile PBS. The plate was incubated overnight at 4°C in moist chamber. After that, the anti-CD3 mAb coated plate was washed 3 times with sterile PBS and 120 μl of sterile 1% BSA-PBS were added to each individual well and incubated for 1 hour at 37°C in the 5% CO_2

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incubator. Finally, the immobilized anti-CD3 mAb plate was washed 3 times with sterile PBS. The plate is ready for T cell proliferation assay.

2.11.2.1.2 Preparation of peripheral blood mononuclear cells

PBMC were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation. Briefly, 10 ml of heparinized blood was diluted with 30 ml of sterile PBS in 50 ml tube. Then, 5 ml of sterile Ficoll-Hypaque solution was underlayered and centrifuged at 400 g for 30 minutes at 25°C. The PBMC were collected and washed 3 times with RPMI-1640 at 600 g for 10 minutes at 25°C. Finally, PBMC were counted and adjusted to $1x10^6$ cells/ml with 10% FCS-RPMI-1640 medium.

2.11.2.1.3 T cell proliferation assay

One hundred microliter of 10% FCS-RPMI-1640 medium was added to anti-CD3 mAb coated plate. Then, 100 µl of $1x10^6$ cells/ml PBMC in 10% FCS-RPMI-1640 medium were added to each well and incubated for 3 days at 37°C in the 5% CO_2 incubator. After that, 50 µl of 20 µCi/ml ^{3}H-thymidine were added to each well and incubated for 18 hours at 37°C in the 5% CO_2 incubator. PBMC were collected from the cultured plate with the cell-harvester. The radioactive incorporation was measured by a liquid scintillation counter.

2.11.2.2 Functional study of CD147 molecule involving the regulation of anti-CD3 mAb induced cell proliferation

2.11.2.2.1 Immobilization of anti-CD3 mAb

On the day before T cell proliferation assay, 96 well flat-bottom microplate was pre-coated with 100 µl of sterile PBS or 20 ng/ml of sterile anti-CD3 mAb in PBS. Then, the plate was incubated overnight at 4°C in moist chamber. After washing the coated plate 3 times with sterile PBS, 120 µl of 1% sterile BSA-PBS was added and the plate was incubated for 1 hour at 37°C in the 5% CO_2 incubator. Finally, anti-CD3 mAb coated plate was washed 3 times with sterile PBS and was ready for T cell proliferation assay.

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2.11.2.2.2 Functional study of CD147 molecule involving the regulation of anti-CD3 mAb induced cell proliferation

One hundred microliters of 0.1, 0.2 and 2 µg/ml of anti-CD147 mAbs in 10% FCS-RPMI-1640 medium or isotype matched control mAbs or 10% FCS-RPMI-1640 medium were added to anti-CD3 mAb-coated plate. Then, 100 µl of $1x10^6$ cells/ml PBMC in 10% FCS-RPMI-1640 medium were added to each well. The plate was incubated for 3 days at 37°C in the 5% CO_2 incubator. After that, 50 µl of 20 µCi/ml ^{3}H-thymidine were added to each well and the mixture was incubated for 18 hour at 37°C in the 5% CO_2 incubator. Finally, PBMC were collected from cultured plate by the cell harvester. Radioactive incorporation was measured by the liquid scintillation counter.

2.11.2.2.3 Titration of PHA for induction of T cell proliferation

One hundred microliter of various concentrations (1.25 to 10 µg/ml) of PHA in 10% FCS-RPMI-1640 medium or 10% FCS-RPMI-1640 medium alone were added to 96 well flat-bottom microplate. Then, 100 µl of $1x10^6$ cells/ml PBMC in 10% FCS-RPMI-1640 medium were added into each well and incubated for 3 days at 37°C in 5% the CO_2 incubator. To each well, 50 µl of 20 µCi/ml ^{3}H-thymidine were added and incubated for 18 hour at 37°C in the 5% CO_2 incubator. Finally, PBMC were collected from the cultured plate by the cell harvester. Radioactive incorporation was measured by a liquid scintillation counter

2.11.2.2.4 Functional study of CD147 molecule involving the regulation of PHA induced cell proliferation

One hundred microliters of 0.1, 0.2 and 2 µg/ml of anti-CD147 mAbs in 10% FCS-RPMI-1640 medium or isotype matched control mAbs or 10% FCS-RPMI-1640 medium alone were added into 96 well flat-bottom microplate. Then, 50 µl of 2.5 µg/ml of PHA in 10% FCS-RPMI-1640 medium or 10% FCS-RPMI-1640 medium were added. Then, 50 µl of $2x10^6$ cells/ml PBMC in 10% FCS-RPMI-1640 medium were added into each well. Plate was incubated for 3 days at 37°C in 5% CO_2 incubator. To each well, 50 µl of 20 µCi/ml ^{3}H-thymidine was added and incubated for 18 hours at 37°C in the 5% CO_2 incubator. Finally,

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PBMC were collected from the cultured plate by the cell harvester. Radioactive incorporation was measured by the liquid scintillation counter.

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