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 Preparation of immunogen

2.2.1 Immunoprecipitated-bead technique

2.2.1.1 Biotinylation and preparation of cell lysates

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation. Briefly, 20 ml of heparinized blood was diluted with 20 ml of PBS in 50 ml tube. Then, 10 ml of Ficoll-Hypaque solution was underlayered and centrifuged at 400 g, 25°C for 30 minutes. The PBMCs were collected and washed three times using PBS. For biotin labeling, the obtained cells were adjusted to a final concentration of 2×10^7 cells in 1 ml of 5 mM Sulfo-NHS-LC-biotin in PBS and incubated on ice for 1 hour. To stop biotinylation, the cells were quenched by washing once with 1 mM glycine in PBS and then twice with cold-PBS. The 5×10^7 biotinylated cells were lysed on ice for 30 minutes in 1 ml of lysis buffer (50 mM Tris-HCl pH 8.2, 100 mM NaCl, 2 mM EDTA, 0.02% NaN_3) containing 1% NP-40 as detergent and protease inhibitors (1mM phenylmethyl-sulphonylfluoride (PMSF), 5 mM iodoacetamide, 10 µg/ ml aprotinin). The clarified cell lysates were collected by centrifugation at 12,000 g, 4°C for 30 minutes.

2.2.1.2 Preclearing of cell lysates

Five hundred microliters of protein A sepharose 4B beads were coated with 100 µl of normal mouse serum. After rotation at 25°C for 3 hours, the beads were washed five times with PBS. Then, 1 ml of the biotinylated cell lysates were added into the serum coated beads and rotated at 4°C for overnight. The precleared cell lysates were collected by centrifugation at 12,000 g, 4°C for 5 minutes.

2.2.1.3 Immuniprecipitation

One milliliter of precleared cell lysates were added to CD4 mAb-coated beads and rotated at 4°C overnight. After ten times washing with lysis buffer, the protein complexes were dissociated from the beads by boiling in 50 µl of non-reducing or reducing SDS-PAGE sample buffer (see Appendix) for 5 minutes. The precipitated proteins were separated by SDS-PAGE using 10% separating gel and 4% stacking gel at 120 volts (constant volt) and subsequently transferred to a nitrocellulose membrane by semi-dry electrophoretic blotting system at 40 mA (constant ampere) for 2 hours.

The membranes were blocked with 5% BSA in PBS at room temperature for 1 hour. Then, the blocked membranes were rinsed twice with PBS and incubated with horseradish peroxidase (HRP) conjugated streptavidin (1:20,000) in PBS containing 2 % BSA at room temperature for 1 hour. Thereafter, the membranes were washed three times with washing buffer (PBS containing 0.1%Tween 20) and twice with PBS. The proteins were visualized by the chemiluminescence detection system.

2.2.1.4 Chemiluminescence detection system

The membranes were incubated with peroxide-luminol/enhancer solution for 5 minutes at room temperature. Then, the membranes were wrapped with enwrap and

exposed with a light sensitive clear blue X-ray film (CL-X Posure Film). Finally, the films were developed with Kodak GBX solution.

2.2.1.5 Preparation of immunoprecipitate-beads for immunization

To prepare immunoprecipitate-beads for immunization, the unlabeled PBMC lysates were prepared as was described in 2.1.1.1 and subjected for immunoprecipitation procedure according to 2.1.1.3. The precipitated beads were washed 10 times with sterile PBS and resuspened in 500 µl of sterile PBS after overnight incubation. The precipitated beads were then used as the immunogen for mouse immunization.

2.2.2 CD4-BCCP bead technique

2.2.2.1 Amplification of CD4 gene by PCR

The polymerase chain reaction (PCR) was performed to amplify CD4 coding sequence. Plasmid vector containing full length CD4 coding sequence, designed CD4-DNA, was kindly provided by Prof. Dr. H. Stockinger, Medical University of Vienna, Austria and served as template for the amplification. Primers which designed for amplification of the CD4 insert and simultaneously add appropriate sites for restriction endonucleases at either ends of the target sequence. The forward primer contained a restriction site for *Nde* I (5' GAG GAG GAG CTC ATA TGA AGA AAG TGG TC TGG GC 3'), while the reverse primer (5' GAG GAG GAG CTG AAT TCC TCA GCA GAC ACT GCC AC 3') contained a *EcoR* I restriction site. CD4 cDNA was annealed with 250 ng of each described primer in 100 µl of amplification reaction mixture containing of 25 ng CD4-DNA , 5U ProofStart DNA polymerase, 0.3 mM of

each dNTP and ProofStart amplification buffer with 1 mM $MgSO_4$. The PCR cycling condition starts as one cycle at 95 °C for 5 minutes followed by 34 cycles of denaturation at 94 °C for 50 seconds, annealing at 50 °C for 50 seconds, and extension at 72 °C for 1 minute. After 35 amplification cycles, the mixture was incubated at 72 °C for 10 minutes. The amplified product was then analyzed by 1 % agarose gel electrophoresis. Briefly, the amplified product was loaded in 1% agarose gel and separated at 120 volt. In order to visualize the DNA in the agarose gel, the gel was stained with 1% ethidium bromide (EtBr) for 20 minutes. Then the gel was destained with distilled water for 5 minutes. Consequently, the DNA bands were observed by UV transilluminator.

2.2.2.2 Construction of plasmid encoding CD4-BCCP

To isolate the desired PCR product from nonspecific amplification products, the reaction products were separated in an agarose gel as describe in 2.2.2.1.The DNA fragment of 1100 bp was cut out of the gel using a new razor blade and was then purified using QIAquick Gel Extraction kit in accordance with the recommended protocol. Subsequently, 1.5 µg of purified PCR product and 5 µg of pAK400CB vector (previously described by Santala and Lamminmaki, 2004) were digested with 30 U of restriction enzymes *Nde* I and *EcoR* I at 37 °C for 18 hours. The digested PCR product was purified by using QIAquick PCR purification kit, following the manufacturer's instruction, whereas the digested vector of 4 kb was purified using QIAquick Gel Extraction kit. The purified products were proved by agarose gel electrophoresis.

The digested PCR products were cloned into the digested pAK400CB vector using T4 DNA ligase. Five units of T4 ligase was added to the ligation mixture

containing 100 ng of digested amplified PCR product and 128 ng of digested vector. The ligation mixture was then incubated at 4 °C for 18 hours. The produced plasmid vector was named pAK400CB-CD4.

2.2.2.3 Transformation

The pAK400CB-CD4 was incubated with 200 µl of competent *E. coli* XL-1 Blue on ice for 1 hour. Then, *E. coli* were shocked at 42°C for 90 seconds and further incubated on ice for 1 minute. The transformed *E. coli* were cultured in 3 ml of non-antibiotic Luria Bertani (LB) broth with 120 rpm shaking at 37°C for 3 hours. Then, the cells were collected by centrifugation at 450 g, 25°C for 10 minutes. The transformed *E. coli* were then spreaded on LB agar containing 25 µg/ml chloramphenicol and 10 µg/ml of tetracycline. Subsequently, the plates were incubated for overnight at 37°C.

2.2.2.4 Plasmid preparation by alkaline lysis method

The transformed *E. coli* colonies were selected from the cultured plates and inoculated in 3 ml of LB broth containing 50 µg/ml chloramphenicol. The bacterial cells were incubated in a 37°C shaking-incubator at 200 rpm overnight. On the next day, 1.5 ml of bacterial suspension were added into the microcentrifuge tube and centrifuged at 13,000 g, room temperature for 1 minute. One hundred microliters of precooled 1x glucomix-lysozyme were added into the bacterial pellet and resuspended by vortexing. Then, Two hundred microliters of freshly prepared NaOH/SDS were added and mixed by inverting the tube rapidly 2-3 times. Afterward, 150 µl of potassium acetate were added and gently mixed by vortex. The solutions were centrifuged 10,000 g at 4°C for 5 minutes and the supernatants were collected and transferred to the new microcentrifuge tubes. Nine hundred microliters of analytical

grade absolute ethanol were added into the tubes and kept on ice for 2 minutes. The DNA was spun down at 10,000 g, 4°C for 5 minutes and the supernatants were discarded. The DNA pellets were reconstituted by adding 100 µl of sterile DW, followed by adding 50 µl of 7.5 M ammonium acetate, and incubated at -70 °C for 10 minutes. After centrifugation at 10,000 g, 4°C for 5 minutes, the supernatants were collected and transferred to the new microcentrifuge tubes. Subsequently, 300 µl of absolute ethanol were added to the supernatant and incubated at -70 °C for 10 minutes. The solutions were spun down and the pellets were cleaned up with 1 ml of 70% ethanol by centrifugation at 10,000 g, 4°C for 5 minutes. The DNA pellets were dried at 37°C and reconstituted with 30 µl of sterile DW and store -20°C.

To verify the inserted gene in the purified plasmid DNA, the purified plasmid from individual clone was characterized by restriction fragment analysis with restriction enzymes, *Nde* I and *EcoR* I, reamplification by PCR and DNA sequencing.

2.2.2.5 Expression of biotinylated CD4-BCCP protein

The plasmid DNA were transformed into *E. coli* strain Origami B according to the described protocol in 2.2.2.3. The transformed *E. coli* were then selected on LB agar containing 25 µg/ml chloramphenicol and 10 µg/ml of tetracycline and 15 µg/ml kanamycin. To identify bacterial colonies that contain the pAK400CB-CD4 plasmid, the transformed *E. coli* colonies were grown in LB broth containing 10 µg/ml tetracycline, 25 µg/ml chloramphenicol and 15 µg/ml kanamycin. Then, the plasmids from individual clone were purified and characterized by restriction fragment analysis with restriction enzymes, *Nde* I and *EcoR* I, and reamplification by PCR.

E. coli Origami B containing the pAK400CB-CD4 plasmid were cultured in 10 ml SB medium (30 g/l tryptone, 15 g/l yeast extract, and 10 g/l MOPS, pH 7.0)

supplemented with 0.05% glucose and 4 μM biotin, 10 μg/ml tetracycline, 25 μg/ml chloramphenicol, 15 μg/ml kanamycin. The bacterial culture was shaken 300 rpm at 37°C until an optical density at the wavelength of 600 nm ($O.D._{600}$) reached 0.5. The bacterial suspension was subsequently transferred to 100 ml of the same media and was further shaken at 37°C. The culture was grown until the $O.D._{600}$ was 1.0. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 100 mM. The culture was incubated at 25°C, 180 rpm for 22 hours.

For protein extraction, the bacteria were harvested by centrifugation at 400 g, 4°C for 10 minutes. The cultured supernatant was discarded and the wet weight of bacterial pellet was determined. The cell pellet was resuspended in BugBuster Protein extracting reagent by gentle vortexing, using 5 ml reagent per gram of wet cell paste. The mixture was incubated at room temperature for 10-20 minutes with shaking at rpm. The insoluble cell debris was removed by centrifugation at 16,000 g, 4°C for 20 minutes. The clarified extract was collected. The bacterial extract containing biotinylated CD4-BCCP were stored at -70 °C until use.

2.2.2.6 Analysis of biotinylated CD4-BCCP protein

2.2.2.6.1 Detection of biotinylated CD4-BCCP protein by indirect ELISA

To determination biotinylated CD4-BCCP in bacterial extract, indirect ELISA was employed. Each wells of ELISA plate was coated with 50 μl of 10 μg/ml avidin or CD4 mAbs (Leu3a or MT4) in carbonate/bicarbonate buffer, pH 9.6 at 4 °C for 18 hours. The coated plates were blocked with blocking buffer (2% skimmed milk in PBS, pH 7.2) by incubation for 1 hour at room temperature. After four times washing with washing buffer (0.05% Tween 20 in PBS pH 7.2), 50 μl of various dilutions of the bacterial extract containing CD4-BCCP or CD147-147 in blocking buffer were

added. After 1 hour of incubation at room temperature, the plates were washed for four times using washing buffer follows by adding 50 µl of 10 µg/ml CD4 mAbs (Leu3a or MT4), CD147 mAb (M6-1B9) or BCCP mAb (BCCP-2). Then, the plates were incubated at room temperature for 1 hour. The antigen-antibody complexes were monitored by adding of 50 µl HRP-conjugated rabbit anti-mouse immunoglobulins at dilution 1:2000 or HRP-conjugated streptavidin at dilution 1:5000. After 1 hour of incubation and three times washing, 50 µl of tetramethylbenzidine (TMB) were added to each well and incubated at room temperature in the dark. The reaction was stopped by adding 100 µl of 1N HCl. The intensity of developed color was determined by measuring absorbance using ELISA reader at 450 nm.

2.2.2.6.2 Detection of biotinylated CD4-BCCP protein by Western blot

Bacterial extract containing CD4-BCCP or CD147-BCCP were separated by SDS–PAGE and transferred to nitrocellulose membrane by semi-dry blotter. The membranes were then blocked with 5% skimmed milk in PBS. The blocked membranes were incubated with CD4 mAbs or BCCP mAb. After five times washing with 0.1% Tween 20 in PBS, the membranes were incubated with HRP-conjugated rabbit anti-mouse immunoglobulins at dilution 1:5000 or HRP-conjugated streptavidin at dilution 1:20,000 for 1 hour at room temperature. Thereafter, the membranes were washed three times with 0.1% PBS-Tween 20 and twice with PBS. The reactive bands were visualized by the chemiluminescence detection system as was described in 2.2.1.4.

2.2.2.7 Magnetic sorting

To separate biotinylated CD4-BCCP from bacterial components, the magnetic sorting was performed. Five hundred microliters of bacterial extract were incubated with $2.5x10^8$ streptavidin-coated magnetic beads at room temperature for 30 minutes. After three times washing with sterile PBS, the beads were resuspend in 500 µl of sterile PBS. The obtained beads were named CD4-BCCP beads and stored at 4 °C. The CD4-BCCP beads were then used as immunogen for mouse immunization. The presences of CD4–BCCP on the beads were determined by direct and indirect immunofluorescence staining and flow cytometry analysis.

$2.5x10^6$ CD4-BCCP beads, CD147-BCCP beads or streptavidin-coated magnetic beads were stained with phycoerytrin (PE) or FITC labeled CD4 mAbs by direct immunofluorescence. After incubation at room temperature for 30 minutes in the dark, the beads were then washed for three times and resuspended in 500 µl PBS containing 1% paraformaldehyde. The fixed beads were further analyzed by FACSCalibur flow cytometer.

For indirect immunofluorescence staining, $2.5x10^6$ CD4-BCCP beads, CD147-BCCP beads or streptavidin-coated magnetic beads were incubated with 20 µg/ml of CD4 mAb and 20 ug/ml of BCCP mAb for 30 minutes at room temperature. After twice washing with 1% BSA in PBS/NaN_3, FITC-conjugated sheep anti-mouse immunoglobulins were added and incubated for 30 minutes at room temperature. The beads were then washed for three times and resuspended in 500 µl PBS containing 1% paraformaldehyde. The fixed beads were further analyzed by flow cytometer.

2.2.3 CD4-COS cell expression technique

2.2.3.1 Plasmid DNA preparation

cDNA encoding CD4 protein, which was inserted into an eukaryotic expression vector, πH3M (designated CD4-DNA) was kindly provided by Prof. Dr. H. Stockinger, Medical University of Vienna, Austria. The CD4-DNA were transformed into competent bacteria *E.coli* (MC1061/P3) by the same procedure as described in 2.2.2.3. The transformed *E.coli* were then selected on LB agar containing 15 μg/ml of ampicillin and 10 μg/ml of tetracycline.

To prepare a large amount of CD4-DNA, the MC1061/P3 *E.coli* containing CD4-DNA was 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. CD4-DNA was isolated by using QIAGEN plasmid maxi kits. After purification process, the concentration and the purity of the obtained DNA preparation was determined by measuring the absorbance at 260/280 nm by UV-spectrophotometer. The concentration of the plasmid DNA was determined by the following equation:

The concentration of plasmid DNA (μg/ml) = O.D. at 260 nm x 50

The obtained CD4-DNA was proved The obtained CD4-DNA were proved for the expression of CD4 protein by using COS cells expression system and indirect immunofluorescent staining using CD4 mAb MT4.

2.2.3.2 COS cell transfection

CD4-DNA were transfected into COS cells by the DEAE-dextran transfection method. A day before transfection, COS cells were harvested from culture flask using 0.5 mM EDTA-PBS. After twice washing with MEM medium, the cell concentration was adjusted to $1x10^6$ cells in 4 ml of 10% FCS-MEM, plated into 6-cm tissue culture dishes and cultured at 37°C in a 5% CO_2 incubator. The medium were discarded on the next day. Then, the cells were incubated in 2 ml of transfection solution (MEM containing 250 µg/ml DEAE-dextran, 400 µM chloroquine diphosphate and 2 µg cDNA encoding CD4 protein) for 3 hours at 37°C in 5% CO_2 incubator. Thereafter, the transfection solution was removed and the cells were treated with 2 ml of 10% DMSO-PBS for 2 minutes at room temperature. Then, the solution were discarded and washed once with 3 ml of MEM medium. After overnight incubation at 37°C in 5% CO_2 incubator, the medium was removed and 3 ml of 10% FCS-MEM was replaced. The transfected cells were cultured for another 2 days to allow expression of the CD4 proteins.

2.2.3.3 Determination of COS cells expressing CD4 proteins

To determine the percentage of COS expressing CD4 protiens after transfection, the transfected COS cells were stained with CD4 mAb by indirect immunofluorescence staining. At the third day after COS cells transfection, cells were removed from tissue culture dishes by incubation with 0.5 mM EDTA-PBS for 1-2 minutes. The cells were washed three times with PBS, counted and adjusted to a final concentration of $1x10^7$ cells/ml in 1% BSA-PBS-NaN_3. Fifty microliters of suspension cells were then incubated with 50 µl of 20 µg/ml CD4 mAb on ice for 30 minutes. After twice washing with IFS buffer, 25 µl of FITC-conjugated $F(ab')_2$ fragment of sheep anti-mouse immunoglobulins antibody were subsequently added

into the cell suspension and incubated on ice for 30 minutes. Finally, cells were washed three times with 1% BSA-PBS-NaN_3 and fixed with 1% paraformaldehyde-PBS. Membrane fluorescence was analyzed by fluorescence microscope. The percentage of CD4 expressing cells was determined.

2.2.3.4 Immunomagnetic cell sorting

To isolate COS cells expressing CD4 protein, two milliliters of $1x10^7$ cells/ml transfected COS cells in sterile PBS were incubated with 200 µl of CD4 mAb coated ferrous beads at room temperature for 30 minutes. The suspension cells were then placed on the magnetic field for 2 minutes. The supernatant were discarded and the bead bound cells were washed three times with PBS using a magnet. The obtained cells were then used as immunogen for mouse immunization. The percentage of CD4 expressing cells in obtained cells was calculated as described in 2.2.3.3

2.3 Immunization

BALB/c mice were intraperitoneally immunized with three different immunogens, i.e. Immunoprecipitated-beads, 5 x 10^7 CD4-BCCP beads and 2 x 10^6 CD4-COS cells in 500 µl of sterile PBS at one-week intervals. Mice sera were collected before each injection.

2.4 Determination of antibody response in the immunized mice

2.4.1 Indirect immunofluorescence staining

Antibody responses in the immunized mice were determined by indirect immunofluorescence staining using PBMC and CD4 –DNA transfected COS cells as antigens. To block Fc receptor, human AB serum was added to the cell suspension at final dilution of 1:10 and incubated on ice for 30 minutes before staining. The blocked

cells were stained with various dilutions of tested sera or control CD4 mAb by the method as was described in 2.2.3.3. Membrane fluorescence was analyzed by flow cytometer or fluorescent microscope.

2.4.2 ELISA

Antibodies against CD4-BCCP in mice sera were determine by indirect ELISA as described in 2.2.2.6.1 using CD4-BCCP bacterial extract as antigen.

2.5 Hybridoma production

2.5.1 Preparation of splenocytes

The immunized mice were sacrified and spleens were aseptically removed and placed in 6-cm tissue culture dish containing 5 ml of Iscove's Modified Dulbeco's Medium (IMDM) medium. Any contaminating tissue from the spleen was trimmed off and discarded. After rinsing, the spleens were transferred to the new tissue culture dish containing 5 ml of IMDM medium. Splenocytes were collected by crushing the spleen in IMDM. The cell suspensions were transferred to 50 ml centrifuge tube and let the cell debris settle out approximately 10 minutes. Then, cell suspensions were collected and centrifuged at 550 g for 5 minutes. The supernatant were discarded. Red blood cells were lysed by 0.83% NH_4Cl hypotonic solution. To dilute the lysing solution, 20 ml of IMDM were added. Thereafter, Splenocytes and P3X63Ag8.653 mouse myeloma cells were washed twice with IMDM. The number and viability of the cells were counted in a hemacytometer using Turk's solution and 0.2% trypan blue.

2.5.2 Cells fusion

Both splenocytes and the mouse myeloma cells were mixed together at 2:1 ratio (2 splenocytes: 1 myeloma) and spun at 400 g for 10 minutes. The cell pellets were incubated at 37°C for 5 minutes before fusion by standard hybridoma technique using 50% polyethylene glycol (PEG). All PEG and medium were added in a dropwise fashion, continuously and gently shake. The sequence of the addition of reagents was as follows: 1.5 ml of warm 50% PEG was added in a dropwise fashion with gently shaking for over 1 minute followed by continuously shaking for another minute. Then, 1 ml of warm IMDM was added into the tube within 1 minute. Three ml of warm IMDM was then added into the tube for over 1 minute. Finally, the 16 ml of IMDM was added into the tube for 2 minutes. After centrifugation at 400 g for 10 minutes, the cell pellet was placed in a 37°C water-bath for 5 minues. The supernatant was then removed carefully. Subsequently, the fused cells were resuspended in 100 ml HAT medium. One hundred microliters of the cell suspension were seeded into each well of 96-well plate and cultivated at 37°C in 5% CO_2 incubator. After five days of cultivation, 150 µl of HT medium were added into each well. The plates were incubated at 37°C in 5% CO_2 incubator to expand the cells.

2.5.3 Hybridoma screening

2.5.3.1 Screening of hybridomas by indirect immunofluorescence analysis

To screen hybridoma producing CD4 antibody, supernatants from well containing hybridoma cells were collected and analyzed for antibody reactivity against PBMC using indirect immunofluorescence staining as described in 2.2.3.3 and analyzed by flow cytometry. To block the non specific-Fc receptor, human AB serum was added to the cell suspension at final dilution of 1:10 and incubated on ice for 30

minutes before staining. For flow cytometric analysis, individual populations of leukocytes were gated according to their forward and side scatter characteristics.

The supernatants which showed positive reactivity with lymphocyte subpopulations but negative or weakly positive with monocytes were further screened for specific antibody to CD4 protein by the same technique but using CD4-DNA transfected COS cells as antigen and analyzed under a fluorescent microscope. The single clone of hybridomas from positive wells was then cloned by limiting dilution (see 2.5.4).

2.5.3.2 Screening of hybridomas by ELISA

Antibody reactivity against CD4-BCCP in supernatants from well containing hybridoma cells were determined by indirect ELISA as described in 2.2.2.6.1 using the optimal dilution of bacterial extract as antigen. The supernatants which had shown positive reactivity with CD4-BCCP but negative or weakly positive with CD147-BCCP were further screened for antibody against PBMCs and specific to CD4 protein by indirect immunofluorescent analysis as described in 2.5.3.1

2.5.4 Limiting dilution

To obtain the single clone of hybridomas, the limiting dilution was carried out. The hybridomas from the positive wells were counted and cell concentration was adjusted to 4, 2, and 1 cells per 150 µl of 10% BM condimed H1 in 10% FCS-IMDM. Then, 150 µl of each dilution were added in to 96-well plate. Then, cells were expanded by cultivation at 37°C in 5% CO2 incubator. After 7-10 days of cultivation, cell growth was checked under an inverted microscope. Wells which contained a single clone were marked. Supernatant from the well containing single clone of hybridomas were collected and used for PBMCs staining by indirect

immunofluorescence follows by flow cytometry. The positive clones were grown to obtain a large number of cells. The clones, which secrete antibody reacting with lymphocytes subpopulation but negative or weakly positive with monocytes were rechecked further for specificity to CD4 protein using CD4-DNA transfected COS cells by the techniques described in 2.2.3.3.

2.6 Characterizations of monoclonal antibodies

2.6.1 Characterization of CD4 mAbs by Western blotting

Unlabeled PBMC lysate were prepare as described in 2.2.1.1. Non-reducing buffer or reducing buffer 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 subsequently transferred to a nitrocellulose membrane by semi-dry electrophoretic blotting system at 40 mA (constant ampere) for 2 hours. The membrane was blocked with blocking buffer. After 1 hour incubation in blocking buffer, the blocked membranes were probed with the generated monoclonal antibodies as was described in 2.2.2.6.2. Immunoreactivity were visualized using chemiluminescence detection system as described in 2.2.1.4

2.6.2 Determination of isotypes of monoclonal antibodies

The isotype of mAbs were determined by capture ELISA. Each well of the ELISA plate was coated with 50 µl of goat anti-mouse immunoglobulin isotypes (IgA, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgM) a dilution of 1:1000 in carbonate bicarbonate buffer (pH 9.6). After incubation at 4°C overnight, the plate was washed with washing buffer (0.05% Tween 20 in PBS) 4 times. Then, 60 µl of PBS containing 2% bovine serum albumin (2% BSA-PBS) was added in each well and incubated at 37°C

for 1 hour. After blocking step, 50 µl of 10 µg/ml generated mAbs was applied and incubated at 37°C for 1 hour. Afterward, the plate was washed with washing buffer 4 times. The antigen-antibody complexes were monitored by adding 50 µl of HRP-conjugated rabbit anti-mouse immunoglobulins at dilution 1:2000 and incubated at 37°C for 1 hour. After washing 4 times with washing buffer, 50 µl of TMB substrate was added to each well and incubated at room temperature in the dark for 15-30 minutes. The reaction was stopped by adding 50 µl of 1 N HCl and the absorbance was determined by ELISA reader at 450 nm.

2.6.3 Immunoprecipitation

The generated mAbs were used to precipitate corresponding protein in biotinylated cell lysates as described in 2.2.1.3. Proteins were visualized using chemiluminescence detection system as described in 2.2.1.4

2.6.4 Enumeration of the percentage of CD4 cells in peripheral blood lymphocytes

The generated mAbs were used for the enumeration of $CD4^+CD3^+$ cells in peripheral blood lymphocytes of 3 healthy subjects by lysed whole blood immunofluorescence staining. The percentage of $CD4^+CD3^+$ cells in lymphocyte population was analyzed by flow cytometry and compared to those using CD3-FITC/CD4-PE standard reagent purchased from Becton Dickinson.

For enumeration of $CD4^+CD3^+$ cells by using mAbs MT4/2 and MT4/4, 50 µl of K_3EDTA blood were incubated at room temperature with 50 µl of MT4/2 or MT4/4 hybridoma cultured supernatant and 20 µg/ml CD3 mAb OKT3 for 30 minutes. After washing with 1% BSA-PBS-NaN_3, The PE labeled anti-mouse IgM and FITC labeled anti-mouse IgG were added. After incubation at room temperature

for 30 minutes, 1 ml of FACS™ lysing solution was added and let stand at room temperature in the dark for 10 min. Cells were then washed twice with 1% BSA-PBS-NaN_3. After washing, fluorescence intensity of the stained cells was analyzed by a flow cytometry.

For enumeration of $CD4^+CD3^+$ cells by using MT4/3 mAb, 50 µl of K_3EDTA blood were incubated at room temperature with 50 µl of MT4/3 hybridoma culture supernatant for 30 minutes. After twice washing with 1% BSA-PBS-NaN_3, the PE labeled anti-mouse IgG was added and incubated at room temperature for another 30 minutes. Cells were washed twice with 1% BSA-PBS-NaN_3 and counter stained with FITC labeled CD3 mAb OKT3. After incubation at room temperature for 30 minutes, 1 ml of FACS™ lysing solution was added and let stand at room temperature in the dark for 10 min. Cells were then washed twice with 1% BSA-PBS-NaN_3. and fluorescence intensity was analyzed by a flow cytometry.