CHAPTER II

MATERIALS AND METHODS

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

2.2 Hybridoma cultivation and culture supernatant production

Hybridomas producing anti-CD4 mAb clones MT4, MT4/2, MT4/3, and MT4/4 were propagated in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS), gentamycin 40 μg/ml and fungizone 5 mg/ml (10% FBS-IMDM) at 37°C in a 5% CO_2 incubator. Culture supernatants containing anti-CD4 mAbs were collected when the hybridoma cells become confluence and stored at -20^{o}C until used.

2.3 Determination of specific reactivity of anti-CD4 mAbs in culture supernatants by COS cell transfection technique

2.3.1 COS cell transfection

Plasmid DNA encoding CD4 and CD8 proteins were kindly gifts from Prof. Dr. Hannes Stockinger, Medical University of Vienna, Vienna, Austria. The plasmid DNAs were transfected into COS cells by DEAE-dextran transfection method. COS cells were maintained with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, gentamycin 40 μg/ml and fungizone 5 mg/ml (10%

FBS-DMEM). One day before transfection, COS cells were harvested from culture flasks using phosphate buffered saline (PBS) containing 0.5 mM EDTA (0.5 mM EDTA-PBS). After twice washing with DMEM, cell concentration was adjusted to $1x10^6$ cells in 4 ml of 10% FBS-DMEM, plated into 6-cm tissue culture dishes and cultured at 37°C in a 5% CO_2 incubator. The medium was discarded on the next day. Then, the cells were incubated in 2 ml of transfection solution (DMEM containing 250 µg/ml DEAE-dextran, 400 µM chloroquine diphosphate and 2 µg plasmid DNA encoding CD4 or CD8 proteins) for 3 hours at 37°C in a 5% CO_2 incubator. Thereafter, the transfection solution was removed and the cells were treated with 2 ml of PBS containing 10% v/v DMSO (10% DMSO-PBS) at room temperature for 2 minutes. Then, the solution was discarded and washed once with 4 ml of DMEM. After overnight incubation at 37°C in a 5% CO_2 incubator, the medium was removed and 4 ml of 10% FBS-DMEM was replaced. The transfected cells were cultured for another 2 days to allow expression of the corresponding proteins.

2.3.2 Indirect immunofluorescent assay of transfected COS cells with anti-CD4 mAbs

The CD4 and CD8 transfected COS cells were stained with culture supernatants containing anti-CD4 mAbs (clones MT4, MT4/2, MT4/3, and MT4/4) by indirect immunofluorescent staining. At the third day after COS cells transfection (see 2.3.1), COS cells were removed from tissue culture dishes by incubation with 0.5 mM EDTA-PBS for 2 minutes. The cells were washed three times with PBS, and adjusted to $1x10^7$ cells/ml in PBS containing 1% bovine serum albumin (BSA) and 0.02% sodium azide (1% BSA-PBS-0.02% NaN_3). Fifty microliters of COS cell suspension were then incubated with 50 µl of tested culture supernatants on ice for 30 minutes.

After twice washing with 1% BSA-PBS-0.02% NaN_3, 50 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 in dark place. Finally, the cells were washed three times with 1% BSA-PBS-0.02% NaN_3. Membrane fluorescence was analyzed by a fluorescence microscope.

2.4 Large scale production and purification of anti-CD4 mAbs

2.4.1 Large scale production of anti-CD4 mAbs

Hybridoma cells were cultured in 10% FBS-IMDM at 37°C in a 5% CO_2 incubator. After the hybridoma cells growing up, cells were collected and washed three times with PBS. Hybridoma cells were then used for induction of ascitic fluids. For ascitic fluid production, BALB/c mouse (male, 6–8 weeks old) was intraperitoneally injected with 0.5 ml of pristane (Tetrametyl-pentadecane). A week after pristane injection, $1x10^7$ hybridoma cells were injected into mouse's peritoneal cavity. One to two weeks after hybridoma inoculation, mouse developed ascitic fluid. The ascitic fluid was drawn from the induced mouse and centrifuged at 20,000 g, 4°C for 30 minutes to collect the clarified supernatants and stored at -20°C.

2.4.2 Purification of anti-CD4 mAbs by affinity chromatography

Ascitic fluid containing large amount of mAbs (see 2.4.1) was re-clarified by centrifugation at 20,000 g, 4°C for 30 minutes. Then, mAbs were purified out from the clarified ascitic fluid by affinity chromatography. Hitrap® IgM and protein G affinity columns were employed for purification of anti-CD4 mAbs having IgM and IgG isotype, respectively. Clarified ascites were applied into the affinity columns and purified mAb fractions were eluted and collected by AKTA® prime fraction collector.

The obtained mAbs were dialyzed against PBS at 4°C, overnight and determined the protein concentration by measuring at optical density (OD) 280 nm. The purified mAbs were stored at -20°C. The purity and reactivity of obtained purified mAbs were further determined.

2.4.3 Determination of the purity of purified anti-CD4 mAbs

The purity of the purified mAbs was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, 5 µg/ml of purified anti-CD4 mAbs were mixed with reducing and non-reducing sample buffer and boiled for 5 min. Then, the samples were loaded into each lane of 10% SDS-PAGE. The electrophoresis was performed at 120 Volts for 2 hours. The heavy chain and light chain of mAbs were migrated according to their molecular weight. Proteins on the separating gel were visualized by staining with Coomassie Brilliant Blue.

2.4.4 Determination of the reactivity of purified anti-CD4 mAbs by COS cell transfection technique

COS cells were transfected with plasmid DNA encoding CD4 and CD8 proteins by DEAE-dextran transfection as described in 2.3.1. Thereafter, the transfected COS cells were stained with 20 µg/ml of purified anti-CD4 mAbs by indirect immunofluorescent staining as described in 2.3.2. The stained cells were then visualized by a fluorescent microscope.

2.5 Immunoprecipitation of CD4 protein by anti-CD4 mAbs

2.5.1 Biotinylation of cell surface molecules

Sup T1 human cell line (CD4 positive cell line) were maintained in 10% FBS-RPMI 1640 at 37°C in a 5% CO_2 incubator. For biotin labeling, cells were washed

three times with PBS and adjusted to $1x10^8$ cells in 1 ml of 5 mM Sulfo-NHS-LC-biotin in PBS and incubated on ice for 1 hour. To stop biotinylation, cells were quenched by washing once with 1 mM glycine in PBS and then twice with cold-PBS.

2.5.2 Preparation of cell lysates and immunoprecipitation

Biotinylated cells (from section 2.5.1) were added with purified anti-CD4 mAbs to obtain the final concentration of 20 µg/ml and rotated at 4°C for 1 hour. Then the cells were washed three times with PBS. Thereafter, the cells were lysed on ice for 30 minutes with 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 20,000 g, 4°C for 30 minutes.

For precipitation of CD4 molecules, cell lysates were rotated with protein G sepharose (for IgG isotype) or rabbit anti-mouse IgM coated protein G sepharose (for IgM isotype) at 4°C overnight. On the next day, sepharose beads were washed five times with 0.1 M Tris-HCl pH 7.5 and another five times with cold-PBS. Then, the protein was dissociated from the beads by boiling in 50 µl of non-reducing or reducing SDS-PAGE buffer (see Appendix) for 5 minutes. The precipitated protein was separated by SDS-PAGE using 10% separating gel and 4% stacking gel at 120 Volts (constant volt) and subsequently transferred to nitrocellulose membranes by a semi-dry electrophoretic blotting system at 40 mA (constant ampere) for 2 hours. The membranes were then blocked with 5% BSA-PBS at room temperature for 1 hour. The blocked membranes were rinsed twice with PBS and incubated with horseradish peroxidase (HRP) conjugated streptavidin (1:30,000) in 2% BSA-PBS 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 protein was visualized by the chemiluminescence detection system.

2.5.3 Chemiluminescence detection system

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

2.6 Epitope mapping of anti-CD4 mAbs by cross blocking analysis

2.6.1 Preparation of FITC-conjugated anti-CD4 mAbs

To prepare 10 mg/ml of FITC in DMSO solution, 0.001 gram of FITC powder was added into DMSO to give a concentration of 10 mg/ml. For IgG mAb, 1M $NaHCO_3$ solution was added into the antibody at 1:10 ratio. This step was not performed in the case of IgM mAb. Then, an amount of 10 mg/ml of FITC-DMSO solution was added into mAb solution. The volume of the FITC-DMSO was calculated from equation below:

Volume of FITC-DMSO (ml) =

[(mg of mAb x 0.1)/ MW of mAb)] x (MW of FITC x constant factor)

MW of IgG	=	146 kDa
MW of IgM	=	190 kDa
Constant factor for IgG	=	10
Constant factor for IgM	=	20

The mixture was rotated at room temperature for 90 minutes in dark place. The obtained solution was then dialyzed against PBS at 4°C, overnight. OD of protein was measured at 280 nm and FITC was measured at 495 nm. Ratio of OD of FITC to protein was further calculated. The optimal FITC/protein ratio should be at 0.3-1.2.

2.6.2 Peripheral blood mononuclear cells (PBMCs) preparation

Blood samples were collected from healthy donors using EDTA as anti-coagulant. PBMCs were isolated from blood samples using Ficoll-hypaque density gradient centrifugation. Briefly, 10 ml of EDTA-blood were diluted with 10 ml of PBS. Then, 20 ml of diluted blood were overlaid on 10 ml of Ficoll-hypaque solution and centrifuged at 400 g, 25°C for 30 minutes. The PBMCs fraction was collected and washed three times with PBS. The cells were adjusted to 1×10^7 cells/ml in 1% BSA-PBS-0.02% NaN_3.

2.6.3 Cross blocking experiment

PBMCs were blocked Fc receptors by incubating with 10% human AB serum on ice for 30 minutes. Thereafter, 50 µl of purified anti-CD4 mAbs (concentration 200 µg/ml) or without mAb were added into 50 µl of the blocked cells and incubated on ice for 30 minutes. After twice washing with 1% BSA-PBS-0.02% NaN_3, cells were resuspended with 50 µl of 1% BSA-PBS-0.02% NaN_3. Subsequently, 50 µl of FITC-conjugated anti-CD4 mAbs (see section 2.6.1) (concentration 200 µg/ml) were added into the cell suspension and incubated on ice for 30 minutes in dark place. Finally, the cells were washed three times with 1% BSA-PBS-0.02% NaN_3 and fixed with 1% paraformaldehyde-PBS. The stained cells were then analyzed by a flow cytometer. Fluorescence intensity of each tube was measured. The percent inhibitions of mean fluorescence intensity between each anti-CD4 mAbs paired were calculated.

2.7 Reactivity of anti-CD4 mAbs with CD4 molecules expressed on lymphocytes and monocytes

2.7.1 Determination of saturated concentration of anti-CD4 mAbs

The purified anti-CD4 mAbs were adjusted to obtained the concentrations at 400, 200, 100 and 50 µg/ml in 1% BSA-PBS-0.02% NaN_3. PBMCs (see 2.6.2) were blocked Fc receptor by incubating with 10% human AB serum on ice for 30 minutes. Fifty microliters of purified anti-CD4 mAbs (concentrations 400, 200, 100, and 50 µg/ml) were then added into 50 µl of the blocked PBMCs and incubated on ice for 30 minutes. After twice washing with 1% BSA-PBS-0.02% NaN_3, cells were resuspended with 50 µl of 1% BSA-PBS-0.02% NaN_3 and 50 µl of FITC-conjugated F(ab')2 fragment of sheep anti-mouse immunoglobulins antibody were subsequently added and incubated on ice for 30 minutes in dark place. Finally, the cells were washed three times with 1% BSA-PBS-0.02% NaN_3 and fixed with 1% paraformaldehyde-PBS. The stained cells were then analyzed by a flow cytometer. Lymphocytes and monocytes were gated according to their granularity (SSC) and size (FSC). FITC-fluorescence (FL-1) of each cell population was determined.

2.7.2 Determination of anti-CD4 mAbs reactivity with lymphocytes and monocytes

PBMCs (see 2.6.2) were blocked Fc receptor by incubating with 10% human AB serum and incubated on ice for 30 minutes. Fifty microliters of the saturated concentration of purified anti-CD4 mAbs (see 2.6.1) were then added into 50 µl of the blocked cells and incubated on ice for 30 minutes. After twice washing with 1% BSA-PBS-0.02% NaN_3, 50 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 in dark place. Finally, the cells were washed three times with 1% BSA-PBS-0.02% NaN_3 and fixed with 1% paraformaldehyde-PBS. The stained cells were then analyzed by a flow cytometer. Lymphocytes and monocytes were gated according to their granularity (SSC) and size (FSC). FITC-fluorescence (FL-1) of each cell population was determined.

2.8 Study the effect of anti-CD4 mAbs on the regulation of anti-CD3 induced PBMCs proliferation

2.8.1 Immobilization of anti-CD3 mAb

Anti-CD3 mAb clone OKT3 at a concentration of 60 ng/ml in sterile PBS were prepared. To immobilize the anti-CD3 mAb on culture plates, 100 µl of OKT3 mAb were added to each well of the 96-well tissue culture plates. For the unstimulated control, wells were added with 100 µl of sterile PBS. Then, the plates were incubated at 4°C, overnight.

2.8.2 Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling of PBMCs

PBMCs were isolated from heparinized blood by Ficoll-hypaque gradient centrifugation (see 2.6.2) and adjusted to 1×10^7 cells/ml in PBS. The CFSE was diluted in sterile PBS to give a final concentration of 100 µM. Five microliters of the diluted CFSE were added into 1×10^7 cells/ml of PBMCs to give a final concentration of 0.5 µM and mixed gently by pipetting cells up and down. The PBMCs were then incubated at 37°C in water-bath for 10 minutes. After incubation, 10 ml of cold 10% FBS-RPMI 1640 were added and immediately centrifuged at 600 g, 4°C for 10 minutes. The cells were then twice washing with 10% FBS-RPMI 1640. Finally, the

cells were adjusted to 1×10^6 cells/ml in 10% FBS-RPMI 1640. The CFSE labeled PBMCs were checked for the labeling capacity by flow cytometry.

2.8.3 Proliferation assay

Before adding the CFSE labeled PBMCs, anti-CD3 mAb immobilized plates (see 2.8.1) were washed twice with 200 µl/well of sterile PBS and blocked each well with 100 µl of sterile 1% BSA-PBS at 37°C for 1 hour. The anti-CD3 mAb immobilized plates were washed twice with sterile PBS 200 µl/well. Then, purified anti-CD4 mAbs (MT4, MT4/2, and MT4/3), media control, IgM isotype matched control antibody (FE-1H10), IgG isotype matched control antibody (13M-1F), and inhibitory control antibody (anti-CD147 mAb, M6-1E9) were added into each well to give a final concentration at 20 µg/ml in 10% FBS-RPMI 1640. Thereafter, the CFSE labeled PBMCs (see 2.8.2) were added into each well to have the total volume of 200 µl. The cells were cultured at 37°C in a 5% CO_2 incubator. At the third, fifth and seventh day of cultivation, the cells were harvested and assessed for CFSE fluorescence by a flow cytometer.

2.9 Study the effect of anti-CD4 mAbs on the regulation of anti-CD3/CD28 induced monocyte-depleted lymphocyte proliferation

2.9.1 Immobilization of anti-CD3 mAb

Culture plates were immobilized with anti-CD3 mAb OKT3 as described in 2.8.1.

2.9.2 CFSE labeling of monocyte-depleted lymphocytes

PBMCs were isolated from heparinized blood by Ficoll-Hypaque gradient centrifugation as described in 2.6.2. PBMCs at 1×10^7 cells/10 ml in sterile 10% FBS-

RPMI 1640 were plated into 10-cm glass dishes and incubated at 37°C in a 5% CO_2 incubator for 1 hour. Then, non-adherent lymphocytes were harvested. To completely remove out of monocytes, the adhesion process was repeated. The non-adherent cells (lymphocytes) were harvested and adjusted to 1×10^7 cells/ml in PBS. The obtained lymphocytes were then labeled with CFSE as described in 2.8.2.

2.9.3 Proliferation assay

Before adding the CFSE labeled monocyte-depleted lymphocytes, anti-CD3 mAb immobilized plates (see 2.9.1) were washed twice with 200 µl/well of sterile PBS and blocked each well with 100 µl of sterile 1% BSA-PBS as described in 2.8.3. Then, purified anti-CD4 mAbs, media control, IgM isotype matched control antibody (FE-1H10), IgG isotype matched control antibody (13M-1F), and inhibitory control antibody (anti-CD147 mAb, M6-1E9) were added into each well to give a final concentration of 20 µg/ml in 10% FBS-RPMI 1640. Thereafter, the CFSE labeled monocyte-depleted lymphocytes (see 2.9.2) and anti-CD28 mAb were added into each well to give a final concentration of anti-CD28 mAb at 5 µg/ml and total volume of 200 µl. The cells were cultured at 37°C in a 5% CO_2 incubator. At the third, fifth and seventh day of cultivation, the cells were harvested and assessed by a flow cytometer.

2.10 Study the effect of anti-CD4 mAbs on monocyte oxidative burst induction

2.10.1 Phorbol myristate acetate (PMA) activation (Control group)

Fifty microliters of phorbol myristate acetate (PMA) were added into 100 µl of EDTA-blood to give a final concentration of 400 ng/ml. Cells were incubated at 37°C in a water-bath for 15 minutes. After the incubation, 50 µl of dihydroehidium (DHE) were added into the samples to give a final concentration of 500 ng/ml and incubated

at 37°C in a water-bath for 15 minutes. Thereafter, the samples were washed twice with 1% BSA-PBS-0.02% NaN_3. Red blood cells (RBCs) were lysed with 100 µl of RBCs lysis buffer (PBS containing diethylene glycol and 37% formaldehyde) at room temperature for 15 minutes and 1 ml of distilled water were then added and strongly vortex. Finally, the cells were washed three times with 1% BSA-PBS-0.02% NaN_3 and fixed with 1% paraformaldehyde-PBS. The stained cells were analyzed by a flow cytometer. The fluorescence (FL-2) of the oxidized DHE was determined.

2.10.2 Effect of anti-CD4 mAbs on monocyte oxidative burst

(Experimental group)

Fifty microliters of purified anti-CD4 mAbs were added into 100 µl of EDTA -blood to give a final concentration of 20 µg/ml and incubated at 37°C in a water-bath for 30 minutes. After the incubation, 50 µl of DHE were added into the samples as described in 2.10.1. Thereafter, the samples were washed twice with 1% BSA-PBS-0.02% NaN_3. RBCs were lysed with 100 µl of RBCs lysis buffer as described in 2.10.1. Finally, the cells were washed three times with 1% BSA-PBS-0.02% NaN_3 and fixed with 1% paraformaldehyde-PBS. The stained cells were analyzed by a flow cytometer. The fluorescence (FL-2) of the oxidized DHE was determined.