# CHAPTER II

# MATERIALS AND METHODS

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

## 2.2 Production of Anti-CD99 monoclonal antibodies

### 2.2.1 Generation of CD99 expressing COS7 cells

A mammalian expression plasmid vector containing cDNA encoding the CD99 protein, CD99-DNA, was constructed in our laboratory (Kasinrerk *et al.* 2000). CD99-DNA plasmids were transfected into COS7 cells by the DEAE-dextran transfection method. Briefly, COS7 cells ($1x10^6$ cells) were seeded into 6-cm tissue culture dishes and cultured at $37^oC$ in a 5% $CO_2$ incubator overnight before transfection. The media were discarded the next day. Then, the cells were cultured in 2 ml of transfection solution (MEM containing 250 μg/ml DEAE-Dextran, 400μM chloroquine diphosphate and 2 μg CD99-DNA) for 3 hours at $37^oC$ in a 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 and washed twice with incomplete MEM medium. The transfected cells were cultured for another 2 days to allow surface expression of CD99 proteins.

### 2.2.2 Hybridoma production

A BALB/c mouse was intraperitoneally immunized three times at 2-week intervals with $1x10^7$ CD99 transfected COS7 cells. After three immunizations, the mouse was intravenously boosted with $1x10^6$ of the transfectants. Five days after being boosted, the immunized mouse was sacrificed and spleen was aseptically removed. Splenocytes were collected by crushing the spleen in Iscove's Modified Dulbeco's Medium (IMDM) medium. The splenocytes were mixed together with P3X63Ag8.653 mouse myeloma cells at 2:1 ratio (2 splenocytes: 1 myeloma) and spun at 400 g for 10 min. The cell pellets were incubated at 37°C for 5 min before fusion. After incubation, cells were fused by standard hybridoma technique using 50% polyethylene glycol (PEG). For cell fusion, 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 min followed by continuously shaking for another minute. Then, 1 ml of warm IMDM was added into the tube within 1 min. Three milliliters 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 min. After centrifugation at 400 g for 10 min, the cell pellet was placed in a 37°C water-bath for 5 min. 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. The developed hybridoma cells in each well were observed under an inverted microscope.

After HAT medium selection, culture supernatants were collected from hybridoma containing wells. The obtained culture supernatants were analyzed for antibody reactivity by indirect immunofluorescence analysis using CD99 transfected COS7 cells (see 2.2.3) and analyzed under a fluorescent microscope. The supernatants which showed positive reactivity with CD99 transfected COS7 cells but negative with untransfected COS7 cells were then cloned by limiting dilution (see 2.2.4). The positive hybridoma clones were grown to obtain a large number of cells.

### 2.2.3 Indirect immunofluorescence staining of COS7 cells

CD99 transfected and non-transfected COS7 cells (1x $10^7$cell/ml) were pre-incubated with 10% human AB serum at 4$^o$C for 30 min to block nonspecific Fc-receptor-mediated binding of mAbs. For staining of the surface membrane, 50 µl of the cell suspension were incubated with an equal volume of hybridoma culture supernatants or 20 µg/ml mAb of interested at 4$^o$C for 30 min. The cells were washed twice with 1% bovine serum albumin in PBS containing 0.02% sodium azide (1%BSA-PBS-$NaN_3$) and then incubated with FITC conjugated sheep $F(ab')_2$ anti-mouse immunoglobulins antibodies at 4°C for another 30 min. The stained cells were then washed for three times and analyzed by a fluorescent microscope.

### 2.2.4 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 cell(s) 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$^o$C in 5% CO2 incubator. After 7-10 days of cultivation,

cell growth was checked under an inverted microscope. Wells contained a single clone were marked. Supernatants from the well containing single clone of hybridomas were collected for the detection of specific antibody production by indirect immunofluorescence analysis (see 2.2.3)**.**

### 2.3 Western blotting of cell lysates

The cells ($5x10^7$ cells/ml) were solubilized for 30 min on ice in 1 ml lysis buffer (1% NP-40, 50mM Tris-HCl pH 8.2, 100mM NaCl, 2mM EDTA, 5mM iodoacetamide, 1mM PMSF, 2 μM pepstatin A and 10μg/ml aprotinin). Fifty microliters of cell lysates were mixed with an equal volume of 2x non-reducing or reducing sample buffer and boiled for 5 min. Proteins were then resolved by 10% SDS-PAGE followed by transfer to a nitrocellulose membrane. The membrane was blocked overnight in 5% skimmed milk in PBS at $4^oC$. The blocked membrane was then incubated with the mAb of interest for 1 hr at room temperature. After being washed five times with 0.1% Tween-20 in PBS, the membrane was incubated with HRP conjugated rabbit $F(ab')_2$ anti-mouse immunoglobulins antibodies, HRP-conjugated anti-mouse IgG, light chain specific, HRP conjugated anti-mouse IgM, μ chain specific or HRP-conjugated Mouse IgG TrueBlot for 1 hr. The membrane was then washed five times. The specific proteins were then visualized by the chemiluminescent detection system.

For chemiluminescent detection, 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. Finally, the films were developed with Kodak GBX solution.

### 2.4 Immunoprecipitation and Western immunoblotting of cell surface molecules

The cells were twice washing with PBS. The cells ($5x10^7$ cells) were solubilized for 30 min on ice in lysis buffer (20mM Tris-HCl pH 7.5, 100mM NaCl, 2mM EDTA, 50mM NaF, 1mM $Na_3VO_4$, 5mM iodoacetamide, and protease inhibitors) containing either detergent 1% Brij-58 or 1% Lauryl maltoside (LM). The cell suspension was clarified by centrifugation at 10,000×g for 30 min at 4°C. The clarified cell lysates were precleared with protein G Sepharose beads (Pierce) coated with mouse immunoglobulins. One milliliter of precleared lysates was then incubated with 10 µg of purified mAbs for 2 hr at 4°C. For IgG isotype mAbs, 30 µl Protein G Sepharose beads were then added. While IgM isotype mAbs, 30 µl of protein G sepharose beads were firstly coated with 10 µg of goat anti-mouse IgM antibody before adding. The mixtures were rotated overnight at 4°C. After immunoprecipitation the precipitated proteins were resolved in 10% SDS-PAGE and transferred onto a nitrocellulose membrane followed by western blotting as described in 2.3.

### 2.5 Indirect immunofluorescence staining of cell surface molecules

The tested cells ($1x10^7$cells/ml) were pre-incubated with 10% human AB serum at 4°C for 30 min to block nonspecific Fc-receptor-mediated binding of mAbs. For staining of the surface membrane, 50 µl of the cell suspension were incubated with an equal volume of hybridoma culture supernatants or 20 µg/ml mAb of interested at 4°C for 30 min. The cells were washed twice with 1% bovine serum albumin in PBS containing 0.02% Sodium azide (1%BSA-PBS-$NaN_3$) and then incubated with FITC

conjugated sheep $F(ab')_2$ anti-mouse immunoglobulins antibodies at 4°C for another 30 min. The stained cells were then washed for three times and resuspended in 500 µl PBS containing 1% paraformaldehyde. Membrane fluorescence of the stained cells was analyzed by flow cytometry.

For staining of peripheral blood leukocyte surface molecules, lysed whole blood staining method was carried out. 50 µl of whole blood sample (using EDTA as anti-coagulant) were stained with mAbs as was described above. Then, 1 ml of FACS™ lysing solution was added for lysis of RBC and let stand at room temperature in the dark for 10 min. The remained leukocytes were then washed twice with 1% BSA-PBS-$NaN_3$. Membrane fluorescence was analyzed on a flow cytometer. Individual populations of blood cells were gated according to their forward and side scatter characteristics.

### 2.6 Coimmunoprecipitation of biotinylated cell surface proteins

The tested cells (PBMCs, Jurkat cells, U937 cells, Molt4 cells and K562 cells) were labeled with 5mM Sulfo-NHS-LC-Biotin at 4°C for 1 hr. The biotinylation was quenched by washing once with 1mM glycine in PBS and twice with PBS. The biotinylated cells ($5x10^7$ cells) were solubilized for 30 min on ice in 1 ml lysis buffer (20mM Tris-HCl pH 7.5, 100mM NaCl, 2mM EDTA, 50mM NaF, 1mM $Na_3VO_4$, 5mM iodoacetamide, and protease inhibitors) containing either detergent 1% Brij-58 or 1% LM. The biotinylated cell lysates were immunoprecipitates as described in 2.4.

After immunoprecipitation the precipitated proteins were resolved in 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% BSA in PBS overnight at 4°C. The blocked membrane was then

incubated with HRP conjugated streptavidin at room temperature for 1 hr. After washing five times with 0.1% Tween-20 in PBS, the reactive protein bands were visualized by the chemiluminescent detection system (see 2.3).

**2.7 Coimmunoprecipitation and amino acid sequencing**

The Jurkat cell lysates were immunoprecipitates using mAb MT99/3 as described in 2.4. After immunoprecipitation the precipitated proteins were resolved in 10% SDS-PAGE and stained either with silver or Colloidal Coomassie-blue dye. The bands containing protein which was not found in the immunoprecipitation of isotype match control were excised from Colloidal Coomassie-stained SDS-PAGE gels were subjected to tryptic digestion and MALDI-MS/MS protein identification which had done by Petr Pompach, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic. The identities of the peptides were determined by comparison with the nonredundant NCBI data base using the Mascot search engine.

**2.8 Production of monoclonal antibodies against CD99 associated molecules**

To prepare the immunogen, we used the immunoprecipitated beads method developed by us earlier (Pata *et al.* 2009). Briefly, the Jurkat cell lysates were precleared by incubation at 4$^{o}$C overnight with Sepharose beads coated with mouse immunoglobulins. The precleared cell lysates were then added to CD99 mAb MT99/3 coated beads and rotated at 4$^{o}$C overnight. Subsequently, the precipitated beads were washed 10 times with PBS and resuspened in 500 µl of sterile PBS. The precipitated beads were then used as the immunogen for mouse immunization. The BALB/c mice were intraperitoneally immunized with CD99-immunoprecipitated-beads at one-week intervals. After three immunizations, the mice were intravenously boosted with the

immunogen. Five days later, the immunized mice were sacrificed and splenocytes were collected and fused with P3-X63Ag8.653 myeloma cells by a standard hybridoma technique using 50% polyethylene glycol (see 2.2.2). After HAT medium selection, culture supernatants obtained from hybridoma containing wells were analyzed for antibody reactivity by indirect immunofluorescence staining using Jurkat cells as an antigen (see 2.5) and Dot blot assay using CD99-immunoprecipitated proteins as an antigen (see 2.4). The supernatants which showed positive reactivity were further screened by Western blotting using CD99 immunoprecipitated proteins as an antigen (see 2.4). The hybridomas which showed positive reactivity were then cloned by limiting dilution (see 2.2.4). The positive clones were grown to obtain a large number of cells.

### 2.8.1 Dot blot assay

The immunoprecipitated proteins using MT99/3 mAb and isotype matched control mAb were prepared according to the method described in 2.4. Then, two microliters of the immunoprecipitated proteins were spotted onto the nitrocellulose membrane. The membranes were blocked with 5% skimmed milk in PBS at $4^{o}C$. The blocked membranes were then incubated with the hybridoma culture supernatants for 1 hr at room temperature. After being washed five times with 0.1% Tween-20 in PBS, the membranes were incubated with HRP conjugated rabbit $F(ab')_2$ anti-mouse immunoglobulins antibodies for 1 h. After washing step, the immunoreactivity was visualized using TBM blotting substrate solution.

## 2.9 Immunomagnetic cell sorting

Ramos wild type cells or transduced cells were stained with anti-CD99 mAb MT99/3 for 30 min on ice. Then, FITC-conjugated anti-mouse immunoglobulins antibodies were added and incubated for 30 min on ice. After incubation, cells were washed and incubated with anti-FITC MicroBeads according to the manufacturer's instructions. Cells were washed and resuspended in MACS sorting buffer (0.5% BSA, 2 mM EDTA in PBS) and sorted with an AutoMACS cell sorter. The obtained positive and negative cell fractions were collected and cultured in RPMI-1640 medium supplemented with 10% FBS, 40 mg/ml gentamicin and 2.5 mg/ml amphotericin B in a humidified atmosphere of 5% $CO_2$ at 37°C. CD99 expression on the sorted cells was verified by flow cytometric analysis. All cell lines in this study were established as polyclonal cultures.

## 2.10 Plasmid DNA construction and transformation

### 2.10.1 Amplification of gene encoding DDI2, CD99 short form and CD99 long form

Total cellular RNA was purified from the Jurkat cells using Trizol reagent according to the Invitrogen manufacturer's protocol. First strand cDNA synthesis was performed with 1 μg of total RNA using a cDNA synthesis kit. The coding region of human DDI2 was amplified from Jurkat cell cDNA library using the primer 5′-TTTTAGATCTAGTGAC-TCACTGAGCGTGTG-3′ and 5′-GCAGAGCGTCAGAAGCCATGAGAATTCTTT-3′. The PCR product was cloned

into EcoRI site of pBluescript SK vector and sequenced. This plasmid DNA was used as a template for construction of plasmid DNA encoding Myc-tagged DDI2. The Myc-tagged DDI2 construct was generated by PCR using the reverse primer 5′-GATGCAGAGCGTCAGAAGCCAACGCGTGAGCAGAAACTCATCTCTGAAG AGGATCTGTAAGAATTCTTTT-3′ containing sequence for Myc.

For amplification of cDNA encoding CD99 short and long forms, plasmid DNA encoding full-length CD99 cDNA (Kasinrerk *et al.* 2000) was used as a template by the polymerase chain reaction (PCR). The forward primer was 5′-TTTTGA-ATTCGCCATGGCCCGCGGGGCTG-3′ and reverse primers were, 5′-GACGGTACCT-TTCTCTAAAAGAGTACGCTGAACAG-3′ and 5′-CTTGGTACCTCCATCATTTTCTT-TGAAGCATAGCTTCTTTTTC-3′ for the CD99 long- and short-form cDNA respectively,

The amplified PCR product was 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 min. Then the gel was destained with distilled water for 5 minutes. Consequently, the DNA bands were observed by UV transilluminator.

### 2.10.2 Plasmid DNA preparation and transformation

The PCR product was cloned into *EcoR* I site of retroviral vector pMSCV using T4 DNA ligase. The ligation mixture was then incubated at 4 °C for 18 hr. The ligation mixture or plasmid DNA were incubated with 200 µl of competent *E. coli* TOP10 on ice for 1 hr. Then, the *E. coli* were shocked at 42$^{o}$C for 1 min and further incubated on ice for 2 min. The transformed *E. coli* were cultured in 1 ml of non-

antibiotic Luria Bertani (LB) broth with 120 rpm shaking at 37°C for 1 hr. The transformed *E. coli* were then spreaded on LB agar containing 100 μg/ml amplicilin. Subsequently, the plates were incubated overnight at 37°C. The plasmid DNA was then isolated from the transformed *E. coli* by Qiagen chromatography columns. The inserted genes in the constructed plasmids were checked by restriction fragment analysis using corresponding restriction enzymes and DNA sequencing.

To prepare a large amount of plasmid DNA, the *E.coli* containing plasmid DNA was grown in 1 ml of LB broth supplemented with ampicillin by shaking at 180 rpm for 4 hr at 37°C. Then, 1 ml of cultured broth was added to 100 ml of LB broth and the mixture was shaken at 180 rpm overnight at 37°C. The bacterial cells were harvested by centrifugation at 2,400 g for 30 min at 4°C. The plasmid DNA was isolated by using QIAGEN plasmid midi 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.

**2.11 Transfection**

For expression of protein of interest, the plasmid DNAs were transfected into the cells using Lipofectamine 2000™ reagent according to Invitrogen manufacturer's instructions. Briefly, HEK293 or Phoenix Ampho cells were plated at a density of $5x10^5$ cells per well in a six-well plate overnight. The plasmid DNA and the Lipofectamine reagent were diluted in Dulbecco's modified Eagle's medium (DMEM). An equal volume of diluted DNAs and diluted Lipofectamine reagents were gently mixed together and incubated for 30 min to allow DNA-liposome complexes to form. The diluted DNAs-liposome complexs were added to the cells.

Following 6 hr of incubation, cells were rinsed with fresh medium and grown in normal growth medium containing 10 % FBS.

**2.12 Transduction and selection of stable clones**

To prepare retroviruses (RV) harboring plasmid carrying protein of interest, the plasmid DNA were transfected into Phoenix-Ampho cells using Lipofectamine as described above. At 48 hr post-transfection, RV-containing supernatants were collected and clarified by centrifugation. The RV were then used to spin-infect (1200×g, 90 min at 32$^{o}$C) the Ramos wild type cells or Ramos CD99 negative cells in the presence of polybrene (10 μg/ml). Cells were allowed to expand in culture and sorted by immunomagnetic cell sorting to isolate the CD99 expressing cells (see 2.9). Stable transfectant clones with high CD99 expression were identified by flow cytometric analysis. All cell lines in this study were established as polyclonal cultures**.**

**2.13 Colocalization analysis**

Raji B cell line and PBMCs (1x10$^{7}$cells/ml) were pre-incubated with 10% human serum (blood group AB) at 4$^{o}$C for 30 min. For staining of the surface membrane proteins, 50 μl of the cell suspension were incubated with an equal volume of 20 μg/ml anti-MHC class I, anti-MHC class II, anti-CD81 mAbs, or isotype matched control PB-1 mAb at 4$^{o}$C for 30 min. The cells were washed twice with PBS containing 1% BSA and 0.02% $NaN_3$ (1% BSA-PBS-$NaN_3$) and then incubated with Alexa Fluor 488-conjugated goat F(ab')$_2$ anti-mouse IgG antibodies at 4°C for 30 min. After twice washing, the cells were then incubated with mouse immunoglobulins at 4$^{o}$C for 30 min to neutralize the reactivity of the adding conjugates. Subsequently, CD99 molecules were stained using anti-CD99 mAb MT99/1 (IgM isotype) at 4$^{o}$C for

30 min, followed by Alexa Fluor 568-conjugated goat $F(ab')_2$ anti-mouse IgM antibodies at 4°C for 30 min. Finally, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and plated on poly-D-lysine coated slides. Cell nuclei were visualized with Hoechst 33258 dye. For evaluation of colocalization, cells were visualized and images were acquired using a confocal laser scanning microscope (LSM 700; Zeiss, Le Pecq, France).

### 2.14 Immunological synapse and confocal microscopic analysis

Raji B cells ($1x10^7$cells/ml) were loaded with 1 μg/ml of staphylococcal enterotoxin B (SEB) for 15 min at 37°C. Jurkat T cells ($1x10^7$cells/ml) were labeled with CellTrace™ Far Red DDAO-SE. After washing, an equal number of Jurkat cells were mixed with Raji cells and incubated at 37°C for 15 min. Cell mixtures were placed on poly-D-lysine coated slides, fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 5 min on ice. Cells were then incubated with anti-CD99 mAb MT99/3 for 30 min at room temperature. Alexa Fluor 488-labeled goat anti-mouse IgG secondary antibody and TRITC-phalloidin were added and incubated for 30 min. Cell nuclei were stained with Hoechst 33258 dye. The stained cells were analyzed and images were acquired using a confocal laser scanning microscope (LSM 700; Zeiss, Le Pecq, France).

### 2.15 Determination of CD99 in lipid rafts

Lipid rafts were isolated according to the method described elsewhere. Briefly, cells ($1x10^8$) were resuspended in 0.4 ml of ice-cold hypotonic buffer (10 mM HEPES pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, and protease inhibitors), incubated on ice for 15 min and then passed 10 times through the 30-gauge needle. The suspension was

centrifuged for 5 min, 300×g, at 2°C to remove nuclei. The supernatant was re-centrifuged for 10 min, 25,000×g, at 2°C to sediment the membranes. Membranes were then lysed in 0.2 ml of lysis buffer containing 1% Brij-98 or 1% LM for 30 min on ice, and spun at 10,000×g for 30 min. The clarified lysates (0.1 ml) were applied at the top of a 1 ml Sepharose 4B column and sequentially washed with 0.1 ml of the lysis buffer. The fractions (0.1 ml) were collected at 4°C and analyzed for the presence of CD99 by SDS-PAGE and Western immunoblotting.

### 2.16 Proliferation assay

PBMCs were isolated from heparinized whole blood of healthy donors by Ficoll-Hypaque density gradient centrifugation. For staining with carboxyfluorescein diacetate, succinimidyl ester (CFSE) the cells at $1\times10^7$ cells/ml in PBS were incubated at 37 °C for 10 min with the final concentrations of CFSE at 0.5 μM. Staining was terminated by adding cold 10% FCS-RPMI, the cells washed twice and resuspended in 10% FCS RPMI. The CFSE-labeled cells were either left unstimulated or stimulated with Anti-CD3 mAb OKT3. For stimulation, the $1\times10^5$ CFSE-labeled cells were added into each well of Anti-CD3 mAb, OKT3, immobilized the 96-well plates. Then, the mAbs, MT99/3 or isotype matched control 13M, at a final concentration of 20 μg/ml were added. The mixture was cultured at 37°C in a 5% $CO_2$ incubator. At the third day of cultivations, the cells were harvested and assessed by flow cytometry.

### 2.17 Apoptosis assay

Jurkat cells were harvested, washed and resuspended in 10 % FCS-RPMI to a final concentration of $2x10^6$cells/ml. One hundred microliters of the cell suspension were plated into 24 well plates. To each well was added 10% FCS-RPMI in the

presence or absence of mAbs (final concentration of 10 μg/ml) to obtain a final volume of 1 ml. The cells were then incubated in a $CO_2$ incubator for 30 min. After incubation, cells were harvested and resuspended in 100 μl of buffer containing propidiumiodide (PI) and FITC conjugated annexin V at a final concentration of 5 μg/ml. After incubation in a dark room for 15 min, percent of cell death via necrosis and apoptosis were measured using a flow cytometer**.**

### 2.18 Analysis of protein phosphorylation

Cells (5 x $10^7$cells/ml) were stained with 10 μg/ml of purified CD99 mAb MT99/3 or isotype-matched irrelevant control mAb for 30 min on ice. After twice washing with PBS, the cross-linking antibodies, anti-mouse IgG, were added and incubated at 37°C, at each time point, 0.1 ml of cell suspension will be mixed with equal volume of 2X sample buffer, sonicated and further denatured for 3 min at 95°C. The cell lysates were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Phosphorylated proteins were detected by Western blotting using mouse anti-phosphotyrosine, anti-phosphoserine and anti-phosphothreonine mAbs according to the described method in 2.3. Subsequently, the membranes were re-probed with Rabbit anti-GAPDH antibodies as a loading control.