CHAPTER III

RESULTS

3.1 Production of monoclonal antibodies against CD99 molecules

3.1.1 Hybridoma technique for production of anti-CD99 monoclonal antibodies

To produce mAbs to CD99, in this study, the COS cell expression system was employed to prepare recombinant CD99 proteins and used as the immunogen for mouse immunization. Plasmid DNA encoding CD99 protein (Kasinrerk *et al.* 2000) was transfected into COS7 cells and the CD99 expressing COS7 cells were used for intraperitoneal immunization of a BALB/c mouse. Splenocytes from the immunized mouse were then fused with mouse myeloma cells by standard hybridoma technique. Specificity of the obtained mAbs was examined by indirect immunofluorescence staining using CD99-DNA transfected COS7 cells as an antigen. Using this screening procedure, two mAbs strongly positive for the CD99 expressing COS7 cells, but not with un-transfected control cells, were obtained (Table 1 and Figure 3.1). These mAbs were then designated as MT99/1 and MT99/2.

To confirm whether these mAbs were able to bind to native CD99 expressed on human leukocytes, peripheral blood cells were stained with the established mAbs. We found that the mAbs had reactivity to lymphocytes, monocytes, but not to granulocytes (Figure 3.2). In our laboratory, we have previously produced anti-CD99 mAb, named MT99/3 (Kasinrerk *et al.* 2000). In this experiment, cells were also

stained with the mAb MT99/3. The FACS profiles observed using the known anti-CD99 mAb MT99/3 (Kasinrerk *et al.* 2000) were the same of those obtained by the generated mAbs MT99/1 and MT99/2 (Figure 3.2). In addition to leukocytes, red blood cells (RBCs) from six healthy donors were also examined for their reactivity with mAbs MT99/1, MT99/2 and MT99/3. Four donors showed positive and two donors showed negative results with mAbs MT99/1 and MT99/3 (Figure 3.3). Surprisingly, all tested RBCs were negative with the mAb MT99/2 (Figure 3.3).

The reactivity to CD99 expressing COS cells and the cellular distribution patterns, compared to the reported data, suggest that the new generated MT99/1 and MT99/2 are mAbs specific to CD99.

Table 3.1 Reactivity of the generated mAbs with CD99 transfected and non-transfected COS cells

COS cells	Monoclonal antibody		
	MT99/1	MT99/2	M6-1D4
CD99-transfectant	+	+	-
Non-transfectant	-	-	-

COS7 cells were transfected with the plasmid DNA encoding CD99 protein or kept non-transfection. Cells were then stained with the generated mAbs MT99/1, MT99/2 or isotype matched control, anti-CD147 mAb, M6-1D4, by indirect immunofluoresecent staining technique. The stained cells were then analyzed by a fluorescent microscope.

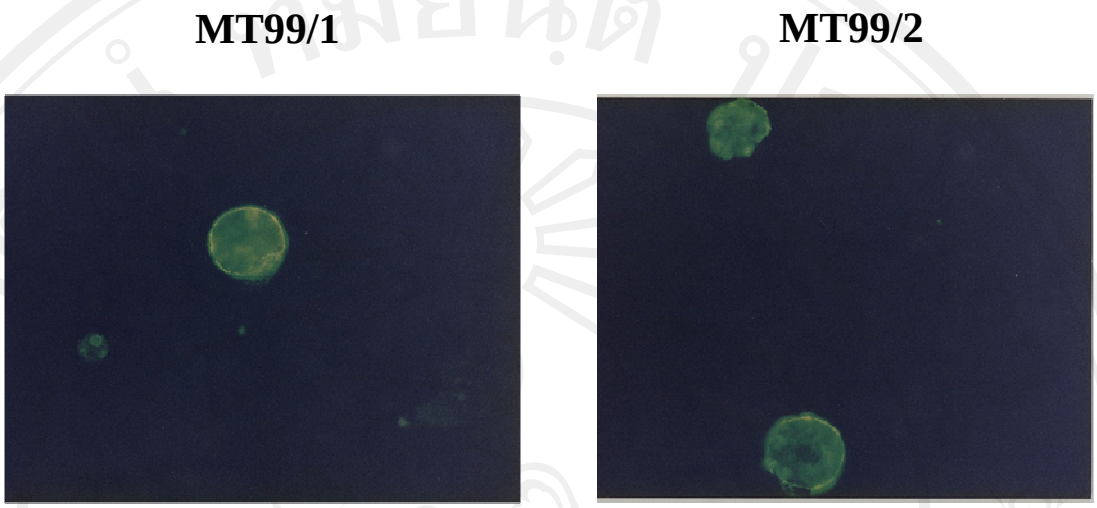

Figure 3.1 Immunofluorescence analysis of reactivity of mAb MT99/1 and MT99/2 with CD99-DNA transfected COS cells.

CD99-DNA transfected COS7 cells were stained with mAbs MT99/1 or MT99/2 by indirect immunofluorescence staining and examined under a fluorescence microscope. The mAb MT99/1 and MT99/2 reacted to the CD99 transfected cells but not to non-transfected COS cells (not shown) (see also Table 1).

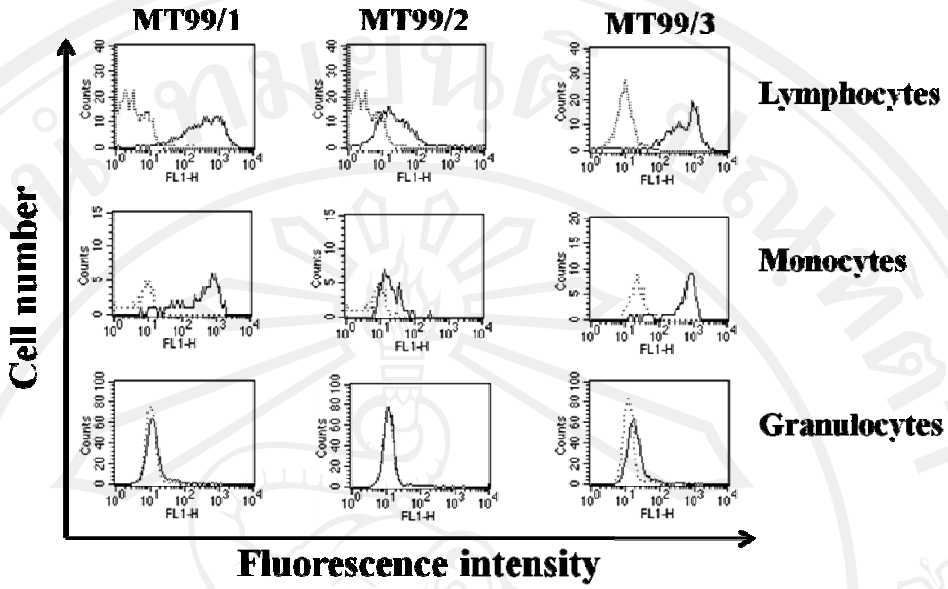

Figure 3.2 Immunofluorescence analysis of the reactivity of mAbs MT99/1, MT99/2 and MT99/3 with peripheral blood leukocytes.

Peripheral blood lymphocytes, monocytes, and granulocytes were stained with mAbs MT99/1, MT99/2 and MT99/3 by indirect immunofluorescence and analyzed by flow cytometry. Conjugated controls are shown as dashed lines, while cells stained with the indicated mAbs are represented by solid lines. A representative result from one of three donors is shown.

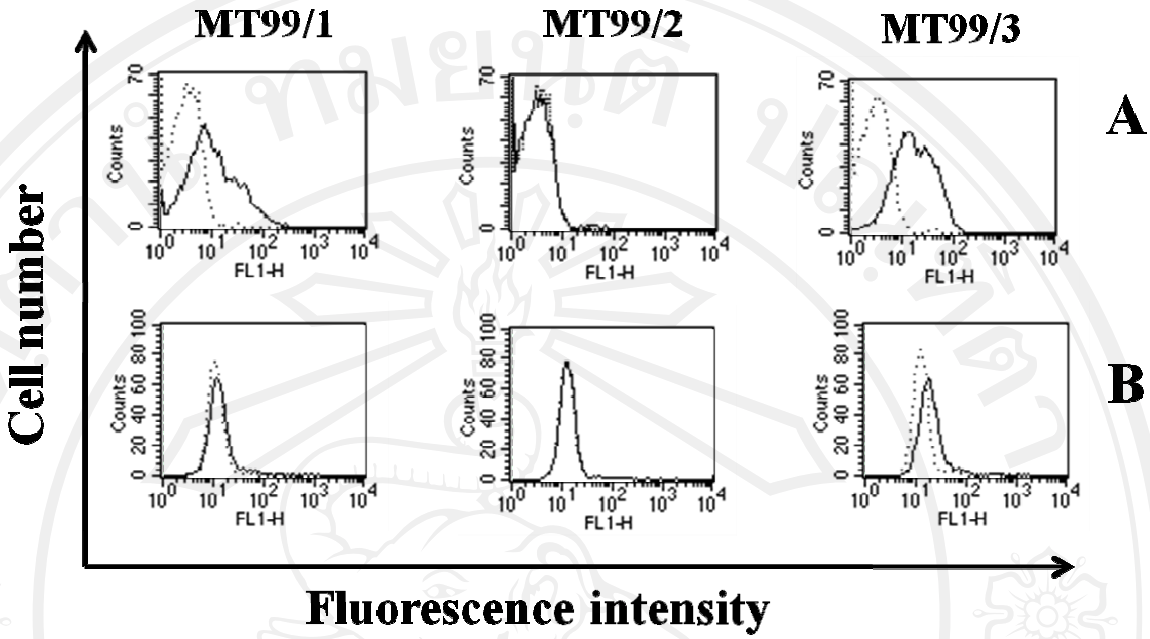

Figure 3.3 Reactivity of mAbs MT99/1, MT99/2 and MT99/3 with red blood cells. RBCs were stained with mAbs MT99/1, MT99/2 and MT99/3 by indirect immunofluorescence staining and analyzed by flow cytometry. (A) FACS profiles of RBCs which show positive reactivity with mAbs MT99/1 and MT99/3, but negative with mAb MT99/2. (B) FACS profiles of RBCs which show negative reactivity with mAbs MT99/1, MT99/2 and MT99/3. Conjugated controls are shown as dashed lines, while cells stained with the indicated mAbs are represented by solid lines.

3.1.2 Western blotting and immunoprecipitation experiments using the generated anti-CD99 mAbs

The specificity of mAbs MT99/1 and MT99/2 was confirmed by Western blotting. The previously produced anti-CD99 mAb MT99/3 was used as positive control and anti-CD4 mAb MT4 was used as an isotype matched control mAb. The mAb MT99/1 recognized two protein bands with molecular weights of 28 and 32 kDa, as was also observed using the known CD99 mAb MT99/3 (Figure 3.4). The mAb MT99/2, however, did not recognize any protein band (Figure 3.4). These findings indicated that the mAbs MT99/1 and MT99/3 recognizes a linear epitope on the CD99 molecule. For mAb MT99/2, as no reactive band could be determined, it is suggested that this mAb reacts to a conformational epitope on the CD99 molecule.

Immunoprecipitation was then employed to confirm the specificity of the generated mAbs. The mAbs MT99/1, MT99/2 and MT99/3 were used to precipitate its recognized molecules from Jurkat cell lysates. Western blotting with anti-CD99 mAb MT99/3 was used to detect the CD99 molecules in the immunoprecipitated protein samples. As shown in Figure 3.5, two forms of CD99 molecule, 28 and 32 kDa, were detected in immunoprecipitation of either mAbs MT99/1, MT99/2 or MT99/3. In contrast, isotype matched control mAbs, MT4 and 4G2, did not precipitate any protein band at the molecular weight corresponding to the CD99 proteins.

Taken together, results from cell staining, Western blotting and immunoprecipitation experiments indicated that the generated mAb MT99/1 and MT99/2 are anti-CD99 mAbs. Therefore, in our laboratory three mAbs, MT99/1, MT99/2 and MT99/3, against CD99 molecule were generated. The mAbs MT99/1

and MT99/3 recognize the linear epitope, whereas the mAb MT99/2 may recognize conformational epitope on the CD99 molecule.

3.2 Characterization of CD99 molecules

3.2.1 Expression of CD99 molecules on various hematopoietic cell lines

To study the expression profile of CD99 molecules on various hematopoietic cell lines, several cell lines were stained with our produced anti-CD99 mAb MT99/3 and analyzed by flow cytometry. It was found that all of the tested cell lines, including Ramos, Raji, Jurkat, SUP-T1, U937 and THP-1 were positive with anti-CD99 mAb (Figure 3.6). Interestingly, among the tested cell lines, only Ramos cells expressed CD99 in heterogeneous manner, i.e., there were CD99 expressing and non-expressing populations.

3.2.2 Biochemical characterization of CD99 molecules on peripheral blood cells and cell lines

On the cell surface, CD99 was demonstrated to express as two distinct isoforms, long form and shot form, depending on the alternative splicing of the encoding gene (Hahn *et al.* 1997). The long form contains 185 amino acid residues with the MW of 32 kDa. The short form (161 amino acid residues) has the MW of 28 kDa. We then, investigated the expression of CD99 isoforms in various hematopoietic cells and cell lines. The cell lysates were resolved by 10% SDS-PAGE under reducing condition. Western blotting using mAb MT99/3 was then performed. CD99 long form was found in all tested peripheral blood cells including thrombocytes, RBCs, monocytes, NK cells, B cells, T cells, except granulocytes (Figure 3.7A), CD99 long form was also

found in all cell lines tested (Raji, Ramos, SUP-T1, Jurkat, THP-1 and U937 cells) (Figure 3.7B). Whereas, CD99 short form was found only in Jurkat and SUP-T1 cell lines (Figure 3.7B). The results indicated that CD99 isoforms are differentially expressed in a cell type-specific manner among hematopoietic cells and cell lines, CD99 long form being predominantly expressed.

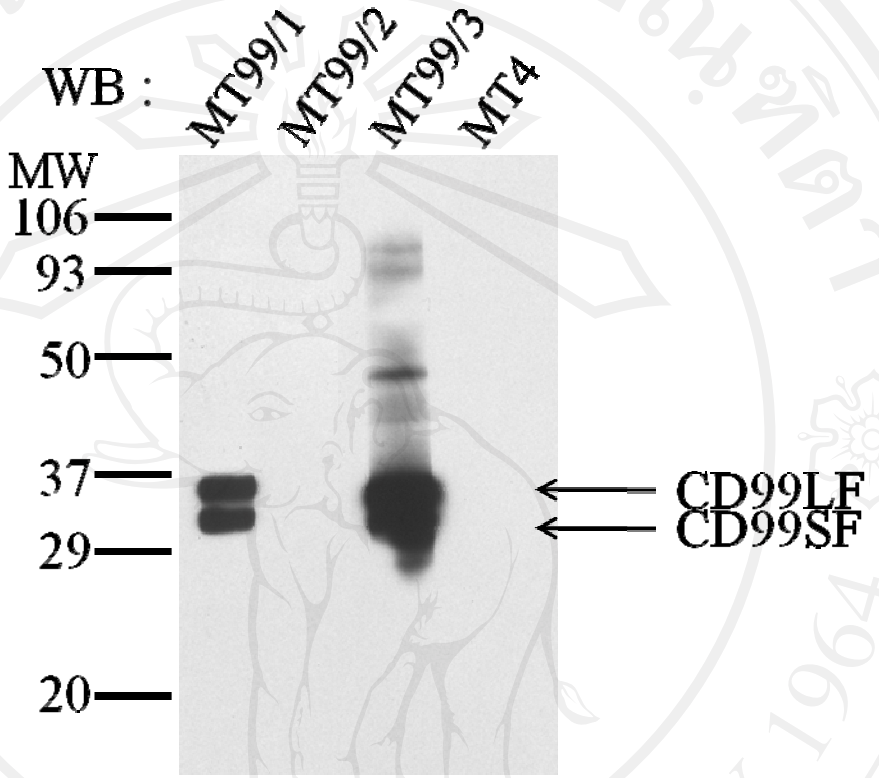

Figure 3.4 Western blotting analysis of cell surface antigen recognized by mAbs MT99/1, MT99/2 and MT99/3.

Jurkat cell lysate was resolved by 10%SDS-PAGE under non-reducing conditions. Western blotting was performed by using mAbs MT99/1, MT99/2 or MT99/3 and isotype matched control mAb MT4. Protein markers with molecular weight indicated in kDa are shown

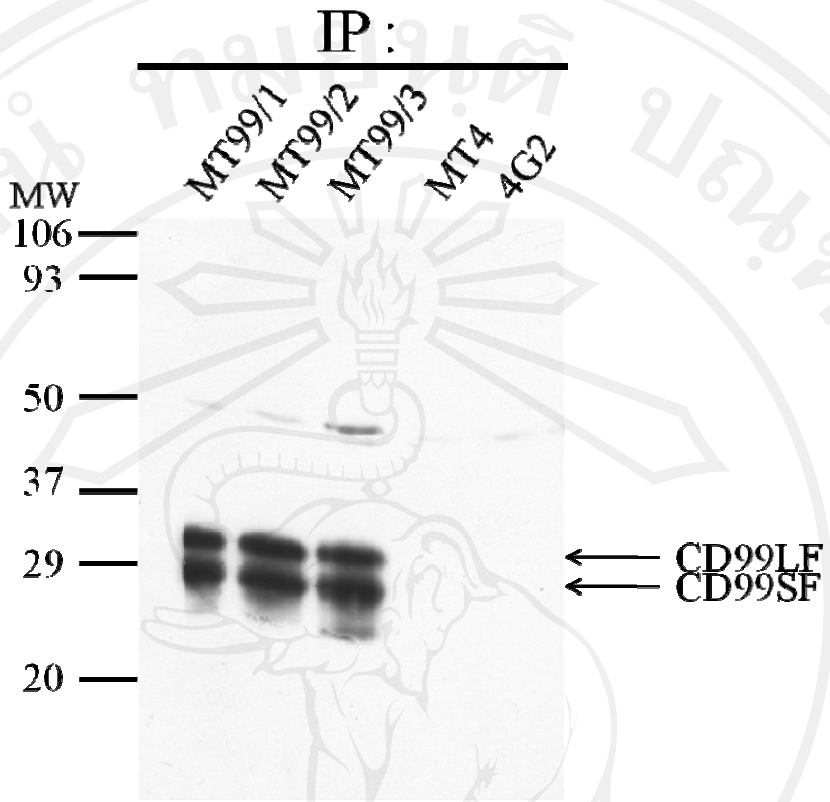

Figure 3.5 Immunoprecipitation analysis of cell surface antigen recognized by mAbs MT99/1, MT99/2 and MT99/3.

The Jurkat cell lysates were subjected to immunoprecipitation using anti-CD99 mAbs MT99/1, MT99/2, MT99/3, isotype matched control anti-CD4 mAb MT4 (IgM) and isotype matched control mAb 4G2 (IgG2a). The immunoprecipitated proteins were resolved by SDS-PAGE under reducing conditions followed by Western blotting using mAbs MT99/3 as CD99 tracer. Protein markers with molecular weight indicated in kDa are shown.

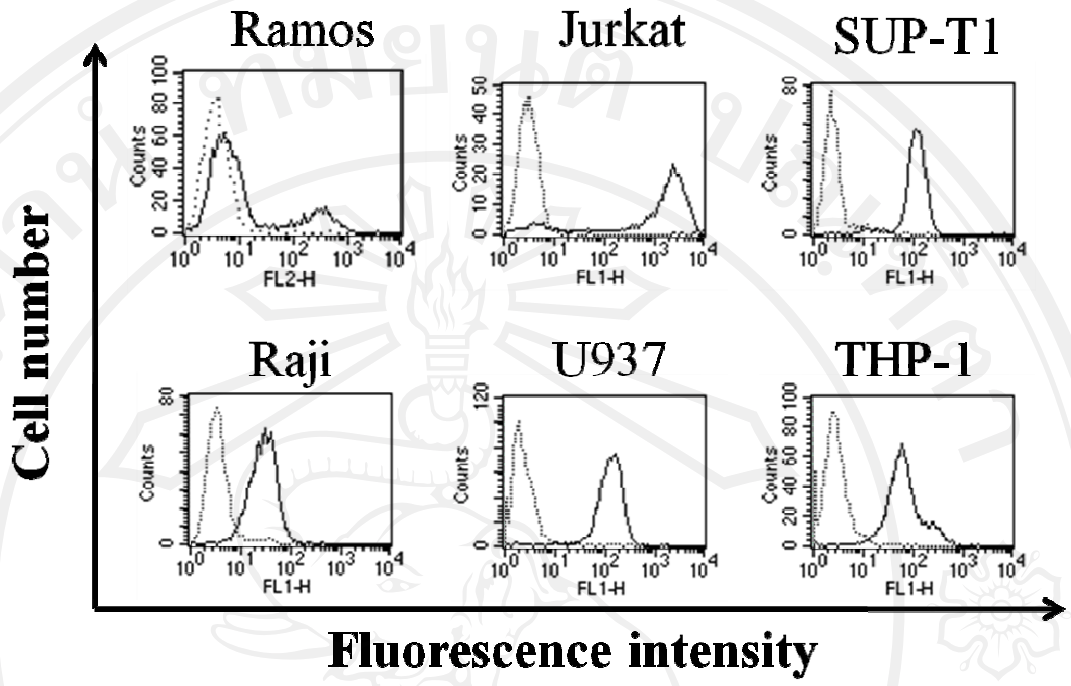

Figure 3.6 Distribution of the CD99 molecule on various hematopoietic cell lines. The indicated cells were stained with anti-CD99 mAb MT99/3 mAb by indirect immunofluorescence and analysed by flow cytometry. Conjugated controls are shown as dashed lines, while cells stained with the indicated mAbs are represented by solid lines.

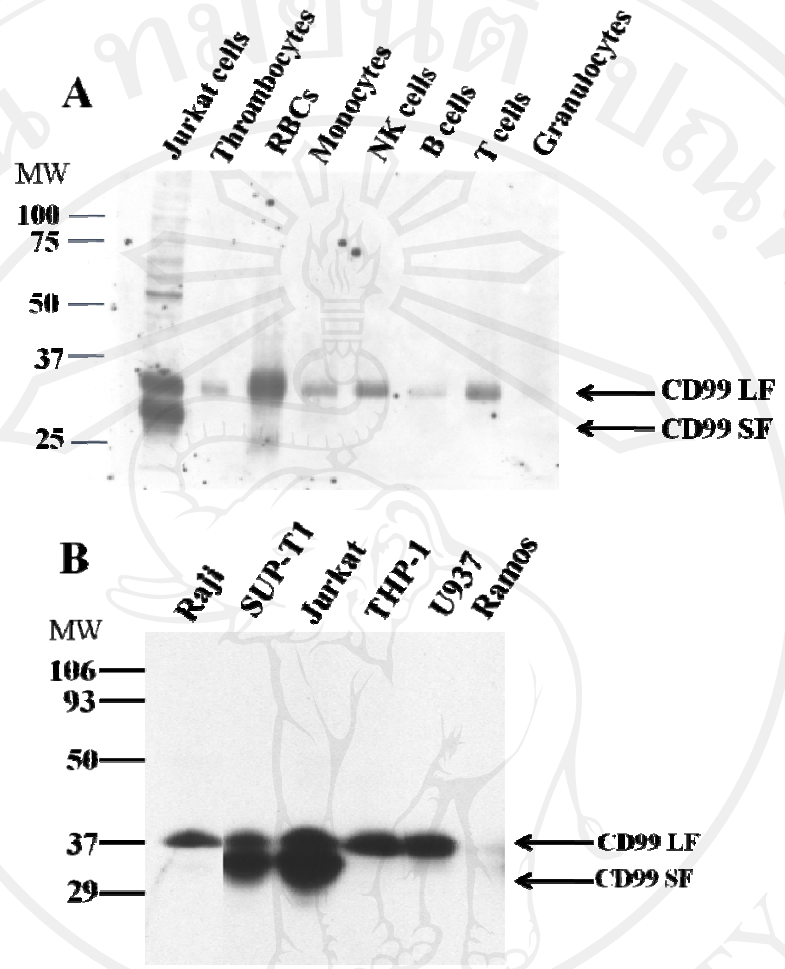

Figure 3.7 Expression of CD99 isoforms on peripheral blood cells (A) and hematopoietic cell lines (B).

The cell lysates obtained from the indicated cells were separated by SDS-PAGE under reducing conditions and blotted onto a PVDF membrane. The membrane was immunostained with anti-CD99 mAb MT99/3. Protein markers with molecular weight indicated in kDa are shown.

3.3 Identification of CD99 associated molecules

Several reports have indicated that CD99 is a multi-functional cell surface molecule and play a key role in several biological processes (Waclavicek *et al.* 1998; Bernard *et al.* 2000; Kasinrerk *et al.* 2000; Schenkel *et al.* 2002; Khunkaewla *et al.* 2007). However, this molecule contains a short intracellular domain (Banting *et al.* 1989). Interaction with other cell surface molecules is, therefore, assumed to be necessary in order to regulate its multiple functions. In this study, we explored the possibility that CD99 may form a microdomain with other proteins on the cell surface

3.3.1 Association of CD99 molecules with various membrane proteins

In an attempt to identify the CD99 interacting molecules, coimmunoprecipitation of surface biotinylated cell lysates using anti-CD99 mAbs (MT99/1, MT99/2 and MT99/3) was employed.

Biotin labeled Jurkat cells were solubilized in the presence of 1% nonionic detergent Brij-58 and subjected to coimmunoprecipitation. As shown in Figure 3.8, a number of cell membrane proteins, at molecular weight 110, 50 and 40 kDa, were co-precipitated with the CD99 molecules whereas none of any proteins at the indicated MW was found in immunoprecipitation of Ig isotype matched control. On the basis of this observation, we hypothesized that CD99 molecules interact directly with several molecules. Furthermore, due to our results, the highest number of CD99 associated molecules was observed in MT99/3 mAb immunoprecipitation, the mAb MT99/3 was selected for further coimmunoprecipitation experiments.

To confirm whether the association of CD99 and other membrane proteins was found also in other cell types, several cell types (including PBMCs, Jurkat, U937, Raji) were subjected for the coimmunoprecipitation experiments. As shown in 3.9, by mAb MT99/3, coimmunoprecipitation between CD99 and the proteins at molecular weight of 110, 50 and 40 kDa were found in all tested cell lysates. These results indicate that association of CD99 and various proteins is observed in both peripheral blood mononuclear cells and various cell lines. In the coimmunprecipitation experiments, the association of CD99 with other proteins may come from the artificial effect of lipid raft association. To exclude the possible artificial effect of lipid raft association, the CD99 containing complexes were immunoprecipitated using raft disrupting detergent 1% LM (Figure 3.10A). Similar results were obtained using mild detergent 1% Brij-58 (Figure 3.10B). Our results indicated that the CD99 interact directly with other cell surface molecules.

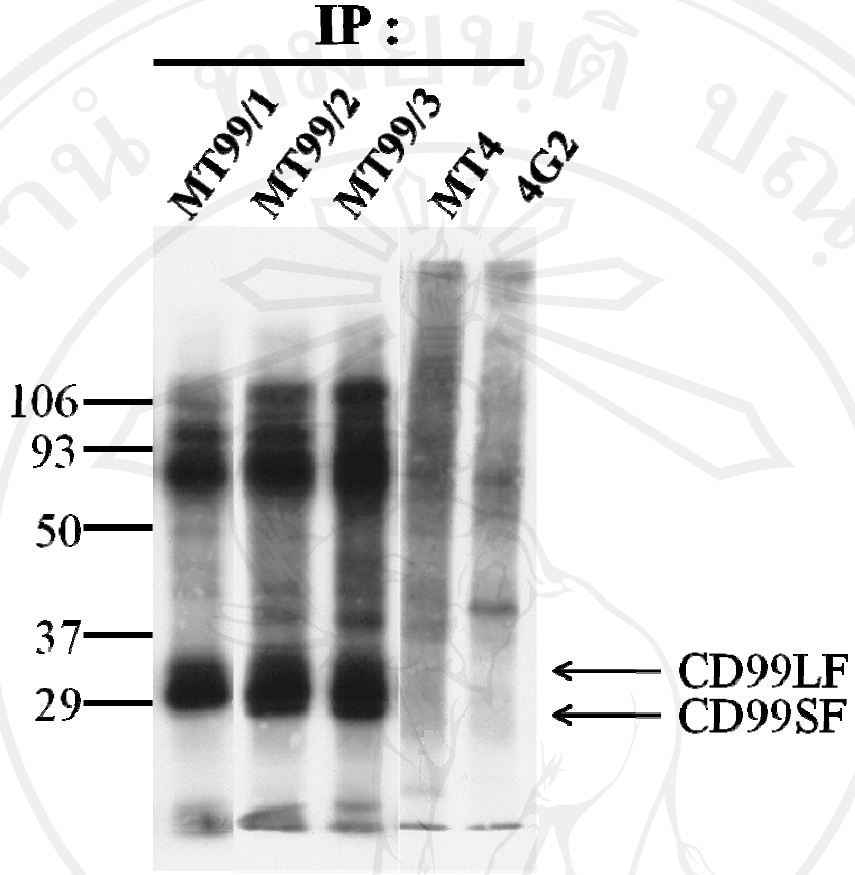

Figure 3.8 Coimmunoprecipitation of CD99 with other membrane proteins.

Biotinylated Jurkat cells were lysed using a mild detergent 1% Brij-58. The cell lysates were immunoprecipitated using anti-CD99 mAb MT99/1, MT99/2, MT99/3 or isotype matched control mAbs, MT4 and 4G2. Electrophoresis was performed under reducing conditions. The positions of molecular mass markers are indicated on the left in kDa. The positions of CD99 molecules are indicated.

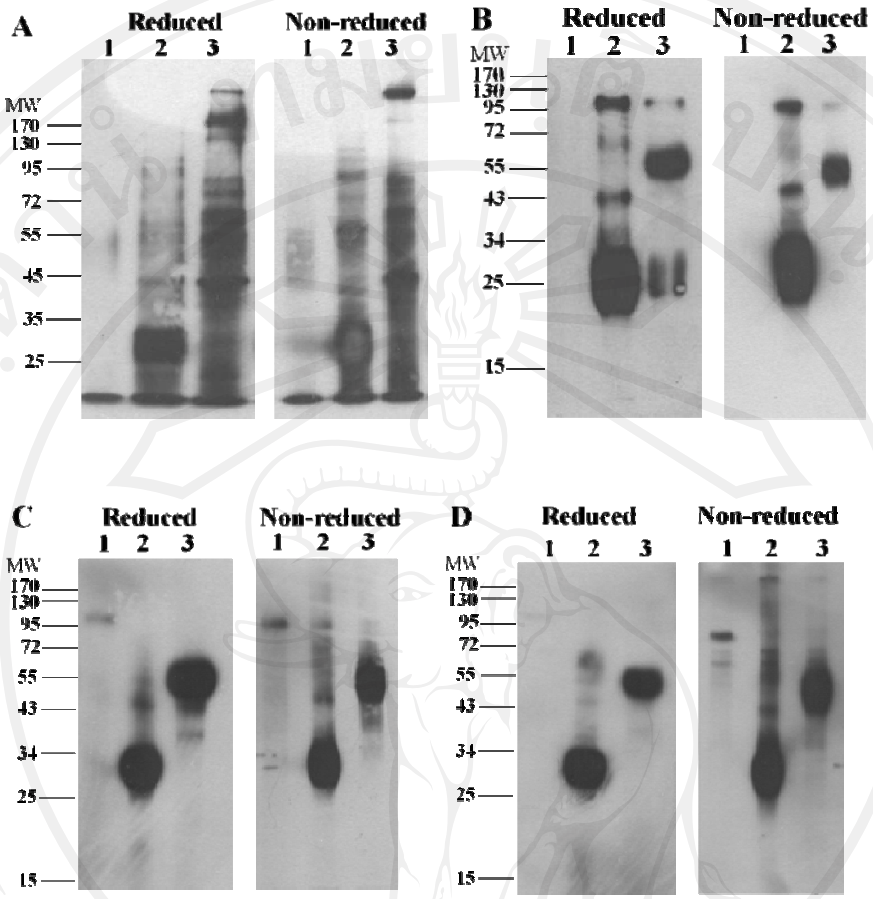

Figure 3.9 Coimmunoprecipitation of CD99 with other membrane proteins using cell lysates of various cell types.

Immunoprecipitation was performed using lysates of biotin labeled PBMCs (A), Jurkat cells (B), U937 cells (C), Raji cells (D) and precipitated by mAb 4G2 (lane 1), anti-CD99 mAb MT99/3 (lane 2), anti-CD147 mAb M6-1E9 (lane 3). Electrophoresis was performed under reducing or non-reducing condition. The positions of molecular mass markers are indicated on the left in kDa.

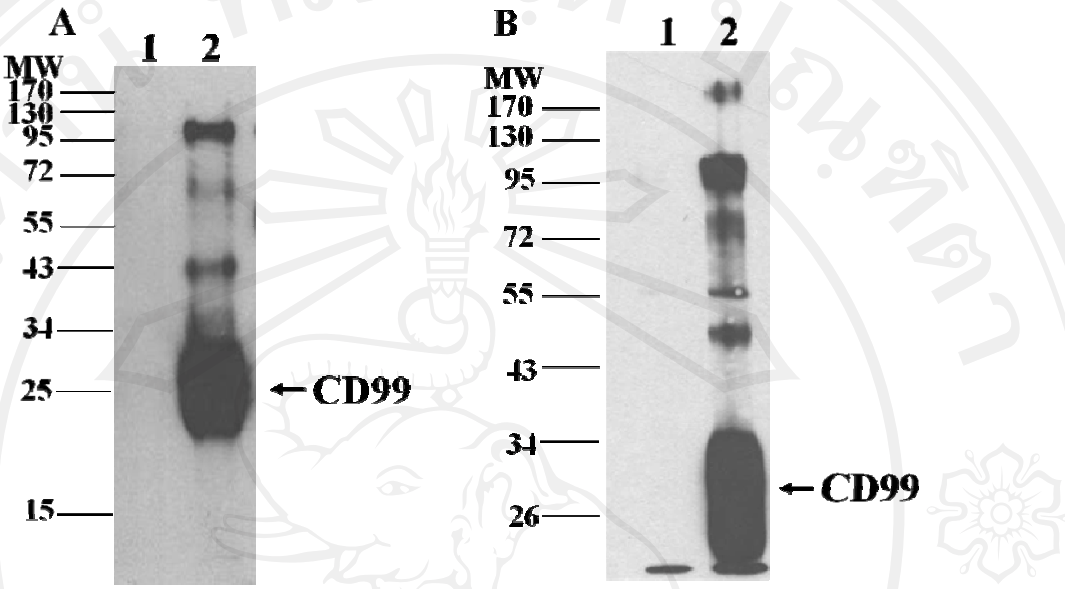

Figure 3.10 Coimmunoprecipitation of CD99 with other membrane proteins using raft-disrupting detergent 1% Lauryl maltoside and mild detergent 1% Brij-58.

Immunoprecipitation of biotinylated Jurkat cell lysates using mild detergent 1% laurylmaltoside (A) or raft-disrupting detergent 1% Brij-58 (B). Cell lysates were precipitated using isotype matched control mAb 4G2 (lane 1) and anti-CD99 mAb MT99/3 (lane 2). Electrophoresis was performed under reducing conditions. The positions of molecular mass markers are indicated on the left in kDa. The positions of CD99 molecules are indicated.

To identify the CD99 interacting molecules, the CD99 interacting molecules were isolated by coimmunoprecipitation technique using CD99 mAb MT99/3 and followed by amino acid sequencing. Aliquots of the immunoprecipitated proteins were loaded on a one-dimensional SDS-PAGE gel, which were then stained by silver dye (Figure 3.11) and Colloidal Coomassie blue dye. The 40 and 50 kDa protein bands, which were not found in the immunoprecipitation of isotype matched control mAb, were excised from Colloidal Coomassie-stained SDS-PAGE gels. The isolated protein bands were subjected to tryptic digestion and MALDI-MS/MS protein identification. The identities of the peptides were determined by comparison with the nonredundant NCBI data base using the Mascot search engine. Analyses of the two protein bands, 40 and 50 kDa, revealed peptides aligned with the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; accession number NP_002037) and the protein DNA-damage inducible 1 homolog 2 (DDI2; accession number NP_115717.3), respectively. In addition, as a control, the band at MW corresponding to CD99 molecule was also excised from Colloidal Coomassie-stained SDS-PAGE gels and subjected to tryptic digestion and MALDI-MS/MS protein identification. As predicted, this protein band revealed peptides aligned with the human CD99 molecule. Therefore, the identified proteins might be the correct CD99 associated molecules.

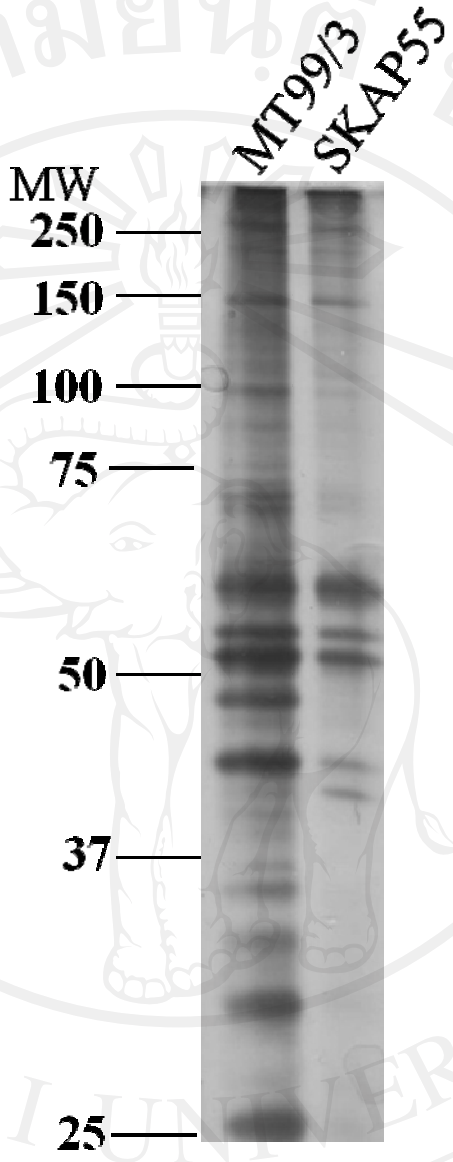

Figure 3.11 Silver stained gel of CD99 immunoprecipitates.

Jurkat cell lysates were immunoprecipitated by using anti-CD99 mAb MT99/3 or isotype matched control mAb SKAP55. The CD99 or control immunoprecipitates were loaded on a one-dimensional SDS-PAGE gel. The gel was then stained by silver dye. The positions of molecular mass markers are indicated on the left in kDa.

3.3.2 Investigation of possible interaction between CD99 and GAPDH

GAPDH is an enzyme of 37 kDa that catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules. In addition to this long established metabolic function, GAPDH has recently been implicated in several non-metabolic processes, including transcription activation, initiation of apoptosis and ER to Golgi vesicle shuttling (Hara *et al.* 2005; Tarze *et al.* 2007). In our previous experiment, we identified the GAPDH as one of the CD99 interacting partners. We speculated that GAPDH may play a role and maintain the CD99 function.

To confirm the interaction of CD99 and GAPDH, CD99-coimmunoprecipitated proteins were analyzed by Western blotting with GAPDH mAb. It was found that the GAPDH was not found only in CD99 immunoprecipitation but also in the immunoprecipitation of isotype match control mAb (Figure 3.12). Therefore, the co-isolation of GADPH with CD99 appears to be non-specific nature.

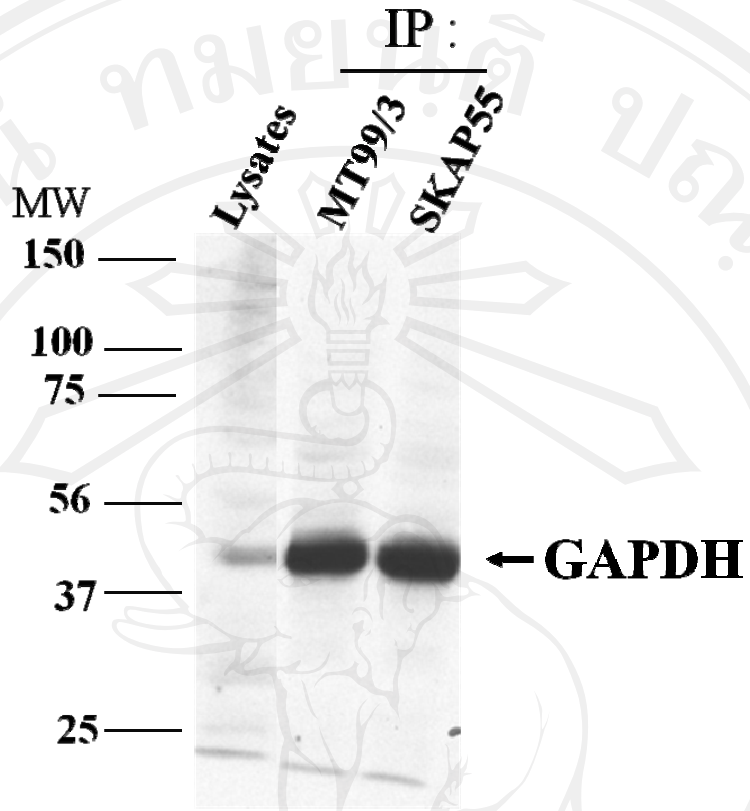

Figure 3.12 Investigation of the interaction between CD99 and GAPDH

Lysates of Jurkat cells were immunoprecipitated with mAb MT99/3 and isotype matched control mAb SKAP55. Jurkat cell lysates and the immunoprecipitates were resolved by SDS-PAGE under reducing conditions followed by Western blotting using anti-GAPDH antibodies. Protein markers with molecular weight indicated in kDa are shown.

3.3.3 Investigation of possible interaction between CD99 and DDI2

3.3.3.1 Generation of plasmid DNA encoding DDI2 Myc tag fusion protein

The DNA-damage inducible 1 homolog 2 (DDI2) belongs to novel family of predicted retroviral-like aspartyl proteases, the DDI1 family (Krylov and Koonin 2001; Su and Lau 2009). This protein family was suggested to play a possible key role in eukaryotic cell cycle control (Krylov and Koonin 2001; Su and Lau 2009). From our previous experiments, the DDI2 was suggested to be a CD99 integrating molecule. For further verification of the interaction of CD99 and DDI2, the DDI2-Myc recombinant protein was generated. In our study, first, we generated the vector encoding DDI2 protein. The Jurkat cDNA libraries was constructed and used as the template for amplification of human DDI2 coding region by PCR. The amplified product of 1200 bp corresponding to cDNA encoding DDI2 protein was demonstrated by agarose gel electrophoresis (Figure 3.13, lane 1), whereas no PCR product was found in the negative control, non reverse transcriptase added template, (Figure 3.13, lane 2). The Myc-tagged DDI2 construct was generated by PCR using the primer containing sequence for Myc. Subsequently, the PCR product was cloned into *EcoR* I site of retroviral vector pMSCV. The resulting vector was named pMSCV-DDI2Myc. The pMSCV-DDI2Myc vector was then transformed into the *E. coli* TOP10 and the amplicilin-resistant colonies were selected for purification of the plasmid vector. The correct insertion of DDI2-Myc gene was verified again by restriction fragment analysis using restriction enzymes *EcoR* I and *Nco* I (Figure 3.13, lane 3 and 4) and by DNA sequencing. The sequence of the inserted sequence in pMSCV-DDI2Myc was completely identical to the nucleotide sequence of the

known DDI2 gene. Hence, the pMSCV-DDI2Myc vector could be use to produce recombinant DDI2-Myc proteins

3.3.3.2 Verification of DDI2-Myc protein expression

To produce DDI2-Myc protein, the pMSCV-DDI2Myc vectors were transfected into HEK293 cells. To determine the DDI2-mys preotin expression, the transfected cell lysates were analyzed by Western blotting using anti-Myc mAb (9B11). As shown in Figure 3.14, a protein band at MW 55 kDa corresponding to DDI2-Myc protein was found in pMSCV-DDI2Myc transfected cell lysates, but not in pMSCV transfected control lysates. The pMSCV-DDI2Myc vector was, therefore, used to prepare Myc-tagged forms of DDI2 in the further experiments.

3.3.3.3 Generation of CD99 expressing and CD99 non-expressing cells

We then studied the association of CD99 and the DDI2 proteins. To obtain more precise results, we first established CD99 expressing and CD99 non-expressing cells. As was shown in Figure 3.6 and Figure 3.15A, Ramos cells express CD99 in a heterogeneous manner (they contain both CD99 expressing and non-expressing populations during cultivation). The Ramos cells were, therefore, selected and separated by immunomagnetic sorting resulting in CD99 positive and CD99 negative populations (Figure 3.15B and C). During long-term cultivation, the CD99 negative cells retained their CD99 negative phenotype, while the CD99 positive population returned to the wild type heterogeneous profile. Hence, the Ramos wild type and CD99 negative cells were employed for further coimunoprecipitation experiments to study the association of CD99 and DDI2.

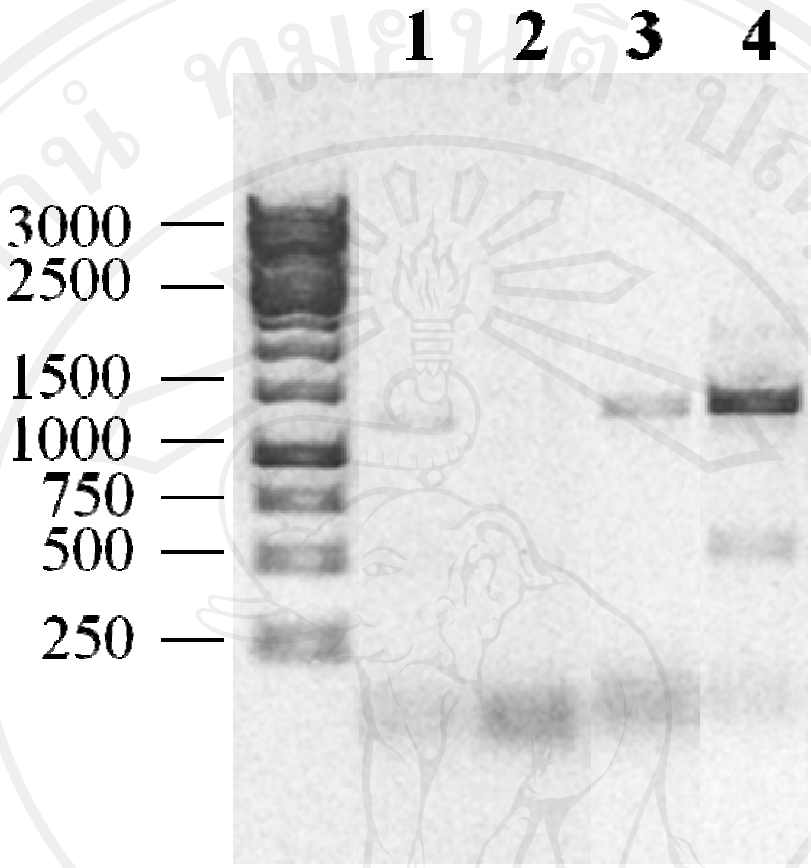

Figure 3.13 Agarose gel electrophoresis of amplified PCR product and restriction fragment analysis of pMSCV-DDI2Myc.

DDI2 gene was amplified from Jurkat cell cDNA library by PCR. Amplified PCR products of DDI2 gene at 1200 bp (lane 1) and product from non reverse transcriptase added template (lane 2). The pMSCV-DDI2Myc was digested with *EcoR* I enzyme (lane 3) and *Nco* I (lane 4). Standard DNA markers (bp) are indicated on the left.

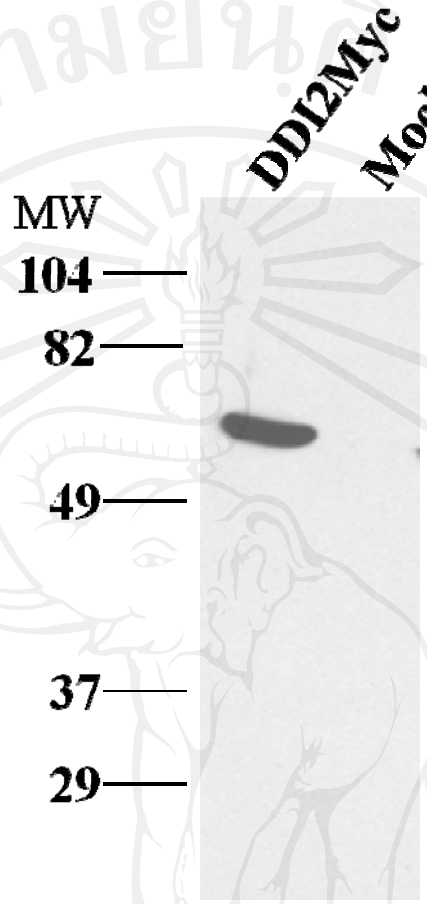

Figure 3.14 Western blot analysis of pMSCV-DDI2Myc transfected HEK293

Plasmid DNA encoding DDI2-Myc fusion protein, pMSCV-DDI2Myc vector, or pMSCV control vector were transfected into HEK293 cells. Lysates of pMSCV-DDI2Myc transfected HEK293 and pMSCV transfected HEK293 were analyzed by Western blotting using anti-Myc mAb. Protein markers with molecular weight indicated in kDa are shown.

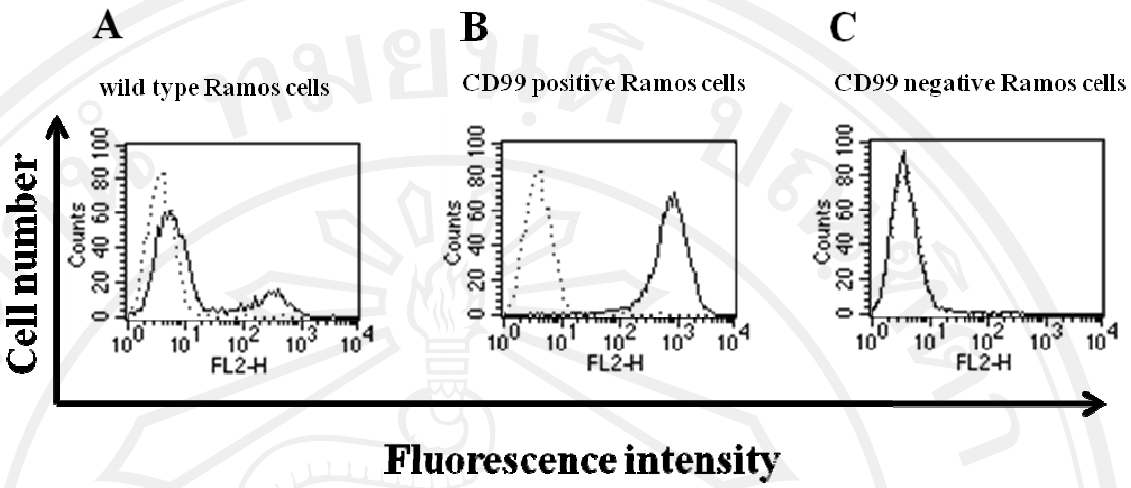

Figure 3.15 Immunofluorescence analysis of CD99 expression on Ramos wild type and CD99 sorted cells.

Ramos cells were chosen for establishment of CD99 expressing and non-expressing cells by an immunomagnetic sorting system using anti-CD99 mAb. The sorted cells were then verified for their surface CD99 expression by immunoflurorescence and flow cytometry. The wild type (A), CD99 positive (B) and CD99 negative (C) cells were stained with anti-CD99 mAbs MT99/3 and PE-conjugated anti-mouse immunoglobulin antibodies. Solid lines represent the immunofluorescence profiles of the cells stained with anti-CD99 mAb and dashed lines represent background fluorescence of conjugate control.

3.3.3.4 Verification of possible interaction between CD99 and DDI2

To confirm the interaction between CD99 and DDI2, we introduced the pMSCV-DDI2Myc vector into Ramos wild type and CD99 negative Ramos cells. Coimmunoprecipitation analysis of the transfected cell lysates using anti-CD99 mAb was then performed. The precipitated proteins were then subjected for Western immunoblotting using anti-CD99 mAb and anti-Myc mAb. In immunoblotting analysis (Figure 3.16), unfortunately, the DDI2-Myc protein was detected in the CD99 precipitated proteins of both DDI2-Myc expressing Ramos wild type and CD99 negative Ramos cell lysates. So far, we still cannot give any explanation on the obtained results. It seems likely that mAb MT99/3 may also bind the DDI2 protein. If this speculation is correct, the appearance of DDI2 in CD99 immunoprecipitated proteins is because of the cross reactivity of the mAb MT99/3. Other experiments are need for proving this assumption. From our results, however, the association of CD99 and DDI2 remains to be clarified.

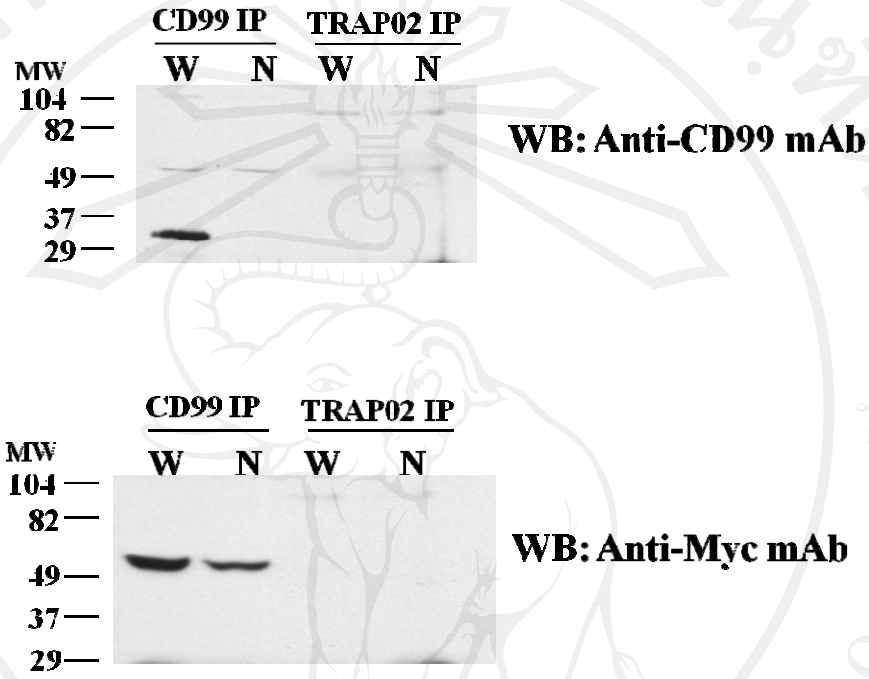

Figure 3.16 Verification of the interaction between CD99 and DDI2 protein.

DDI2-Myc expressing Ramos wild type (W) and CD99 negative Ramos cells (N) were lysed. The cell lysates were immunoprecipitated with MT99/3 mAb and isotype matched control mAb TRAP02. The cell lysates and immunoprecipitates were analyzed by Western blotting with anti-CD99 mAb and anti-Myc mAb. Protein markers with molecular weight indicated in kDa are shown.

3.3.4 Production of mAbs to CD99 interacting molecules

In an attempt to identify further CD99 associated molecules, we then generated mAbs against the CD99 interacting molecules. Recently, we have developed a technique for production of mAbs to immunoprecipitated proteins (Pata *et al.* 2009). The developed technique was then applied for production mAbs to CD99 associated molecules. The BALB/c mice were immunized with CD99-immunoprecipitated beads. Splenocytes from an immunized mouse were then fused with mouse myeloma cells by standard hybridoma technique. Specificity of the obtained mAbs was examined by indirect immunofluorescence staining using Jurkat cells as an antigen and dot blot assay using CD99-immunoprecipitated protein as an antigen. Using this screening procedure, a mAb was identified which recognized a membrane protein of Jurkat cells and were positive by the dot blot assay. These mAbs were further screened by Western blotting using CD99 immunoprecipitated proteins as an antigen. Out of 21 hybridomas, five hybridoma clones were of interest and named CD99-BW1, CD99-BW7, CD99-BM9, CD99-BM52, and CD99-BW42. The reactivities of the generated hybridoma clones are shown in Table 2 and Figure 3.17. The mAb CD99-BW1, CD99-BW7 and CD99-BM9 reacted to a protein with MW of 40 kDa. The mAb CD99-BM52 reacted with 50 kDa protein, whereas the mAb CD99-BW42 reacted with 110 kDa protein. All of the rest clones showed positive reactivity with the protein at MW approximately 25-35 kDa which is corresponding to the CD99 molecule. Additionally, the isotype of mAbs produced by the generated hybridoma clones were determined by capture ELISA. It was found that, the isotype of all generated hybridoma clones are IgM.

Table 3.2 Summary of generated mAbs against CD99 associated molecules

MW of the recognized protein (kDa)	Monoclonal antibody named	Isotype
40	CD99-BW1, CD99-BW7, CD99-BM9	Ig M
50	CD99-BM52	Ig M
110	CD99-BW42	Ig M

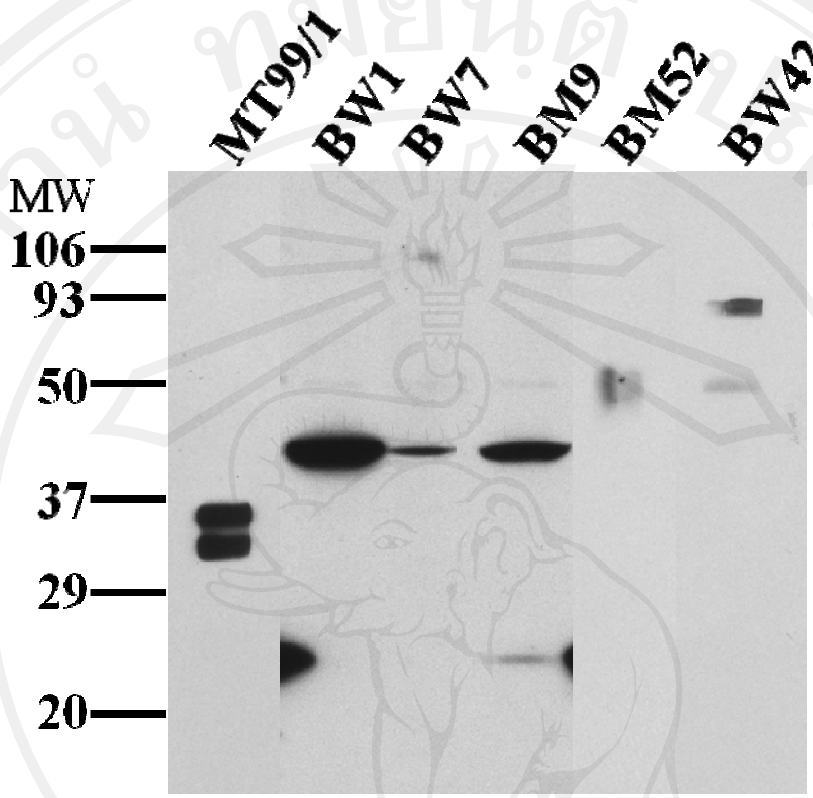

Figure 3.17 Reactivity of the generated mAbs with the CD99 precipitated proteins.

Jurkat cell lysates were immunoprecipitated using mAb MT99/3. CD99 immunoprecipitates were resolved by SDS-PAGE. Consequently, Western blotting was performed by using the generated mAbs, CD99-BW1 (BW1), CD99-BW7 (BW7), CD99-BM9 (BM9), CD99-BM52 (BM52), and CD99-BW42 (BW42) or mAb MT99/1(MT99/1). Protein markers with molecular weight indicated in kDa are shown.

3.3.4.1 Specificity of the generated mAbs

To confirm whether the generated mAbs specifically reacted to CD99 associated molecules, the reciprocal coimmunoprecipitation was carried out. The generated mAbs were used to precipitate the protein from Jurkat cell lysates. Then, the precipitated proteins were separated by SDS-PAGE under reducing conditions. After blotting on nitrocellulose membrane, the membrane was probed with the mAb MT99/3 and isotype matched control mAb 4G2. Two forms of CD99 molecule, 28 and 32 kDa, were observed in immunoprecipitation using mAbs CD99-BM52 and CD99-BW42 (Figure 3.18). The long form of CD99 was found in immunoprecipitation of mAbs CD99-BW1, CD99-BW7 and CD99-BM9. In contrast, in immunoprecipitation using isotype matched control mAb, M6-1D4, no protein band corresponding to CD99 molecule was observed. In addition, by Western blotting using isotype matched control mAb, 4G2, no immune-reactive band corresponding to CD99 molecule was observed in all immunoprecipitated proteins (data not shown). These results indicated that the generated mAbs are the mAbs that specifically react with molecules associated to CD99.

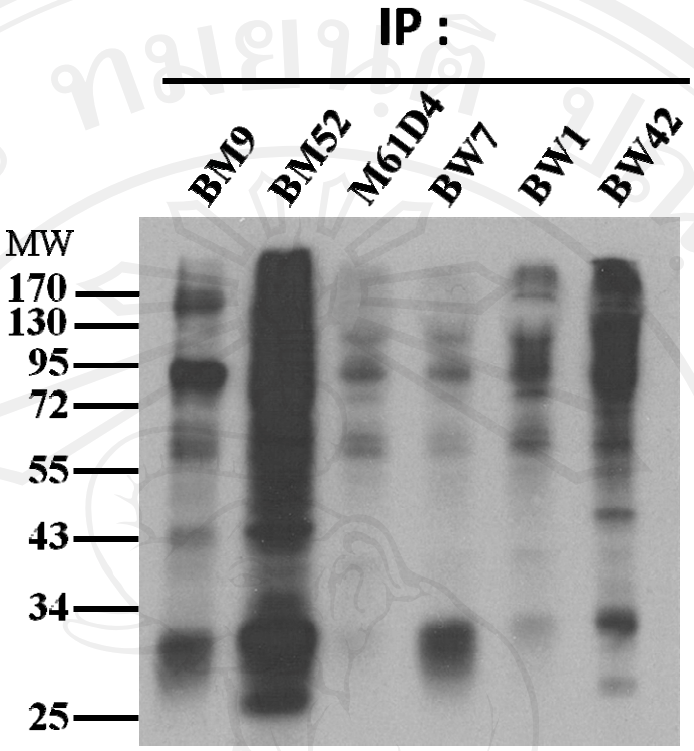

Figure 3.18 Verification of the interaction between the proteins recognized by the generated mAb and CD99 molecules

Jurkat cell lysates were immunoprecipitated using the generated mAb against CD99 associated molecules, CD99-BW1 (BW1), CD99-BW7 (BW7), CD99-BM9 (BM9), CD99-BM52 (BM52), and CD99-BW42 (BW42) or IgM isotype matched control mAb M6-1D4. Subsequently, Western blotting was performed by using CD99 mAb MT99/3. Protein markers with molecular weight indicated in kDa are shown.

3.3.4.2 Isolation of CD99 associated molecules using the generated mAbs

To further identify the CD99 associated molecules, the coimmunoprecipitation experiments using the generated mAbs against CD99 interacting partners were performed. For peptide identification by mass spectrophotometry, the precipitated proteins were separated by SDS-PAGE under reducing condition and non-reducing condition. The gel was then stained with silver dye (Figure 3.19). Unfortunately, we could not detect any protein band that appeared only in the immunoprecipitation using the generated mAbs when compared to the isotype matched control mAb. Therefore, in our immunoprecipitation using the generated CD99 interacting partner mAbs, we could not prepare an appropriated sample for peptide identification. It is likely that the amounts of the molecules immunoisolated by these mAbs were too low for the mass spectrometry.

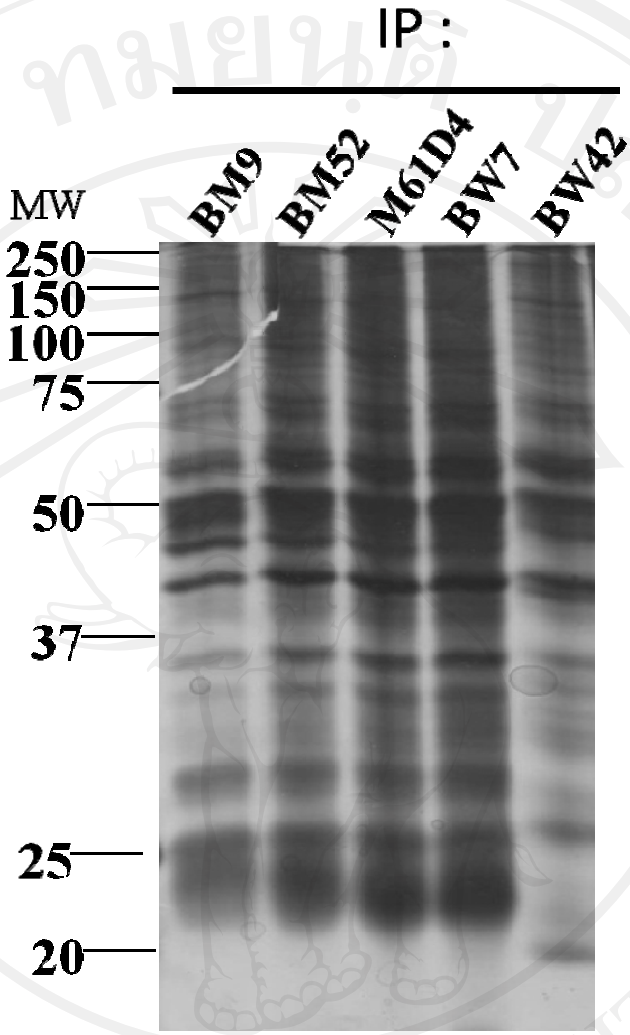

Figure 3.19 Silver stained gel of immunoprecipitated protein using the generated mAbs against CD99 associated molecules.

Jurkat cell lysates were immunoprecipitated using the generated mAbs, CD99-BW1 (BW1), CD99-BW7 (BW7), CD99-BM9 (BM9), CD99-BM52 (BM52), and CD99-BW42 (BW42) or IgM isotype matched control mAb M6-1D4. The immunoprecipitates were loaded on a one-dimensional SDS-PAGE gel, which was then stained by silver dye. Protein markers with molecular weight indicated in kDa are shown.

3.3.5 Association of CD99 with MHC class I and MHC class II and tetraspanin CD81

3.3.5.1 Association of CD99 with MHC class I and MHC class II

In our experiments mentioned earlier, association of CD99 with some surface molecules was demonstrated. As CD99 has been reported to transport MHC class I and class II to the cell surface (Choi *et al.* 1998; Sohn *et al.* 2001), we speculated that CD99 molecules may be associated with the transported molecules. To verify this hypothesis, immunoprecipitation using anti-CD99 mAb was carried out. The immunoprecipitated proteins were then analyzed by Western immunoblotting using the anti-MHC class I and class II mAbs. However, to obtain more precise results, we used the Ramos wild type and CD99 negative Ramos cells, which have been established in our earlier experiments (see section 3.3.3.3), in the coimmunoprecipitation experiments. As shown in Figure 3.20A, when using wild type Ramos cells, MHC class I and MHC class II were co-precipitated with the CD99 molecules. The MHC class II, however, was demonstrated to co-precipitate with CD99 in lower amount than the MHC class I molecules (Figure 3.20A). In contrast, no MHC class I or class II were detected in the immunoprecipitates resulting from CD99 negative Ramos cells. CD99, as expected, was detected only in immunoprecipitates of the wild type, but not of the CD99 negative Ramos cells. These findings indicated that CD99 physically interacted with MHC class I and class II. Western immunoblotting of cell lysates prior to immunoprecipitation were also performed. MHC class I and class II were present in similar amounts in both the wild type and CD99 negative Ramos cell lysates (Figure 3.20A), indicating these

molecules can be normally expressed even in the absence of CD99. In the presence of CD99, nevertheless, the MHC molecules were expressed as CD99-MHC complexes. We note that, in the Western immunoblotting experiment using anti-CD99 mAb, CD99 could not be detected in either wild type or CD99 negative Ramos cell lysates (Figure 3.20A). It is likely that, as the wild type Ramos cells expressed CD99 in a heterogeneous pattern in which a small CD99 positive population was present, a small amount of CD99 molecules are contained in the whole cell lysates. For this reason, the CD99 molecule could not be detected by Western immunoblotting.

To confirm the observed association of CD99 with MHC molecules, reciprocal coimmunoprecipitation experiments were carried out. In accordance with the above results, CD99 was observed in the immunoprecipitates using anti-MHC class II mAb (Figure 3.20B). However, CD99 could not be detected in the immunoprecipitates using anti-MHC class I mAb (data not shown). This may be because of the low affinity of the employed anti-MHC class I mAb itself, resulting in it being inappropriate for immunoprecipitation. Taken together, our findings suggest that CD99 form complexes with MHC class I and MHC class II molecules.

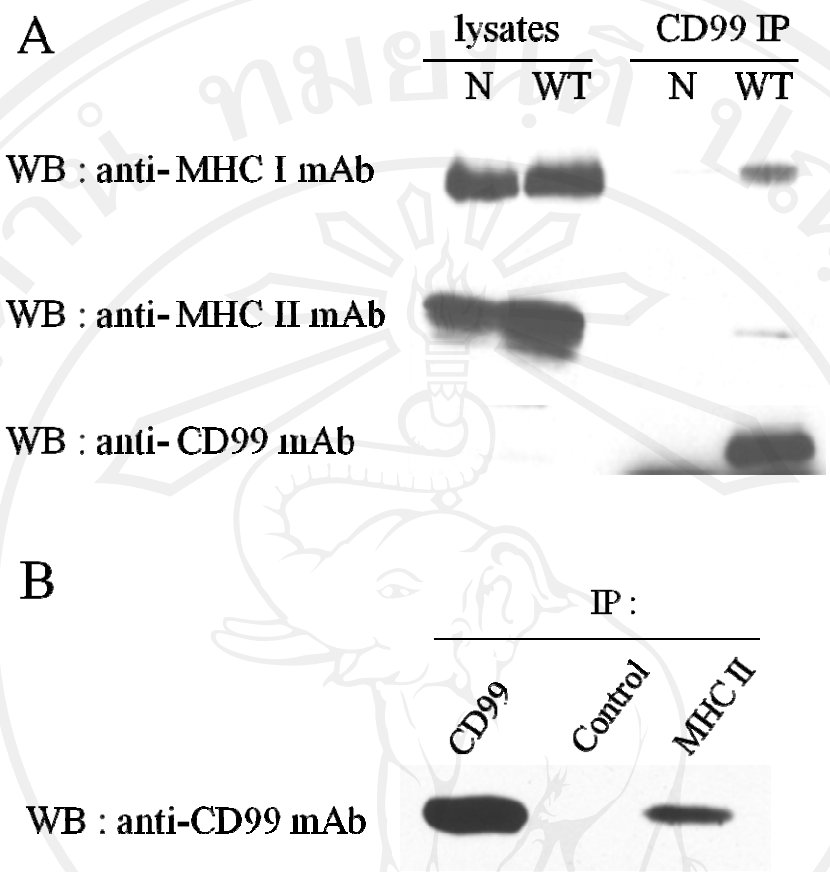

Figure 3.20 Interaction between CD99 and MHC class I and MHC class II.

(A) Cell lysates (lysate) or CD99 immunoprecipitated proteins (CD99 IP) of wild type (WT) or CD99 negative (N) Ramos cells were analyzed by Western immunoblotting using the indicated mAbs. (B) Wild type Raji cell lysates were immunoprecipitated using anti-CD99 (CD99), anti-MHC class II (MHC II) and isotype matched control mAbs (control) and analyzed by Western immunoblotting by using anti-CD99 mAb MT99/3.

3.3.6 Association of CD99-MHC complexes with tetraspanin CD81

Tetraspanins are broadly expressed cell surface proteins that span the cell membrane four times (Boucheix and Rubinstein 2001). All cells of the immune system express tetraspanins (Tarrant *et al.* 2003), which provide a scaffold that facilitates the spatial and temporal engagement of their associated proteins (Tarrant *et al.* 2003; Levy and Shoham 2005). The tetraspanins have been implicated in several cellular functions such as cell growth, differentiation, intercellular adhesion, motility, and intracellular signaling (Boucheix and Rubinstein 2001; Levy and Shoham 2005). Associations of tetraspanins with MHC class I and class II have been previously described (Schick and Levy 1993; Szollosi *et al.* 1996; Boucheix and Rubinstein 2001; Levy and Shoham 2005). CD81, a member of the tetraspanins, is expressed on T and B lymphocytes and functions as a co-stimulatory molecule. The associations of CD81 with MHC molecules have been described (Schick and Levy 1993; Szollosi *et al.* 1996; Boucheix and Rubinstein 2001; Levy and Shoham 2005). Following our finding of an association between the MHC and CD99 molecules, we investigated whether the CD99-MHC complex also contained tetraspanins, CD81. Immunoprecipitated proteins of wild type and CD99 negative Ramos cells were analyzed by Western immunoblotting using mAb against a tetraspanin, CD81. CD81, as well as MHC class II and CD99, were detected in the CD99 immunoprecipitates of the wild type, but not the CD99 negative Ramos cells (Figure 3.21A). To exclude any possible artificial effect of lipid raft association, we verified the presence of a typical lipid raft containing molecule, NTAL, in the CD99 immunoprecipitates. As expected, NTAL was not found in the CD99 immunoprecipitates (Figure 3.21A) indicating that the association observed is not due to the occurrence of lipid raft association.

Correspondingly, using Jurkat cells, CD81and CD99 were observed in the CD99 immunoprecipitates (Figure 3.21B), but not in that using an irrelevant control mAb. Concordantly, the reciprocal coimmunoprecipitation showed that CD99 and CD81 were detected in the CD81 immunoprecipitates (Figure 3.21B). As Jurkat cells express both CD99 long and short forms, Western immunoblotting obtained by using anti-CD99 mAb demonstrated 2 bands corresponding to the 32 kDa long form and the 28 kDa short form. Collectively, our results indicate that tetraspanin CD81 is associated with the CD99- MHC complexes.

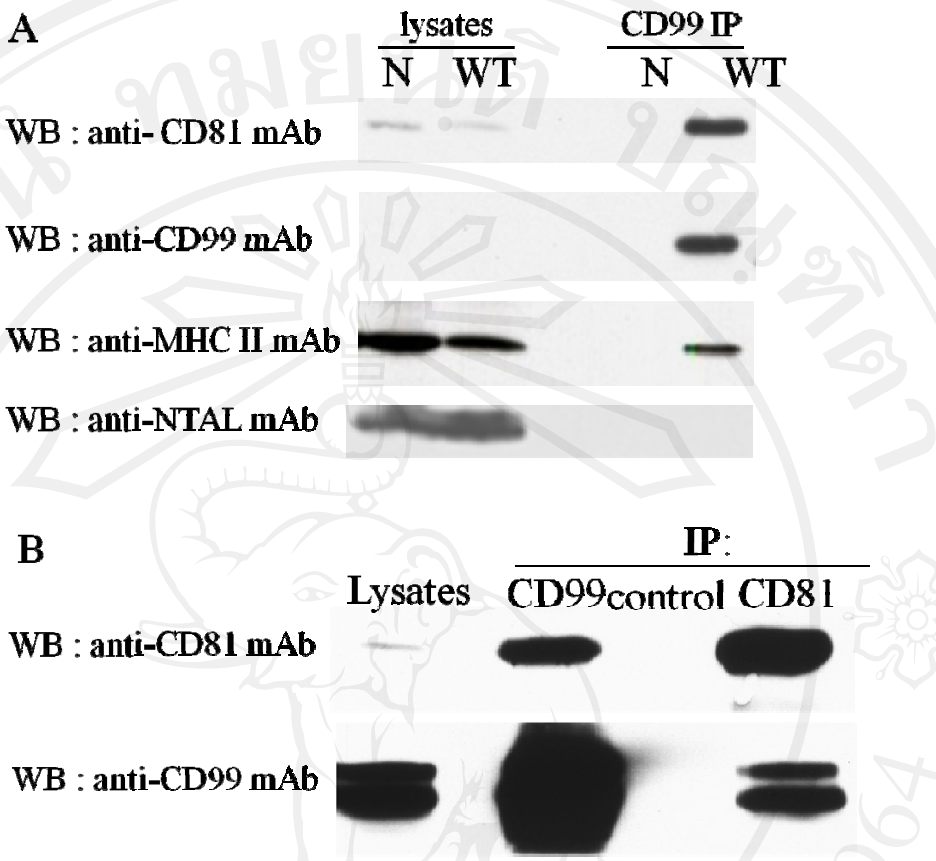

Figure 3.21 Association of CD99-MHC class II complexes with CD81.

(A) Cell lysates (lysate) or CD99 immunoprecipitated proteins (CD99 IP) of wild type (WT) or CD99 negative (N) Ramos cells were analyzed by Western immunoblotting using the indicated mAbs. (B) Jurkat cell lysates were immunoprecipitated with the anti-CD99 mAb (CD99), anti-CD81 mAb (CD81) or isotype matched control mAb (control) and analyzed by Western immunoblotting using the indicated mAbs. The cell lysates (Lysate) prior to immunoprecipitation was also analyzed by Western immunoblotting using the indicated mAbs.

3.3.7 Colocalization of CD99 with MHC class I, MHC class II and tetraspanin CD81

To further elucidate the association of CD99 with MHC and CD81 molecules, colocalization of CD99 and MHC class I, MHC class II or CD81 were visualized by laser confocal microscopy. Colocalizations of CD99 and MHC class I, CD99 and MHC class II as well as CD99 and CD81 were demonstrated in Raji cell line (Figure 3.22A). We note that, in the CD99 and CD81 panel (Figure 3.22A), a CD99 negative/CD81 positive cell was captured. This cell expressed only the green fluorescence of CD81 in all confocal images, indicating no cross-reactivity between conjugates and the primary antibodies used. It is important to mention that not all of the CD99 molecules and MHC or CD81 were associated; some molecules were expressed in an un-associated form. The confocal imaging results, consistent with the co-immunoprecipitation experiments, strongly suggest that CD99 is associated with MHC class I, MHC class II and CD81 on the cell surface.

To demonstrate whether the association of CD99 molecules with its interacting partners is also observed in peripheral blood cells, colocalization of CD99 with MHC and CD81 molecules on PBMCs' membrane was determined by confocal microscopic analysis. As shown in Figure 3.22B, colocalization of CD99-MHC class I, CD99-MHC class II and CD99-CD81 was observed. The colocalizations were observed only in cells expressing CD99 and MHC or CD81. In IgG control, anti-CD54 mAb was used instead of anti-MHC class I, class II or CD81 and no colocalization between the CD99 and CD54 molecules was found indicating no cross-reactivity between conjugates and the primary antibodies used. These results indicated that association of CD99 with MHC and CD81 is exists occurred in peripheral blood cells.

3.4 Investigation of the association of CD99 short and long forms with MHC class I, MHC class II and CD81

CD99 molecules are expressed on the cell surface in two isoforms, a 28 kDa short form and 32 kDa long form (Hahn *et al.* 1997). As described above, we have demonstrated that MHC class I, MHC class II, and CD81 are associated with the CD99 molecules. To determine whether this association is dependent on the CD99 isoforms, we have developed Ramos cells expressing CD99 short and long forms, using retroviral vectors encoding the respective isoforms.

The cDNAs encoding CD99 short form and long form were amplified by PCR from a plasmid vector πH3M-CD99 (Kasinrerk *et al.* 2000) and subsequently inserted into the retroviral expression vector pMSCV. The constructed plasmid vectors were designed as pMSCV-CD99SF and pMSCV-CD99LF for CD99 short form and long form, respectively. The correct of insertion of corresponding CD99 genes was verified by restriction fragment analysis (Figure 3.23), re-amplification by PCR (Figure 3.23) and subjected to DNA sequencing. The nucleotides of the inserted sequence were completely identical to the nucleotide sequence of the known CD99 short form and CD99 long form genes. Hence, the generated plasmid vector could be use to generate stable cell lines expressing CD99 short form and CD99 long form.

To produce cells expressing CD99 short form and CD99 long form, the pMSCV-CD99SF and pMSCV-CD99LF retroviral vectors were used to produce the retrovirus carrying CD99 short form or long form genes. The retroviral vectors were transfected into amphotropic Phoenix packaging cells. After cultivation, culture supernatants containing retrovirus carrying CD99 short form or long form genes were harvested.

We then infected the CD99 negative Ramos cells with the produced retrovirus containing CD99 short form or long form gene. Cells infected with retrovirus containing an empty pMSCV vector was also prepared and served as the mock cells. The cells were further isolated by cell sorting on the basis of their CD99 surface expression. The expression of CD99 in the generated stable cell lines was verified by flow cytometry (Figure 3.24) and immunoprecipitation (Figure 3.25). Immunoprecipitation of CD99 molecules followed by Western immunoblotting with anti-CD99 mAb demonstrated the expected surface expression patterns on the transduced cells (Figure 3.25).

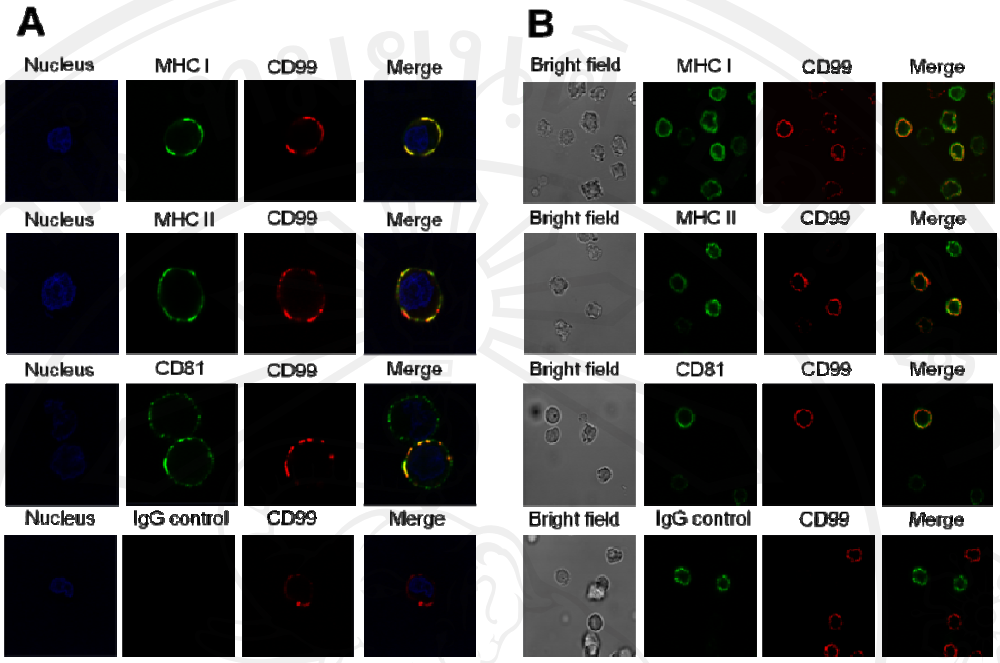

Figure 3.22 Colocalization of CD99 and MHC class I, MHC class II and CD81.

(A) Raji cells (B) PBMCs were stained with anti-MHC class I (MHC I), anti-MHC class II (MHC II), anti-CD81 (CD81) and anti-CD99 (CD99) mAbs as described in Materials and Methods. The stained cells were analyzed for colocalization by a confocal microscope. The green images represent the distribution of MHC class I, MHC class II, CD81 molecules and red images represent the CD99 molecules. Cell nuclei are shown in blue.

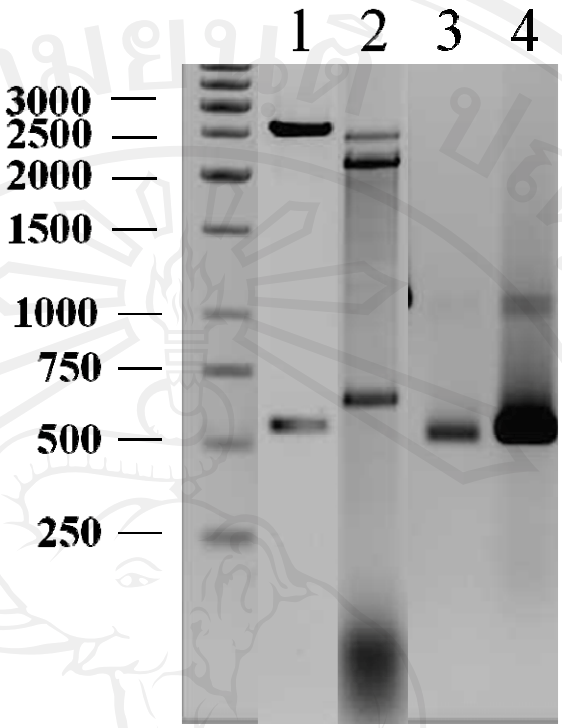

Figure 3.23 Verification of pMSCV-CD99SF and pMSCV-CD99LF.

The isolated plasmid were digested with *EcoR* I and *Nco* I and re-amplified by PCR followed by 1% agarose gel analysis. pMSCV-CD99SF (lane 1) and pMSCV-CD99LF (lane 2) plasmid vector digested with *Nde* I and *EcoR* I enzyme, un-digested plasmid DNA amplified PCR product from pMSCV-CD99SF (lane 3) and pMSCV-CD99LF (lane 4) plasmid vector. Sizes of standard DNA markers (bp) are indicated on the left.

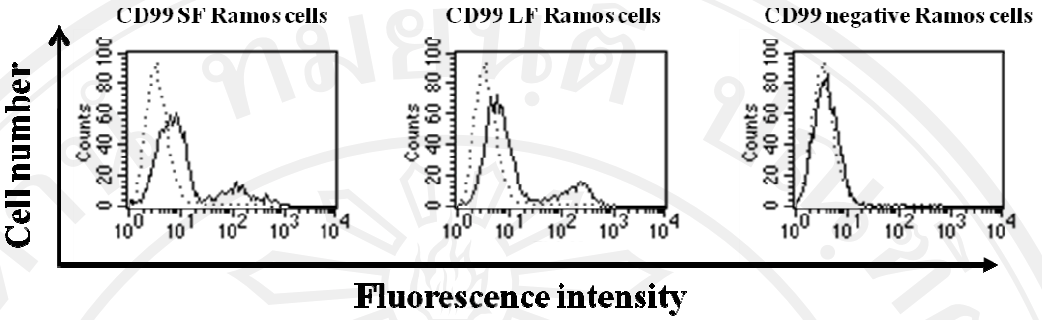

Figure 3.24 Flow cytometric analysis of CD99 expression of Ramos cells expressing CD99 short and long forms.

CD99 negative Ramos cells were transduced with retrovirus carrying CD99 short form (CD99 SF Ramos cells) or CD99 long form (CD99 LF Ramos cells). The transduced cells were stained with the anti-CD99 mAb MT99/3 and counterstained with PE-conjugated anti-mouse immunoglobulins antibodies. Solid lines represent the immunofluorescence profiles of the cells stained with anti-CD99 mAb and dashed lines represent background fluorescence of conjugate control.

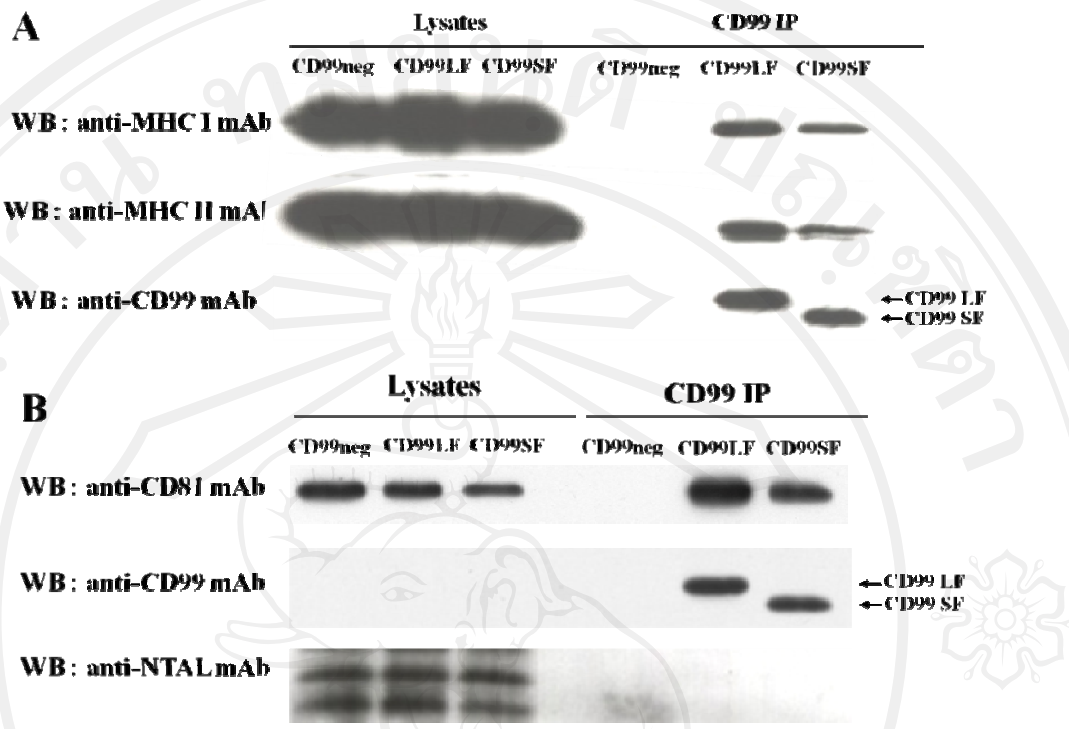

Figure 3.25 Interaction of CD99 isoforms with MHC class I, MHC class II and CD81.

Cell lysates (Lysate) or CD99 immunoprecipitates (CD99 IP) of Ramos cells expressing CD99 short form (CD99SF), CD99 long form (CD99LF) and CD99 negative Ramos (CD99neg) were analyzed by Western immunoblotting using anti-MHC class I, anti- MHC class II and anti-CD99 mAbs (A) or anti-CD81, anti-CD99 and anti-NTAL mAbs (B). The positions of CD99 short and long isoforms are indicated.

3.5 Functional study of the CD99 molecules involving T cell activation

3.5.1 Recruitment of CD99 into the immunological synapse (IS)

Since our results suggested that MHC class I, class II and CD81, which are accessory molecules for T cell activation and are accumulated in the IS, are associated with CD99, we further investigated whether CD99 also appears at the IS during antigen presentation. We loaded Raji B cells with SEB and incubated them with DDAO-SE pre-labeled Jurkat T cells. As shown in Figure 3.26, CD99 accumulated at the T-B cell interface. Colocalization of CD99 with F-actin, a marker of IS formation, was also observed. These data suggest that, upon antigen presentation, CD99 is recruited to the IS.

3.5.2 Investigation the association between CD99 and lipid raft microdomains

Immune cell signaling pathways are at least partially mediated by lipid raft-associated proteins. During T cell activation, lipid rafts are accumulated in the IS and function as the platform for signal molecules (Horejsi 2005; Simons and Gerl 2010). We thus attempted to confirm whether, in the Jurkat T cell line, CD99 is a lipid raft-associated membrane protein. As shown in Figure 3.27, in the presence of mild lipid raft-preserving detergent 1% Brij-98, both short and long forms of CD99 were found in the raft and non-raft fractions. In contrast, in the presence of the raft disrupting detergent 1% LM, CD99 was mostly found in the non-raft fractions. The lipid raft containing protein Lck was included in the experiment as a positive control and was

observed in the lipid raft fractions. These results indicate that CD99 molecules are recruited to the IS and a fraction of CD99 is associated with lipid rafts.

3.5.3 Engagement of CD99 molecules inhibits T cell proliferation

To determine whether CD99 molecule is involved in T cell activation, peripheral blood mononuclear cells (PBMCs) were activated with anti-CD3 mAb OKT3 in the presence of anti-CD99 mAb MT99/3 and cell proliferation was verified. As shown in Figure 3.28, engagement of CD99 by a specific mAb inhibited T cell proliferation. In contrast, the isotype matched control mAb did not show any inhibitory effect. These results indicate that CD99 plays a regulatory role in T cell activation.

3.5.4 Engagement of CD99 molecule induces cell apoptosis

Although the functional properties of the CD99 molecule are not clearly defined, a dominant important function of this surface protein is supposed to be involvement in cell apoptosis. We thus investigated whether engagement of CD99 would induce T cell apoptosis. Jurkat cells were cultured with anti-CD99 mAbs MT99/1, MT99/2 and MT99/3 for 30 mins. Cell apoptosis was examined by staining with FITC conjugated annexinV and PI. As shown in Figure 3.29, mAbs MT99/1 and MT99/3, but not mAb MT99/2, was found to induce cell apoptosis. These results suggest that CD99 is an molecule responsible for controlling of cell death

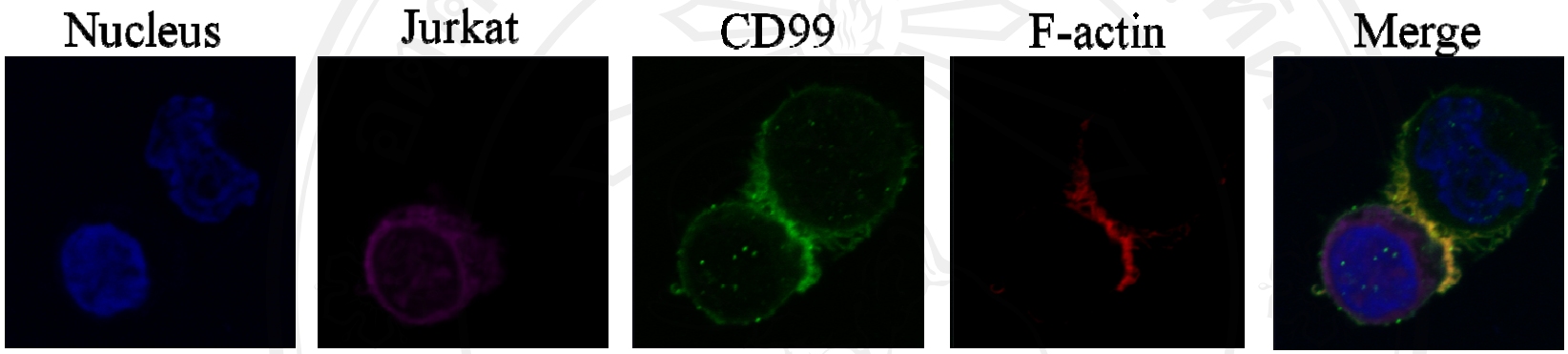

Figure 3.26 CD99 is recruited into the IS

DDAO-SE pre-labeled Jurkat T cells were incubated with SEB-loaded Raji B cells. Cells were then plated onto a cover slip, fixed, stained with anti-CD99 mAb followed by Alexa Fluor 488-conjugated goat anti-mouse IgG antibodies, TRITC-phalloidin for F-actin and Hoechst 33258 for the nucleus. The stained cells were visualized by confocal fluorescence microscopy. The green and red images represent the localization and distribution of CD99 and F-actin, respectively. The nuclei and Jurkat cells are shown in blue and magenta, respectively.

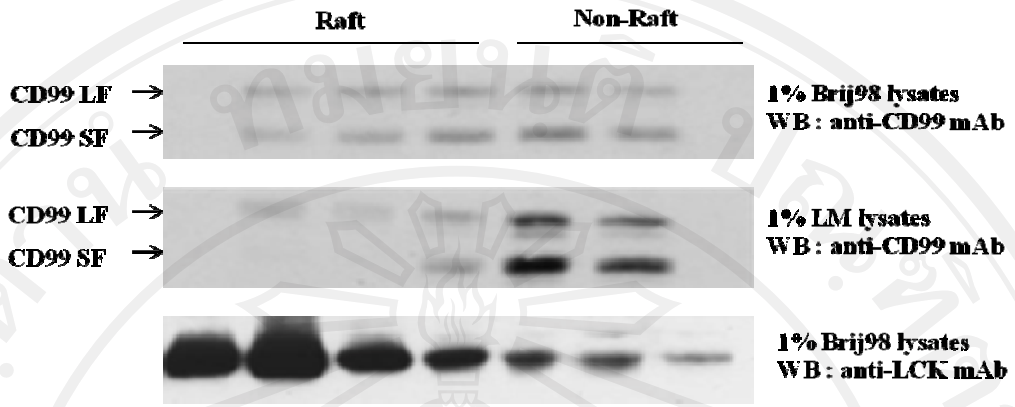

Figure 3.27 The association between CD99 and lipid raft microdomains

Jurkat cells were homogenized to isolate cell membranes, which were then solubilized in lipid raft preserving detergent 1% Brij-98 or lipid raft disrupting detergent 1% laurylmaltoside (LM) and subjected to gel filtration on Sepharose 4B. The obtained fractions were analyzed by Western immunoblotting using anti-CD99 and anti-Lck mAbs.

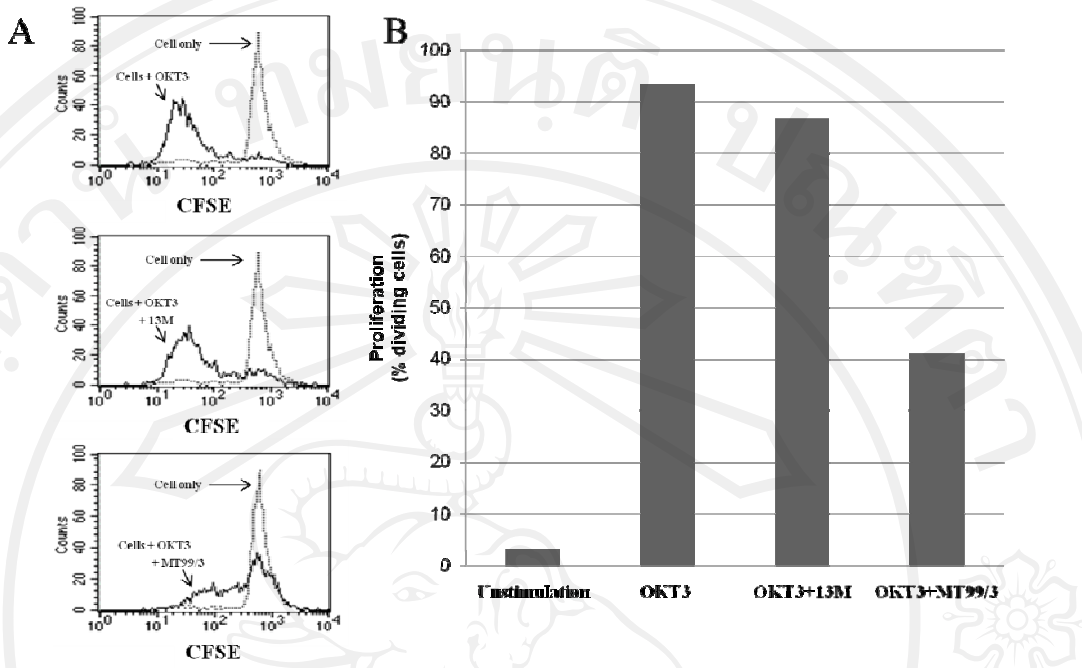

Figure 3.28 Engagement of CD99 inhibits T cell proliferation.

PBMCs were labeled with CFSE and cultured with or without anti-CD3 mAb OKT3 in the absence or presence of anti-CD99 mAb MT99/3 or isotype matched control mAb 13M for 5 days. Cells were then harvested and analyzed by flow cytometry. (A) FACS profiles illustrate the T-cell proliferation levels obtained in the different indicated conditions, as determined by CFSE fluorescence loss. (B) Histogram plots show the level of cell proliferation as determined by the percentage of dividing cells in the indicated culture conditions.

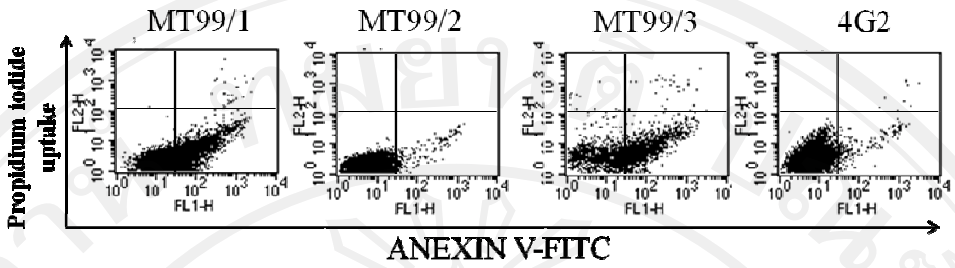

Figure 3.29 mAbs MT99/1 and MT99/3 induce cell apoptosis of Jurkat cells.

Jurkat cells were cultured with the indicated mAb for 30 mins. Cell apoptosis was examined by staining the cells with PI and FITC conjugated annexinV and analyzed by flow cytometer. mAb 4G2 was used as an isotype matched control.

3.5.5 Analysis of CD99 mediated signaling pathway

As described above, our results indicate for the first time that CD99 forms a micromembrane domain with MHC and tetraspanins molecules. Upon T cell activation, the MHC-tetraspanins complexes were demonstrated to involve in T cell signaling. We hypothesized that CD99 may play a role in cell signaling. To explore the intracellular signaling by CD99 molecule, induction of protein phosphorylation upon CD99 engagement was examined. In these experiments, cells were incubated with anti-CD99 mAb MT99/3 and protein phosphorylation was determined by Western blotting using mAbs specific to tyrosine, serine and threonine phosphorylated proteins. Interestingly, the tyrosine phosphorylation levels of proteins at MW 40, 100, 150 and 200 kDa were increased by CD99 activation (Figure 3.30). Furthermore, the levels of serine phosphorylation of two protein bands at MW 50 kDa were also increased after CD99 activation (Figure 3.31). In contrast, increase of protein phosphorylation level was not observed after adding of the isotype matched control mAb (Figure 3.30B and 3.31B). We, however, could not detect any threonine phosphorylated protein upon CD99 engagement (Figure 3.32). In addition, the immunoblottings against GAPDH that serve as a control for equal protein loading were included in all experiments (Figure 3.30, 3.31 and 3.32). Taken together, the results indicate that CD99 is one of the T cell signaling molecules, crosslinking of CD99 molecule significantly elevated tyrosine and serine phosphorylation-dependent activation of signaling components.

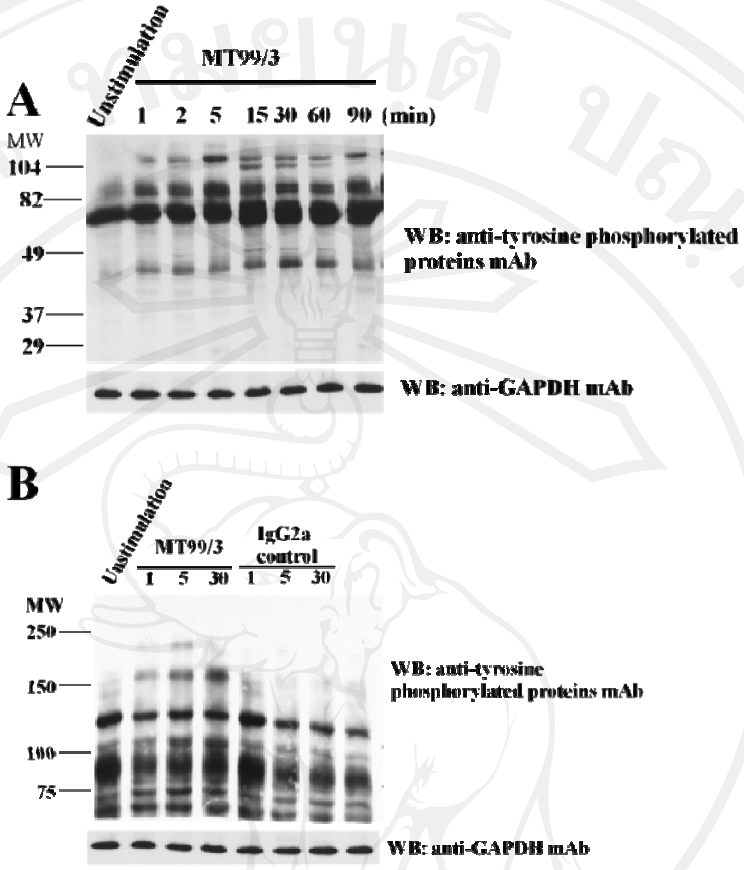

Figure 3.30 Analysis of protein tyrosine phosphorylation upon CD99 engagement.

Serum starved Jurkat cells were treated with anti-CD99 mAb MT99/3 for 30 mins and then cross-linking with goat anti-mouse IgG for the indicated time periods. Phosphorylation levels of the various signaling molecules in cell lysates were determined by immunoblotting analyses using specific antibodies for tyrosine phosphorylated proteins. (A) CD99 activated Jurkat cell lysate was resolved by 12% SDS-PAGE under reducing condition. (B) The cell lysates were resolved by 7% SDS-PAGE under reducing condition. Immunoblotting against GAPDH serve as a control for equal protein loading.

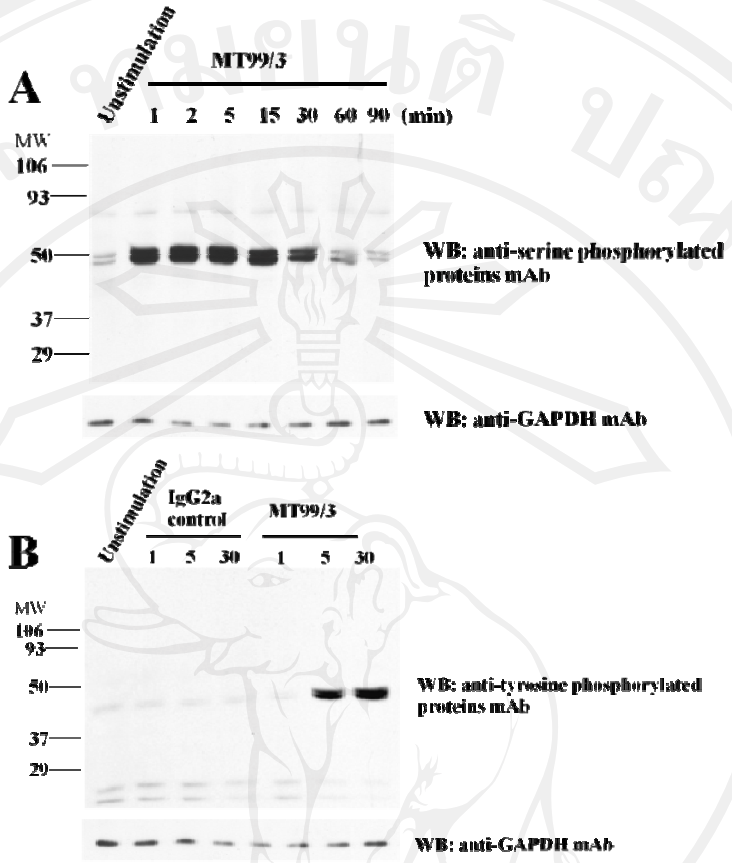

Figure 3.31 Analysis of protein serine phosphorylation upon CD99 engagement. Serum starved Jurkat cells were treated with anti-CD99 mAb MT99/3 for 30 mins and then cross-linking with goat anti-mouse IgG for the indicated time periods. Phosphorylation levels of the various signaling molecules in cell lysates were determined by immunoblotting analyses using specific antibodies for serine phosphorylated proteins. (A) Serum starved Jurkat cells were treated with anti-CD99 mAb MT99/3 for 30 mins and then cross-linking with goat anti-mouse IgG for the indicated time periods. (B) Serum starved Jurkat cell lysate treated with mAb MT99/3 and isotype matched control mAb for 30 mins and then with goat anti-mouse IgG for the indicated time periods. Immunoblotting against GAPDH serve as a control for equal protein loading.

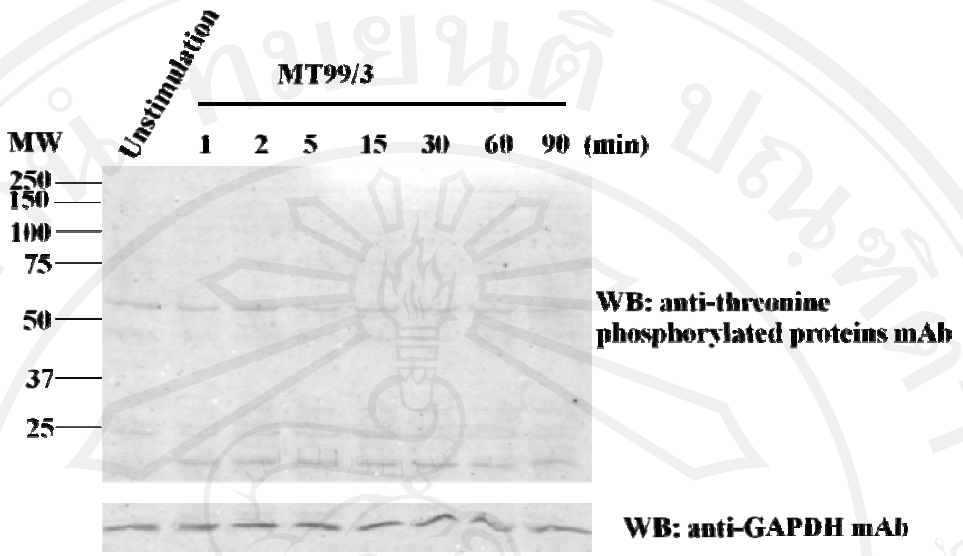

Figure 3.32 Analysis of protein threonine phosphorylation upon CD99 engagement.

Serum starved Jurkat cells were treated with anti-CD99 mAb MT99/3 for 30 mins and then cross-linking with goat anti-mouse IgG for the indicated time periods. Phosphorylation levels of the various signaling molecules in cell lysates were determined by immunoblotting analyses using specific antibodies for threonine phosphorylated proteins. Immunoblotting against GAPDH serve as a control for equal protein loading.