CHAPTER IV

DISCUSSION AND CONCLUSION

CD99 is a transmembrane glycoprotein encoded by the MIC2 gene (Levy *et al.* 1979) with no homology with other known molecules except the Xga protein (Fouchet *et al.* 2000). The gene encoding CD99 is located in the human pseudoautosomal region in the distal short arms of the X and Y chromosomes (Petit *et al.* 1988). Sequence analysis of CD99 cDNA indicates that the CD99 protein is formed by an extracellular domain, which is glycosylated with *O*-linked sugar residues, followed by a transmembrane domain and a 36-amino acid intracytoplasmic domain (Banting *et al.* 1989). CD99 is broadly distributed on many cell types, with particularly strong expression on Ewing's sarcoma cells and peripheral primitive neuroectodermal tumors (Dworzak *et al.* 1999; Dworzak *et al.* 2004; Kovar and Bernard 2006). The function of the CD99 molecule is not yet clearly understood. However, it has been suggested as a key mediator for induction of cell adhesion, cell migration and induction of apoptotic cells (Gelin *et al.* 1989; Bernard *et al.* 1997; Bernard *et al.* 2000; Schenkel *et al.* 2002; Jung *et al.* 2003; Cerisano *et al.* 2004; Imbert *et al.* 2006; Lou *et al.* 2007). In order to better understand role of CD99 , mAbs specific to CD99 are required and employed to develop the functional study. In this study, to obtain more details on the function of CD99 molecules, we have generated several anti-CD99 mAbs. The COS cell expression system was applied in order to obtain CD99 expressing cells. The CD99 expressing cells were then used as

an immunogen for mouse immunization. By standard hybridoma technique, 2 hybridoma clones, named MT99/1 and MT99/3, that produce CD99 specific antibodies were obtained. Western blotting and immunoprecipitation experiments indicated that mAbs MT99/1 recognize the linear epitope, whereas the mAb MT99/2 recognize conformational epitope on the CD99 molecule. In addition to mAbs MT99/1 and MT99/2, an anti-CD99 mAb named MT99/3 was previously produced in our laboratory (Kasinrerk *et al.* 2000). The mAbs MT99/1 and MT99/2 together with MT99/3 were therefore used as a tool for expression profile study, biochemical characterization and functional studies of CD99 molecules.

For cellular distribution study, various cell types were stained with the generated anti-CD99 mAbs by immunofluorescence and flow cytometric analysis. Immunofluorescence staining results suggests that CD99 molecules were broadly expressed on various hematopoietic cell lines. Within peripheral blood cells, lymphocytes and monocytes but not granulocytes expressed CD99 molecules. The FACS profiles obtained by the generated anti-CD99 mAbs were the same as those described by using other CD99 mAbs (Goodfellow and Tippett 1981; Bernard *et al.* 1984; Latron *et al.* 1987; Kasinrerk *et al.* 2000). On red blood cells, CD99 has been reported to be associated with the blood group Xg phenotype and express as a quantitative polymorphism (Petty and Tippett 1995; Kasinrerk *et al.* 2000). In the present study, RBCs were also subjected for immunofluorescence staining. Four out of six healthy donors reacted with mAb MT99/1 indicating the quantitative polymorphism phenomenon. Contrary to expectation, RBCs from all subjects did not react with mAb MT99/2. We suggest that MT99/2 recognize an epitope located very near to the Xg blood group, thus this molecule might prevented binding of MT99/2.

Several reports have suggested that CD99 is a multi-functional cell surface molecule and which plays 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). Signaling pathways triggered by CD99 have been elucidated in several studies. Stimulation of CD99 with agonistic antibodies enhanced the expression of several T cell activation markers on anti-CD3-activating T cells, as well as resulting in elevation of intracellular $Ca2^{+}$ and the tyrosine phosphorylation of cellular proteins (Waclavicek *et al.* 1998; Wingett *et al.* 1999). We have also demonstrated that protein kinase C inhibitor, sphingosine and a protein tyrosine kinase inhibitor, genistein, blocked cell aggregation induced by CD99 engagement (Kasinrerk *et al.* 2000). It has also been reported that CD99 ligation induced differential activation of three mitogen-activated protein kinase (MAPK) members, ERK, JNK and p38 MAPK (Hahn *et al.* 2000). Activation of Src kinase and focal adhesion kinase (FAK) by CD99 molecules was also demonstrated (Lee *et al.* 2002). Although there is evidence that CD99 is involved in signal transduction pathways, there are no reported signaling motifs in the cytoplasmic tail of the CD99 molecule except for a site for PKCα phosphorylation (Alberti *et al.* 2002). Together with the information that CD99 molecule contains a short intracellular domain (Banting *et al.* 1989), it is unlikely that CD99 itself takes part in signaling events. In the cellular context, association of CD99 with other membrane proteins has been suggested to be necessary for exerting its functions.

Cell surface molecules containing a short cytoplasmic tail usually form complexes with other molecules in order to modulate their signal transduction and functions. Identification of the interacting partners of cell surface molecules may lead to a better understanding of cellular function and immune responses. In the present study, we

have identified several interacting partners of CD99. We observed that on the cell surface, CD99 molecules are associated with MHC class I, MHC class II and tetraspanin CD81. The CD99 molecule has been described by several studies as a signaling molecule. Engagement of CD99 induces signal transduction, resulting in the regulation of T cell activation, cell adhesion, cell migration, and cell death. However, CD99 itself contains a short intracellular domain without signaling motifs on the cytoplasmic tail except a site for PKCα phosphorylation (Banting *et al.* 1989; Alberti *et al.* 2002). Signal transduction induced by CD99 is, therefore, incompletely understood. Since we found, in this study, that CD99 forms complexes with other membrane receptors, i.e., MHC class I, MHC class II and tetraspanin CD81, this may help to clarify the precise signaling mechanism of the CD99 molecule and provide better understanding of its functional roles.

MHC molecules are cell surface proteins that play a critical role in antigen presentation. CD99 has been reported to regulate the expression of MHC class I and class II (Choi *et al.* 1998; Sohn *et al.* 2001; Bremond *et al.* 2009). Up-regulation of MHC class I and class II by CD99 results from accelerated intracytoplasmic transportation of MHC molecules to the plasma membrane rather than de novo synthesis of these molecules (Choi *et al.* 1998; Sohn *et al.* 2001). Decreased expression of CD99 resulted in the retention of MHC class I molecules in the Golgi compartment by affecting the transportation of the MHC molecules in the *trans*-Golgi network (Sohn *et al.* 2001; Bremond *et al.* 2009). Moreover, colocalization of CD99 and MHC class I molecules is clearly demonstrated both in the Golgi apparatus and at the cell surface (Bremond *et al.* 2009). Strikingly, it was demonstrated that the CD99 and MHC class I association occurs at the transmembrane domain. Valines located in

the transmembrane region of CD99 are required for the binding to MHC molecules, likely in relation with their hydrophobicity (Bremond *et al.* 2009). In agreement with the previous reports, we have shown in this study, CD99 molecules form complexes with MHC molecules and are co-expressed in microdomains on the cell surface.

In addition to the MHC molecules, we demonstrated the association of CD99 with a tetraspanin, CD81. The tetraspanins are cell surface proteins that are broadly expressed in many cell types. Data from biochemical studies or knockout mice suggest that the tetraspanins play a major role in membrane biology (Boucheix and Rubinstein 2001; Levy and Shoham 2005). One of the most striking features of tetraspanins is their ability to form a network of multi-molecular complexes, known as the tetraspanin web, between each individual tetraspanin and other surface proteins. Within the immune cells, all cells express tetraspanins, which provide a scaffold that facilitates the spatial and temporal engagement of their associated proteins. Tetraspanins and their associated proteins modulate several intercellular immune interactions, including adhesion, migration, synapse formation, as well as assisting in intracellular interactions as organizers of membrane-signaling complexes. They are also involved in intracellular protein transport, endocytosis, and exocytosis, and function as chaperones or stabilizers of lineage-specific molecules. CD81, a member of the tetraspanins, has been reported to be involved in an astonishing range of physiological responses (Levy *et al.* 1998; Boucheix and Rubinstein 2001; Levy and Shoham 2005). Association of CD81 with various surface molecules, including MHC class II, has previously been reported. As our results demonstrated the association of CD99 with CD81 and MHC molecules, we speculate that the CD99-MHC-CD81 complex is a tetraspanin web and plays an important role in the immune response.

CD99 is expressed as two distinct isoforms, a long 32 kDa form (type I) and a short 28 kDa form (type II) resulting from an alternative splicing process of the product of the encoding gene (Hahn *et al.* 1997). The expression profile of CD99 isoforms was also investigated in this study. It was found that CD99 long form is presented in all tested peripheral blood cells including thrombocytes, RBCs, monocytes, NK cells, B cells, T cells, except granulocytes. CD99 long form was also found in all cell lines tested (Raji, Ramos, SUP-T1, Jurkat, THP-1 and U937 cells). In contrast, CD99 short form was found only in Jurkat and SUP-T1 cell lines. 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 which in accordance with previous reports (Bernard *et al.* 1997; Hahn *et al.* 1997). Additionally, both CD99 isoforms have their functional roles, and differential expression can lead to distinct functional outcomes (Wingett *et al.* 1999; Alberti *et al.* 2002; Byun *et al.* 2006). The truncation of the cytoplasmic domain of the CD99 short form may result in a loss of interaction with signaling molecules recognized by the cytoplasmic domain of the long form. It has been demonstrated that the cytoplasmic domain of the long form contains two putative phosphorylation sites, a serine at amino acid residue 168 and a threonine at amino acid residue 181. These potential phosphorylation sites may be important for intracellular signaling events and/or extracellular molecular interactions. Moreover, the S168 of CD99 long form has been reported to be a site for PKCα phosphorylation and is required for the oncosuppressor function (Scotlandi *et al.* 2007). It has also been postulated that truncation of the cytoplasmic domain of the CD99 short form causes an alteration of the three-dimensional structure, leading to different binding

sites for its ligand. However, it is still unknown whether CD99 mediated signaling pathways are modulated by the differential expression of CD99 isoforms (Bernard *et al.* 1997; Hahn *et al.* 1997) or whether each CD99 isoform promote different sets of signaling pathways (Byun *et al.* 2006). To address the mechanisms of diffrent functions of the two CD99 isoforms, we investigated whether the association of CD99 with its partner molecules depended on the distinct isoform. However, we found that both CD99 isoforms interacted with MHC and CD81 molecules. It is tempting to assume that the association of CD99 with MHC and tetraspanin CD81 may bring both CD99 isoforms into the tetraspanin web and into close proximity with the intracellular membrane signaling proteins. The molecular mechanism by which CD99 mediated signaling occurs is likely to reflect the presence of signaling molecules, such as kinases (Yauch and Hemler 2000; Zhang *et al.* 2001) and phosphatases (Carmo and Wright 1995), in the tetraspanin microdomain. This is, however, only speculation and requires further investigation.

The IS is a dynamic structure formed between T cells and antigen presenting cells, and is characterized by lipid and protein segregation, signaling compartmentalization, and bidirectional information exchange through soluble and membrane-bound transmitters (Yokosuka and Saito 2010). The IS is the site where signals are delivered by the T cell receptors, adhesion molecules, as well as co-stimulatory and co-inhibitory receptors. The IS is divided into distinct regions: a central-supramolecular activation cluster (c-SMAC), a peripheral-(p-) SMAC, and a distal-(d-) SMAC (Yokosuka and Saito 2010). It has been demonstrated that the c-SMAC mediates antigen recognition and subsequent T cell activation, whereas the p-SMAC supports T cell-APC conjugation and maintains the architecture of the IS.

Several molecules, including MHC and tetraspanin CD81, have been shown to translocate into the IS during T cell activation (Boucheix and Rubinstein 2001; Mittelbrunn *et al.* 2002; Levy and Shoham 2005). Upon T cell activation, CD81 is redistributed to the contact area of T cell-APC conjugates of both T cells and APCs. Colocalization of CD81 with CD3 at the SMAC of T cells and of CD81 with MHC class II of APCs has been observed in which they function as cell surface co-stimulatory molecules. As CD99 forms complexes with MHC and CD81 molecules, we investigated whether CD99 is translocated into the IS upon TCR triggering. We demonstrated that CD99 is a lipid raft-associated protein and is recruited into the IS, as has been observed for its associated molecules. Engagement of CD99 with agonistic antibody inhibits T cell activation. To our knowledge, this is the first demonstration of the accumulation of CD99 within the IS upon T cell activation and the association of CD99 with the proteins of the SMAC. We speculate that CD99-MHC-tetraspanin CD81 complexes may play an important role in T cell activation.

Apoptosis, or programmed cell death, can be characterized by cell shrinkage, blebbing of the plasma membrane, maintenance of organelle integrity, and condensation and fragmentation of DNA, followed by ordered removal through phagocytosis (Lockshin and Zakeri 2001; Assuncao Guimaraes and Linden 2004). This process is essential for normal development of multicellular organisms and the regulation of tissue homeostasis. Its malfunction may result in malignancies or autoimmune diseases. Induction of apoptosis occurs via cell surface receptors that transmit signals initiated by their specific ligands. The central component of the apoptotic machinery is a family of proteases, the caspases, which participate in a cascade of proteolytic cleavages, finally resulting in the disassembly of the cell.

However, a number of caspase-independent cell death mechanism can be triggered through various cell surface receptors, such as MHC class I and II, CD2 and CD99 (Nagy and Mooney 2003). In order to determine the involvement of CD99 in the apoptosis process, in this study, we applied the mAbs MT99/1, MT99/2 and MT99/3 for induction of cell apoptosis. Exposure of phosphatidylserine on the cell surface was monitored and used as marker of apoptotic cells. In our study, mAbs MT99/1 and MT99/3 could induce cell apoptosis. We hypothesize that mAbs MT99/1 and MT99/3 react with a bioactive domain on the CD99 molecule. Engagement of this bioactive domain induces cell apoptosis. This finding is agreement with the previously reported data that triggering of CD99 by its specific mAbs can induce cell apoptosis (Bernard *et al.* 1997; Sohn *et al.* 1998; Jung *et al.* 2003). Interestingly, the CD99-induced apoptosis was inhibited by engagement of MHC class II (Kim *et al.* 2003). We hypothesize that CD99-MHC complexes also play a role T cell development.

In order to induce various cellular functions, cells often need to communicate and transduce signals from extracellular receptors into the interior of the cells via a downstream signaling pathway. The transduction of a signal in a signaling chain occurs by a sequential activation of the participating proteins, enzymes or connectors to recruit the other proteins in the signaling pathway (Krauss 2003; Lodish *et al.* 2008; Gomperts *et al.* 2009). In this study, the downstream signaling molecules of CD99 were explored. Ligation of CD99 molecules with mAb MT99/3 induces the tyrosine phosphorylation of proteins at MW 40, 100, 150 and 200 kDa and serine phosphorylation of two protein bands at MW 50 kDa. These findings indicate that the signals can be transmitted via CD99 and activation of CD99 molecule significantly

elevated tyrosine and serine phosphorylation-dependent activation of signaling components.

In conclusion, in the present study, we demonstrate that CD99 is associated with MHC class I, MHC class II and a tetraspanin, CD81. The association was observed in both CD99 long and short isoforms. Our data collectively show that upon T cell activation, CD99 is translocated into the IS and involved in regulation of T cell proliferation. Ligation of CD99 molecules induce apoptosis and signaling transduction. The CD99-MHC-CD81 complexes may play an important role in the regulation of the immune responses.