CHAPTER I

INTRODUCTION

1.1 Statement of problems

Immune system is an important system for protection of the body from pathogens and cancers. The immune system can be divided into innate immunity and adaptive immunity. Innate immunity provides the initial defense against infection, whereas adaptive immunity is the immunity which develops more slowly and mediates the later, even more effective, defense mechanisms. The immune system is composed of several types of cells, organs and mediators that work together. Among the immune cells, T lymphocytes play a pivotal role in orchestrating the immune responses. To generate proper immune responses to fight a particular pathogen, T cells with the appropriate receptor specificity for that pathogen must first be activated. This activation is the initial phase for the induction of powerful immunity (Abbas *et al.* 2000; Janeway *et al.* 2004; Chaplin 2010).

Upon T cell activation, T cell stimulation is initiated when a T cell receptor (TCR) encounters specific antigen peptide-MHC complexes expressed on the surface of antigen presenting cells (APCs). The interaction of various co-stimulatory molecules expressed on T cells and APCs is, in addition, involved in the induction of proper T cell responses. These interactions induce the formation of an immunological synapse (IS) at the cell-cell junction between T cells and APCs, resulting in the reorganization of the related cell membrane signaling molecules in a concerted

fashion (Huppa and Davis 2003; Fooksman *et al.* 2010). The IS is proposed to function as a platform for signal transduction and cytoskeleton reorganization, which is essential for the determination of TCR sensitivity and responsiveness. Several co-stimulatory molecules have been shown to translocate into the IS and are crucial in determining antigen-specific T cell activation and tolerance.

CD99 is a type 1 transmembrane glycoprotein encoded by the MIC2 gene, and shares no significant homology with any known protein family (Bernard *et al.* 1988; Goodfellow *et al.* 1988; Aubrit *et al.* 1989; Banting *et al.* 1989; Gelin *et al.* 1989). The CD99 molecule contains an extracellular domain, followed by a transmembrane domain and a short 36-amino acid intracytoplasmic domain (Banting *et al.* 1989). On the cell surface, CD99 is expressed as two distinct isoforms depending on the alternative splicing of the encoding gene, a long 32 kDa form (type I) and a short 28 kDa form (type II). CD99 is broadly distributed among many cell types, both hematopoietic and non-hematopoietic cells (Dworzak *et al.* 1999; Kasinrerk *et al.* 2000; Dworzak *et al.* 2004; Kovar and Bernard 2006; Khunkaewla *et al.* 2007). Although the functional role of CD99 is not yet fully understood, it has been implicated in the regulation of immune responses, cell adhesion and migration, and cell death. CD99 has been described as a T cell co-stimulator and regulator of cytokine production (Waclavicek *et al.* 1998; Oh *et al.* 2007). Engagement of CD99 with agonistic antibodies induced apoptosis of immune cells and tumor cells (Bernard *et al.* 1997; Scotlandi *et al.* 2000; Khunkaewla *et al.* 2007). CD99 ligation was also demonstrated to induce expression of adhesion molecules, including ELAM-1, VCAM-1 and ICAM-1, which are associated with leukocyte adhesion and transendothelial migration (Bernard *et al.* 1995; Hahn *et al.* 1997; Bernard *et al.* 2000;

Kasinrerk *et al.* 2000; Schenkel *et al.* 2002; Yun *et al.* 2006; Khunkaewla *et al.* 2007; Lou *et al.* 2007; Schneider *et al.* 2009). Furthermore, CD99 engagement has been reported to induce the expression of TCR, MHC class I and MHC class II by accelerated mobilization of these molecules from the Golgi compartment to the plasma membrane (Choi *et al.* 1998). Requirement of CD99 expression in IFN-γ induced MHC class I expression has also been observed (Bremond *et al.* 2009). Without CD99, upon IFN-γ stimulation, MHC class I molecules became accumulated within the Golgi apparatus (Bremond *et al.* 2009).

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, elevation of intracellular $Ca2^{+}$ and the tyrosine phosphorylation of cellular proteins (Waclavicek *et al.* 1998; Wingett *et al.* 1999). We have 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 has also been demonstrated (Lee *et al.* 2002).

Although several lines of evidence indicate the multiple functional properties of CD99 and the involvement of CD99 in cell signaling, with its short cytoplasmic tail (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. In this study, in an attempt to understand the molecular mechanisms by which CD99 participates, we analyzed the association of CD99 with other cell surface molecules and its function. Monoclonal antibodies (mAbs) against CD99 molecules were produced and characterized. The produced mAbs were then used to identify CD99 associated partners. Role of CD99 on T cell activation were also determined. The information obtained may lead to a better understanding of immune mechanisms. This knowledge will be the basis for development of new therapeutic approach for various diseases.

1.2 Literature reviews

1.2.1 Immune system

The immune system is the organization of specialized cells and organs that protect our body from outside biological influences. When the immune system is functioning properly, it protects the body against infections, destroying cancer cells and foreign substances (Chinen and Shearer 2005). The immune system consists of innate immunity and adaptive immunity, which work together in protecting the body against foreign invaders as well as removing our own cells that have become diseased (Figure 1.1). Innate and adaptive immune responses are components of an incorporated system of host defense in which numerous cells and molecules function cooperatively (Eales 2003; Chinen and Shearer 2005; Abbas and Lichtman 2006; Chaplin 2010). The basic mechanisms of the innate and adaptive immunity are as follows.

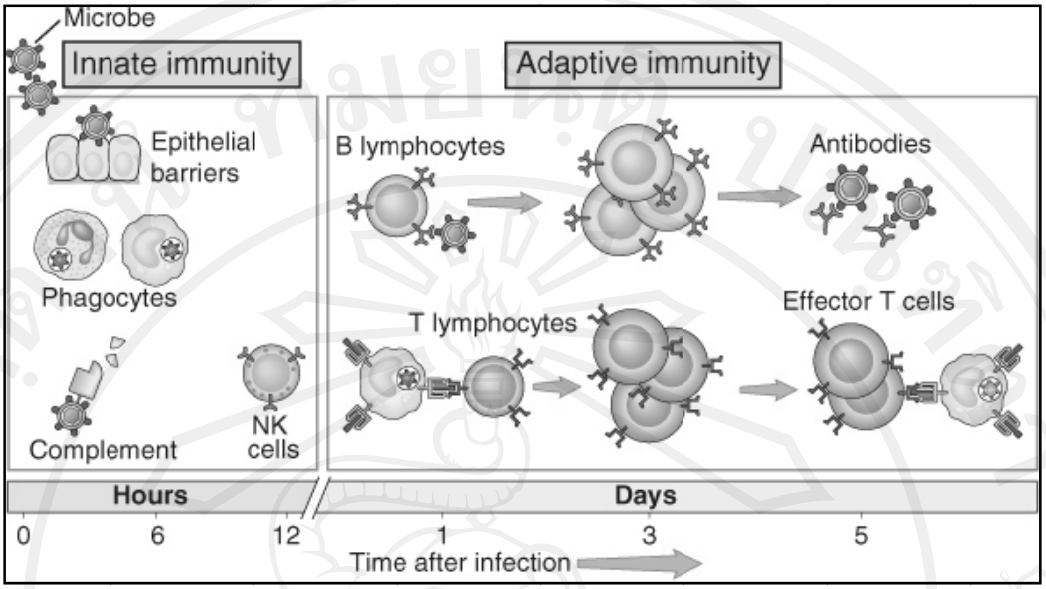

Figure 1.1 Innate and adaptive immunity.

The innate immunity responses the initial defense against infections. Some of the mechanisms prevent infections (e.g., epithelial barriers) and others eliminate microbes (e.g., phagocytes, natural killer (NK) cells, and the complement system). Adaptive immunity develop later and consist of activation of lymphocytes (Abbas and Lichtman 2006).

1.2.1.1 Innate immunity

Innate immunity is the first line of host defense against wide range of microorganism and allows a rapid response to invasion. It refers to the mechanism of host defense that plays a more important role in primitive life forms and host uses immediately or within several hours after exposure to an antigen. The principal components of innate immunity are physical and chemical barriers (skins and epithelia cells), blood protein complements (complement proteins, acute phase protein, and cytokines), and innate immune cells. The major innate immune cells are the phagocytes (macrophages, neutrophils, and dendritic cells), mast cells, eosinophils, basophils, and NK cells. Innate immunity is designed to recognize a few highly conserved structures present in many different microorganisms, which are called pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide, peptidoglycan, lipotechoic acids, mannose, bacterial DNA, double-stranded RNA, and glucans. The recognition of these molecules is mediated by a pattern recognition receptor (PRRs), expressed on the innate immune cells, including immunoglobulin G receptors (FcγR), complement receptors, Toll like receptors, CD14, C-type lectins, mannose receptor, β-glucan receptor (dectin-1), Nod like receptosr, and the scavenger receptors (Janeway and Medzhitov 1998; Medzhitov and Janeway 1998; Areschoug and Gordon 2008; Turvey and Broide 2010). Although innate immunity can effectively combat many infections, microbes that are pathogenic, perhaps, have evolved to resist innate immunity. Defense against these infectious agents is the task of the adaptive immune responses (Abbas *et al.* 2000; Janeway *et al.* 2004; Chinen and Shearer 2005; Chaplin 2010; Turvey and Broide 2010).

1.2.1.2 Adaptive immunity

Adaptive immunity is the next line of host defense after the innate immunity. Adaptive immune responses are triggered by microbes or substances called antigens after they pass through the innate immunity. Adaptive immunity allows the immune system to remember specific pathogens and therefore achieve stronger, faster, and more efficient defense the next time a particular microorganism is encountered. The adaptive immunity also plays an important role in exclusion of tumor cells. Adaptive immune responses are mediated by lymphocytes and their products, such as antibodies and cytokines. There are two types of adaptive immunity, called humoral mediated immunity (HMI) and cell-mediated immunity (CMI). HMI is mediated by antibodies produced by B lymphocytes which recognize microbial antigens, neutralize the infectivity of the microbes, and target microbes for elimination by various effector mechanisms. CMI is mediated by different cells and molecules to provide defense mechanism (Figure1.2) (Abbas *et al.* 2000; Janeway *et al.* 2004; Bonilla and Oettgen 2010; Chaplin 2010)**.**

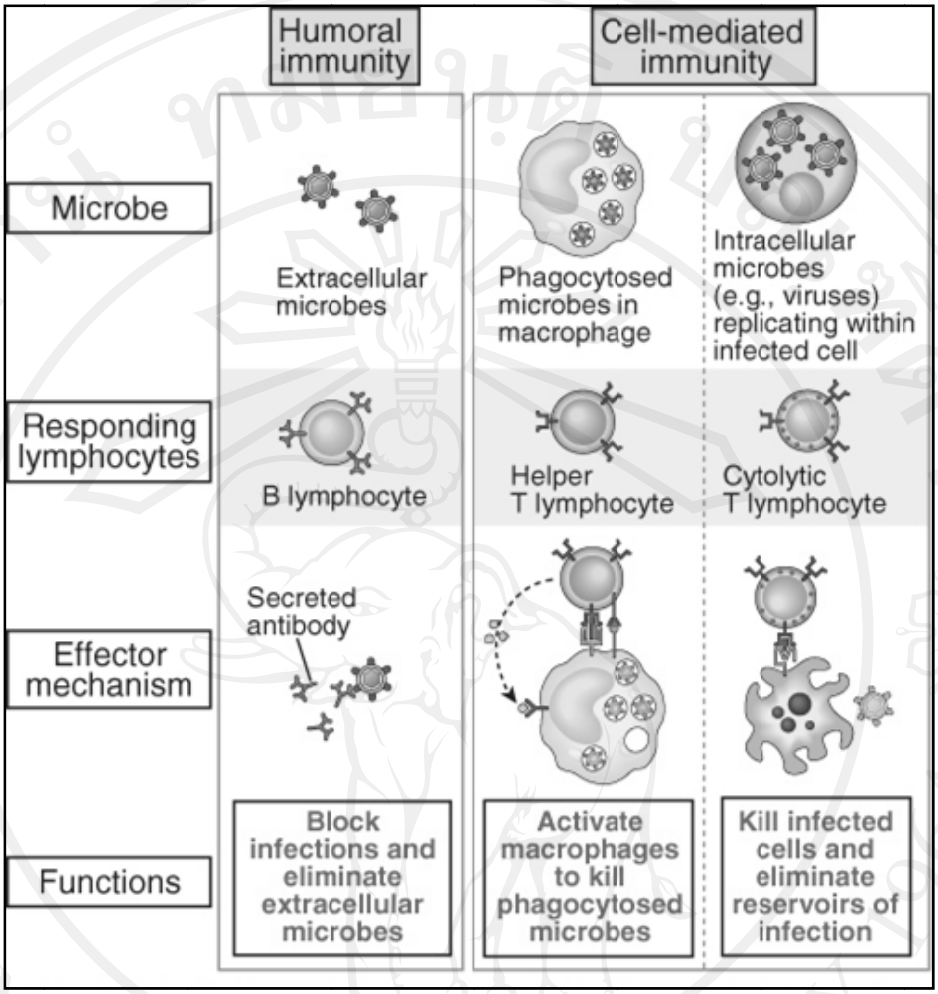

Figure 1.2 Types of adaptive immunity.

In HMI, B lymphocytes secrete antibodies that block infections and eliminate extracellular microbes. In CMI, T lymphocytes eradicate intracellular microbes (Abbas and Lichtman 2006).

1.2.1.2.1 Humoral meditated immunity (HMI)

The main function of the humoral immune response in the body is to destroy extracellular microorganisms and prevent the spread of intracellular infection. HMI is mediated by proteins called antibodies, which are produced by cells called B lymphocytes. A wide variety of antigens, including proteins, polysaccharides, lipids, and small chemicals activate and are recognized by B lymphocytes. The activation of B lymphocytes results in the proliferation of antigen specific cells, also called clonal expansion, and their differentiation into effector cells that actively secrete antibodies. Antibodies are secreted into the circulation and mucosal fluids, and they neutralize and abolish microbes and microbial toxins that are present in the blood and in the lumens of mucosal organs, such as the gastrointestinal and respiratory tracts. One of the most important functions of antibodies is to stop microbes that are present at mucosal surfaces and in the blood from gaining access to and colonizing host cells and connective tissues. In this way, antibodies prevent infections from ever getting established. Antibodies use their antigen-binding (Fab) regions to bind and block, or neutralize the infectivity of microbes and the interactions of microbial toxins with host cells. Other functions of antibodies require the involvement of various components of host defense, such as phagocytes and the complement system. The Fc regions of antibodies, heavy chain constant region, contain the binding sites for Fc receptors and complement, therefore promote the phagocytosis or activate the complement system (Abbas *et al.* 2000; Janeway *et al.* 2004; Bonilla and Oettgen 2010; Chaplin 2010). The actions of the induced antibodies in elimination of the invaders are shown in Figure 1.3.

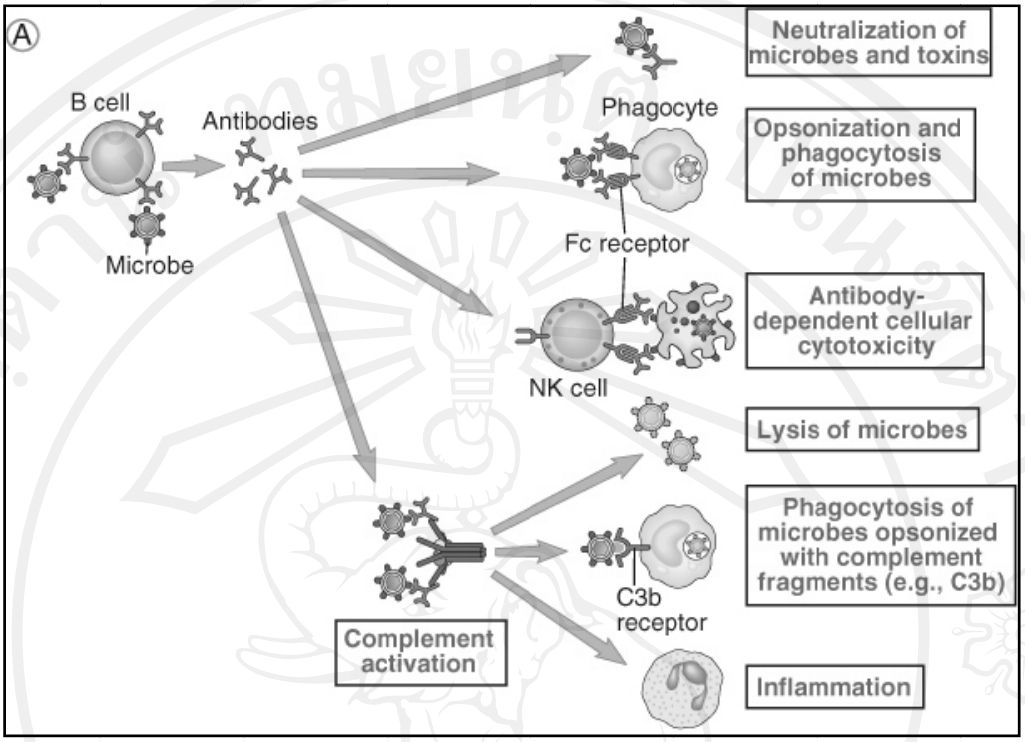

Figure 1.3 The functions of antibodies in elimination of the invaders.

B cell-differentiated plasma cell produce antibodies specifically recognize the antigen. Antibodies neutralize microbes and their toxins, opsonize them for phagocytosis and antibody-dependent cellular cytotoxicity, and activate the complement system (Abbas and Lichtman 2006).

1.2.1.2.2 Cell-mediated immunity (CMI)

CMI is the outcome of effector function of T lymphocytes. The responses of T lymphocytes consist of sequential phases: recognition of cell-associated microbes by naive T cells, expansion of the antigen-specific clones by proliferation, differentiation of some of the progeny into effector cells and memory cells. The biochemical signals triggered in T cells by antigen recognition result in the stimulation of various transcription factors that initiate the expression of genes encoding cytokines, cytokine receptors, and other molecules involved in T cell responses. Consequently, the antigen-specific T cells produce proteins called cytokines, some of which induce proliferation of the antigen-stimulated T cells and others mediate the effector functions of T cells (Figure 1.4). Two major types of T cells have been identified; $CD4^+$ helper T lymphocytes (T_H) and $CD8^+$ cytolytic T lymphocytes (CTLs) are bearing either CD4 or CD8 molecules on their surface, respectively. $CD4^+$ helper T cells differentiate into subsets of effector cells that produce restricted sets of cytokines and perform different functions. For instance, activating mononuclear phagocytes, NK cells, and cytolytic T cells for killing of intracellular microbes and virally infected targets. In addition, cytokines enhance antibody production, as well as a number of aspects of hypersensitivity and parasite-induced immune responses, including eosinophilopoiesis (Janeway *et al.* 2004; Chinen and Shearer 2005; Abbas and Lichtman 2006).

Differentiated $CD8^+$ CTLs recognize class I MHC-peptide complexes on the surface of infected cells and kill these cells, thus eliminating the reservoir of infection. The activation of effector CTLs leads to the exocytosis of the contents of the CTL's granules to the region of contact with the targets. CTLs kill target cells mainly as a

result of delivery of granule proteins, granzymes and perforin, into the target cells. Granzymes are enzymes that cleave and activate caspases of target cells which leads to induction of apoptosis. Perforin conveys granzymes into the cytoplasm of the target cells. Additionally, both perforin and granzymes may enter the target cells by receptor-mediated endocytosis. Perforin may then insert into endosomal membranes and facilitate the movement of granzymes through these membranes and into the cytoplasm. $CD8^{+}$T lymphocytes also produce the cytokine IFN-γ, which activates macrophages to destroy phagocytosed microbes and enhance the recruitment of additional leukocytes (Abbas *et al.* 2000; Janeway *et al.* 2004; Bonilla and Oettgen 2010; Chaplin 2010).

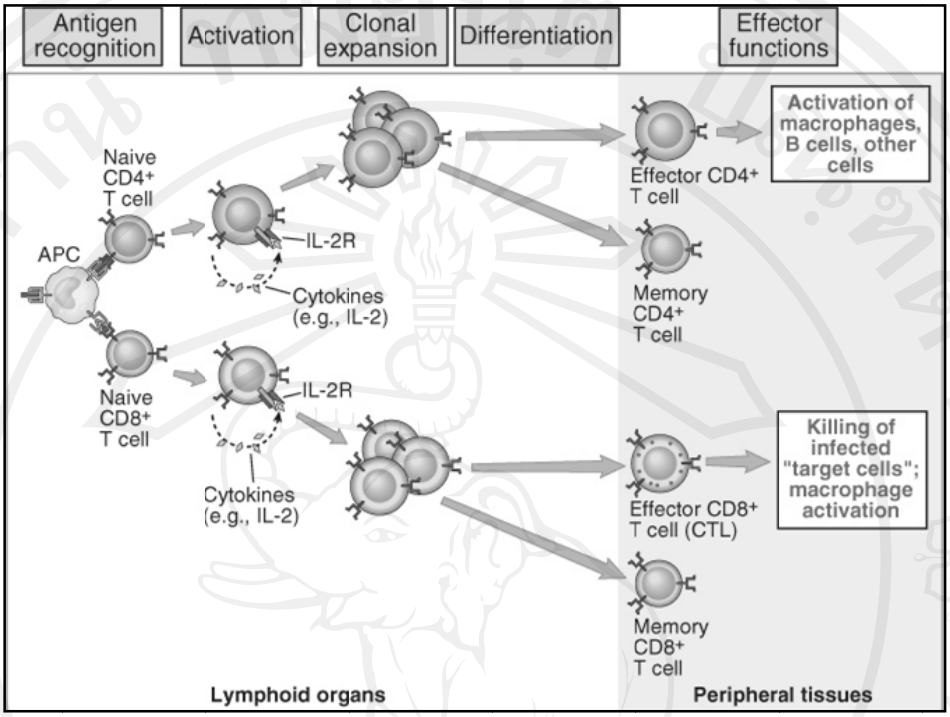

Figure 1.4 The initiation and effector phases of cell-mediated immunity.

T cells are activated on interaction of their TCRs with antigenic peptides complexed with MHC molecule. Subsequently, the activated T cells proliferate and differentiate into effector cells (Abbas and Lichtman 2006).

1.3 Lymphocytes

Lymphocytes, a population of leukocytes, are cellular components of the immune system that participate in and coordinate the immune response. These cells consist of distinct subsets that are different in their function and protein production. Three types of lymphocyte subset are defined: T lymphocytes, B lymphocytes and NK cells. T cells play a central role in orchestrating the immune response. Further, they are instrumental in eliminating intracellular pathogens (viruses, some bacteria) through the generation of cytotoxic T cells. B cells defend against extracellular pathogens by producing antibodies. Natural killer cells are an important component of innate immunity (Abbas *et al.* 2000; Janeway *et al.* 2004; Larosa and Orange 2008; Bonilla and Oettgen 2010; Chaplin 2010).

1.3.1 **T lymphocytes**

T cells develop in the thymus from common lymphoid progenitors coming from the bone marrow or fetal liver. T lymphocytes play a central role in the immune response, both as direct effector cells and as regulatory cells that modulate the functions of numerous other cell types, primarily those that participate in the body's defence mechanisms. This regulatory function is provided either through direct cell-cell contact or via the secretion of various cytokines. Thus, the proper function of T cells is essential for the maintenance of normal homoeostasis within and outside the immune system. Conversely, abnormalities in their function can lead to immunological diseases, e.g. autoimmunity, allergies or immunodeficiencies (Abbas *et al.* 2000; Janeway *et al.* 2004; Larosa and Orange 2008; Bonilla and Oettgen 2010; Chaplin 2010).

The primary event leading to the activation and differentiation of mature T cells is the triggering of their antigen-specific T cell receptor (TCR) by its specific ligand, which consists of a processed antigenic peptide presented in association with MHC molecules on the surface of APCs or appropriate target cells. Interaction of TCR with the peptide-MHC provides only partial signal for cell activation. Complete activation requires additional costimulatory signals provided by costimulatory molecules and their ligands. This event triggers several signal transduction pathways that involve second messengers, protein kinases, protein phosphatases and other enzymes and key intermediates thereby regulating diverse cellular functions such as proliferation, differentiation, and apoptosis (Bonilla and Oettgen 2010; Chaplin 2010; Hwang and Ki 2011).

The key recognition and activation element during physiological T cell responses to antigen is a complex receptor, consisting of an αβ heterodimer of TCR that confers antigen recognition and specificity and the associated, non-polymorphic polypeptides that constitute the CD3 complex (γ, δ, ε, and the disulfide linked ζ-ζ chains). The cytosolic components of these molecules contain a unique motif, the ITAM (immunoreceptor tyrosinebased activation motif). The two tyrosine residues within each ITAM become rapidly phosphorylated upon TCR engagement by the Src family kinases, predominantly Lck and Fyn in T cells. The zeta-associated protein of 70 kDa (ZAP-70), Syk family of tyrosine kinases, is recruited to the phosphorylated ITAMs of CD3 molecules. A tyrosine residue within the activation loop of the ZAP-70 kinase domain is then activated by phosphorylation by Lck and ZAP-70 itself. Activated ZAP-70 induces tyrosine phosphorylation of various downstream effector molecules, such as the adapter molecules linker for activation of T cells (LAT) and

the SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76). These scaffold proteins can associate with several distinct molecules, and this association then regulates activation cascades further downstream pathway, including the Ras/ERK MAPK cascade, the Ca/calcinurin/NF-AT pathway and the PKC/NF-κB pathway (Figure 1.5). Consequently, the genes that control lymphocyte proliferation and differentiation are activated (Razzaq *et al.* 2004; Cronin and Penninger 2007; Bonilla and Oettgen 2010; Chaplin 2010; Nakayama and Yamashita 2010).

1.3.2 **B lymphocytes**

B cells develop in the bone marrow but fully mature in peripheral lymphoid organs. Developmental stages are defined by the successful expression of heavy-chain and light-chain immunoglobulin genes. B cells provide humoral immunity against pathogens through antibody production. Antibodies neutralize pathogens and toxins, facilitate opsonization, and activate complement. In the initial step for B cell activation, B cell receptor (BCR) recognizes antigens in the form of foreign proteins, which exist in soluble, particle-bound or cell-bound forms. In addition, the BCR is able to bind large conformational epitopes alternating in primary structure that include nonpeptide antigens such as polysaccharides and nucleic acids. In most cases, primary infection or vaccination results in prolonged production of high affinity specific antibody, the basis of adaptive humoral immunity (Abbas *et al.* 2000; Janeway *et al.* 2004; Larosa and Orange 2008; Bonilla and Oettgen 2010; Chaplin 2010).

The ligand-binding subunit of the BCR complex is composed of a transmembrane immunoglobulin heavy chain and a covalently linked light chain. A non-covalently associated heterodimer of Igα/Igβ. BCR oligomerization provide the

signal transduction subunit which is induced by antigen binding seems to be sufficient to start the signaling event. After coligation, the cytoplasmic domains of the BCR also cluster. Subsequently, secondary rearrangements occur and a closed conformation then transforms to an open conformation. The open conformation of the activated BCR is dependent on ITAM phosphorylation most likely by the Lyn, one of Src-family kinases. The Syk then binds to phosphorylated ITAMs of Igα/Igβ and becomes activated. Consequently, activated Syk phosphorylates a number of downstream substrates, including adapter molecules, which, in turn, are critical for optimal activation of effector enzymes, e.g. phospholipase Cγ2 (PLCγ2) or phosphoinositide 3-kinase (PI3K). As a result, several transcription factors including cAMP responsive element binding proteins and ELK are activated. This will lead the B cells into cell cycle progression by promoting transcription from key regulatory genes (Figure 1.6) (Kurosaki and Hikida 2009; Bonilla and Oettgen 2010; Chaplin 2010; Hwang and Ki 2011).

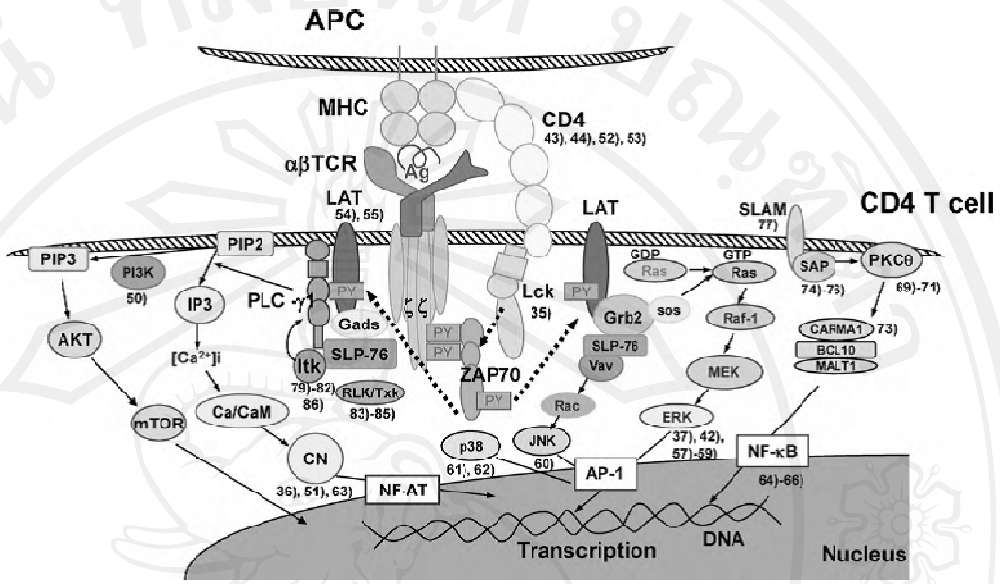

Figure 1.5 TCR activation signaling pathway.

The TCR α and β chains recognize peptide/MHC complexes expressed on APCs, an interaction that is stabilized by the immediate binding of CD8-MHC class I or CD4-MHC class II. The ITAMs are phosphorylated by Src family kinases, leading to recruitment and activation of signaling molecules, including ZAP-70. Activated ZAP-70 mediated downstream signaling molecules phosphorylation resulting in activation of transcription factor (Nakayama and Yamashita 2010).

Figure 1.6 Model for activation of BCRs signaling.

Upon activation after BCR cross-linking, Syk binds to phosphorylated ITAMs of Igα/Igβ, becomes activated and transmitted though downstream signaling molecule lead to activate the transcription factor (Kurosaki and Hikida 2009).

1.3.3 **NK cells**

NK cells are thought to represent a third lineage of lymphoid cells. Activated NK cells have the morphology of a large granular lymphocyte. They develop in the bone marrow under the influence of bone marrow stromal cells and IL-2, IL-15. They represent only a small fraction of peripheral blood cells and a small fraction of lymphoid cells in the spleen and other secondary lymphoid tissues. NK cells do not have T or B cell antigen receptors but instead immunoglobulin (Ig)-like killer-inhibitory receptors (KIRs) or killer-activating receptors (KARs) that recognize HLA class I molecules on target cells.

NK cell functions are induced by ligation of NK cell activating receptors resulting in perforin-dependent cytotoxicity, cytokine production (especially IFN-γ, IL-5, and IL-13), and induction of co-stimulatory molecules. In this ability, NK cells have prominent anti-tumor effects and are effective killers of viral infected cells. Moreover, they can facilitate adaptive immunity. NK cells also have a well defined system of preventing their functions by using several families of inhibitory receptors. These receptors define an essential feature of NK cells, which is the routine sampling of healthy cells in search of abnormal cells that have lost determinants of self (Abbas *et al.* 2000; Janeway *et al.* 2004; Larosa and Orange 2008; Bonilla and Oettgen 2010; Chaplin 2010).

1.4 Immunological synapse (IS)

There are many different cells in the immune system. To mount an effective immune response, they need to communicate with each other. Cells of the immune system communicate both by direct interactions via membrane-bound receptors and

via secreted mediators from one cell to another. Upon T cell activation, APCs present antigenic peptide to T cells by direct cell-cell contact. During T cells-APCs interaction, a specific structure, named immunological synapse, at the interface between T cells and APCs is formed. The immunological synapse is a dynamic structure, formed between T cells and APCs, characterized by lipid and protein segregation, signaling compartmentalization, and bidirectional information exchange through soluble and membrane-bound transmitters (Yokosuka and Saito 2010). The immunological synapse is the site where signals are delivered by the T cell receptors, adhesion molecules, as well as co-stimulatory and co-inhibitory receptors. The immunological synapse is divided into regions: a central-supramolecular activation cluster (c-SMAC), a peripheral-(p-) SMAC, and a distal-(d-) SMAC (Yokosuka and Saito 2010). The major components of the c-SMAC are key molecules for T cell signaling, such as TCR/CD3-MHC, CD28 or cytotoxic T-lymphocyte antigen-4 (CTLA-4)-CD80/CD86, tetraspanin CD81 and protein kinase C θ (PKC θ). In contrast, the p-SMAC consists of cytoskeleton-related or adhesion molecules structurally supporting the immunological synapse, such as leukocyte function-associated antigen-1 (LFA-1)/talin-intracellular adhesion molecule-1 (ICAM-1) and CD2-CD48/CD58. The distal-SMAC (d-SMAC) is defined as a region rich in molecules with long extracellular domains, such as CD45 and CD43 (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 immunological synapse during T cell activation (Figure 1.7) (Tarrant *et al.* 2003; Yokosuka and Saito

2010). Forming of IS and recruitment of various molecules are the crucial events for lymphocyte activation.

1.5 Lipid rafts

Lipid rafts are defined as detergent-resistant membrane microdomains of specific lipid and protein composition. Lipid rafts are enriched in cholesterol, glycosphingolipids, and sphingomyelin creating an environment which attracts certain proteins while excludes others. Many key signaling proteins and receptors including Src-family kinases, GTP-binding proteins and glycosylphosphatidyl-inositol (GPI)-linked receptors are restricted to lipid rafts. Due to their characteristics, lipid rafts resist to solubilization with commonly used detergents such as Triton X-100, NP40 or CHAPS. But, they are easily solubilized, for example, by n-octylglucoside or sodium dodecyl sulfate. They can be easily isolated by equilibrium centrifugation in sucrose gradient, gel filtration or visualized by image or video with immunofluorescent staining and microscopy (Horejsi 2003; Horejsi 2005; Kabouridis and Jury 2008; Simons and Gerl 2010).

Biological functions attributed to lipid rafts include endocytosis, pinocytosis, sorting and transport of proteins, and signal transduction. In addition, many viral pathogen infections take place at the site of lipid rafts. For example, human immunodeficiency virus (HIV), SV40, influenza virus and other viruses use lipid rafts as the injection sites of nucleotide acids, and the sites for assembly and budding as well.

Significantly, it has been indicated that lipid rafts play important roles in the TCR signaling pathways. In resting T cells, the TCR may weakly interact with lipid

rafts. Antigen presentation by APCs strengthens the association of the TCR with lipid rafts and induces a transient redistribution of CD45 to these domains. Dephosphorylation of Lck-Y505 and PAG/Cbp by CD45 in cooperation with dissociation of Csk, increases the activity of Lck. The TCR then gets phosphorylated by the Src family kinase, Lck, and recruits and activates the tyrosine kinase, ZAP70. The main target of ZAP70 is LAT, which initiates further downstream signaling. The TCR clusters are anchored to actin filaments and are transported towards the cSMAC, the central part of the immunological synapse. The TCR microcluster signaling is imagined to take place in a raft platform (Figure 1.8). The conjugation can hold together up to several hours. In the engagement of a cascade of downstream signaling events, raft aggregation promotes tyrosine phosphorylation and recruitment of signaling proteins, but excludes certain proteins, such as the tyrosine phosphatase CD45 and CD43 (Kabouridis 2006; Jury *et al.* 2007; Kabouridis and Jury 2008; Luo *et al.* 2008). Lipid raft clustering is, therefore, an important process for regulation of T cell activation.

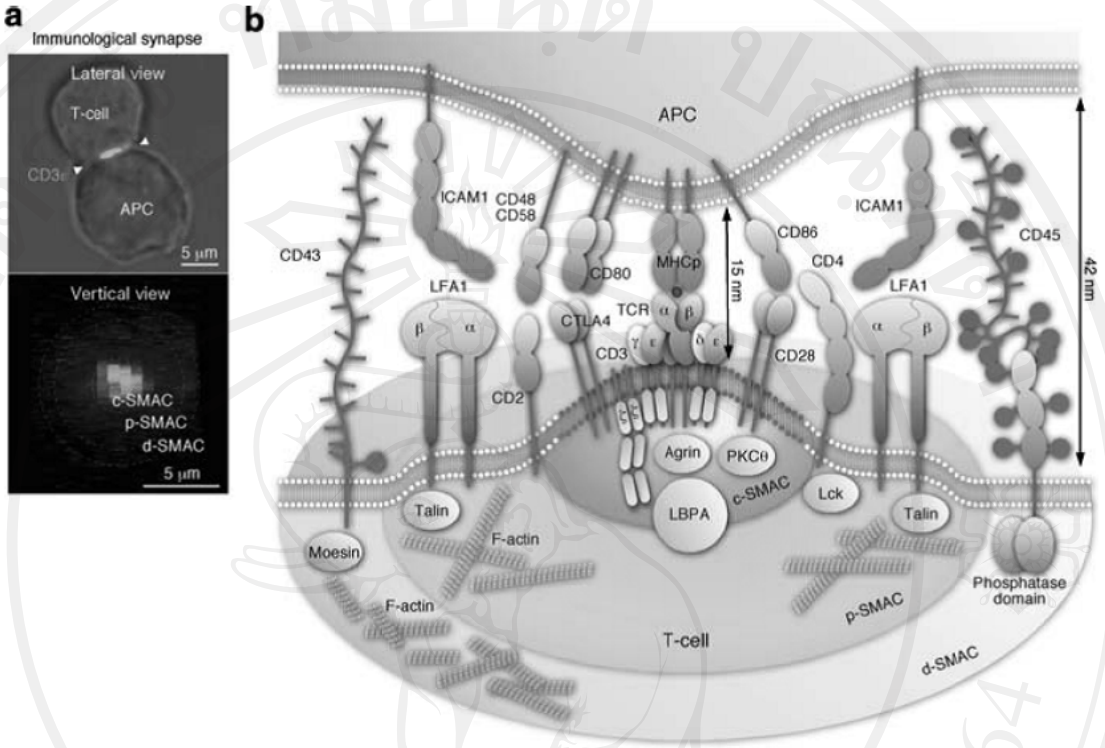

Figure 1.7 Architecture of the conventional immunological synapse.

The IS is conventionally depicted by a "bull's eye" structure between a T cell and an APC. (a) The CD3 core is clearly observed at the stable conjugation between a T cell and an APC by fluorescence-labeled anti-CD3ε antibodies (lateral view, top). The immunological synapse is composed of c-SMAC, p-SMAC, and d-SMAC in the vertical view (bottom). (b) The alignment of the receptors and the adhesion molecules are ordered by size of ectodomain; TCR/CD3 complex-MHC-peptide, CD28/PKC θ - CD80/86, cytotoxic T- lymphocyte antigen-4 (CTLA-4)-CD80/CD86, Agrin, and lysobisphosphatidic acid (LBPA) in the c-SMAC; CD2–CD48/CD58, LFA-1/ICAM-1, F-actin, and CD4/Lck in the p-SMAC; and CD43/moesin, CD45, and F-actin in the d-SMAC (Yokosuka and Saito 2010).

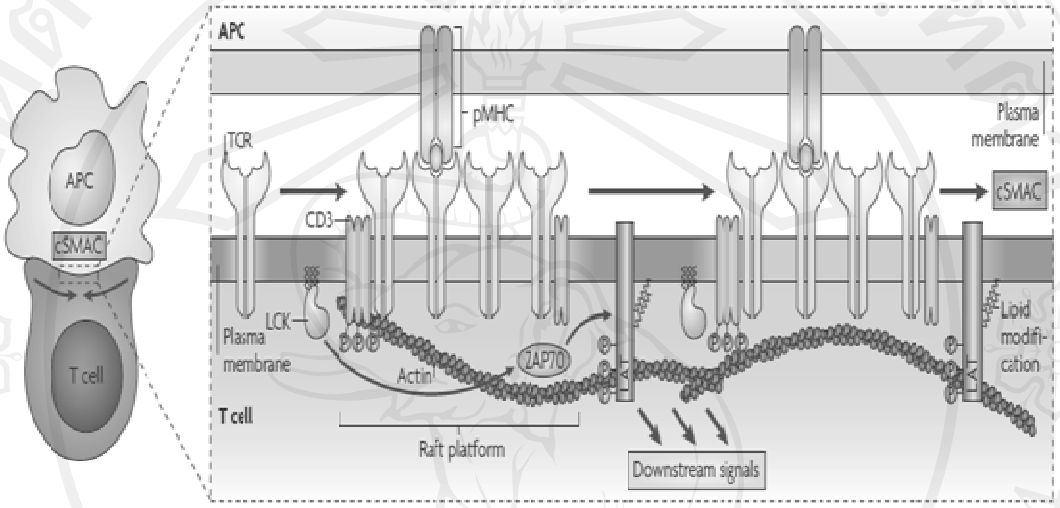

Figure 1.8 The central event of T cell activation occurs in lipid rafts of the conjugation of T cell and APC.

A diagram describes the main components involved in TCR mediated T cell activation, which occurs in the lipid rafts of plasma membrane. T cells are activated by conjugation with cognate APCs. TCRs interact with the antigenic peptide bound to the MHC on APCs, and this leads to the phosphorylation of ITAMs in the TCR multisubunit complex by Lck. Subsequently, the ZAP70 and LAT are recruited and activate, which lead to initiation of further downstream signaling. The TCR clusters are anchored to actin filaments and are conveyed to the cSMAC, the central part of the immunological synapse (Simons and Gerl 2010)**.**

1.6 Tetraspanins

Tetraspanins are cell surface proteins that span the cell membrane four times (Boucheix and Rubinstein 2001). The tetraspanins are characterized by four transmembrane domains delimiting two extracellular regions of unequal sizes, a small extracellular loop (EC1) containing 20–28 amino acids and a large extracellular loop (EC2) containing 76–131 amino acids (Figure 1.9) (Wright and Tomlinson 1994; Maecker *et al.* 1997). Although several protein families have four transmembrane domains, the key features for the tetraspanins are four or more cysteine residues in the EC2 domain, with two in a highly conserved 'CCG' motif. Currently, in human, twenty-six tetraspanins have been identified (Table 1.1). The tetraspanin genes in human are located on different chromosomes; several are located on chromosome 11 (CD81, CD82, CD151 and NAG-2 and Rom-1) and on chromosome 12 (CD9, CD63, C0-029, SAS and NET-5). The conservation of gene structure strongly suggests that these molecules derive from a common ancestor, by duplication. The tetraspanins are broadly expressed in many cell types. 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. 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). 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). Specific tetraspanin interactions with a wide range of leukocyte receptors have been reported, including interactions with CD2, CD4, CD8, CD5, CD19, CD21, Fc receptors and MHC class I and class II. Therefore, tetraspanins have the potential to influence signals transduced through many of the most important receptors in the immune system. Cross-linking tetraspanins at the cell surface can result in tyrosine phosphorylation, calcium fluxes and inositol phosphate turnover. In leukocytes, these signals ultimately regulate such fundamental biological processes as cell proliferation, adhesion and motility. Importantly, ligation of tetraspanin, i.e. CD9, CD53, CD81 or CD82, provides a co-stimulatory signal to T lymphocytes. Furthermore, tetraspanins CD82 and CD81 have been reported to redistribute to the immunological synapse and CD81 clusters to the c-SMAC. Aggregation of tetraspanin microdomains with components of the SMAC, thereby enhancing TCR engagement lead to activation of T cells (Figure 1.9) (Schick and Levy 1993; Szollosi *et al.* 1996; Boucheix and Rubinstein 2001; Levy and Shoham 2005). Although, several functions of tetraspanins have been suggested, the detail mechanism is not precisely known. The structure and function of the "tetraspanin web" is now under investigation in several laboratories around the world. The challenge is to determine how the organization of the "tetraspanin web" modifies the function of its constitutive molecules and consequently influences cellular behavior. The implications may be considerable for the understanding of basic cellular processes such as migration and also of diseases related to loss or mutation of a single tetraspanin.

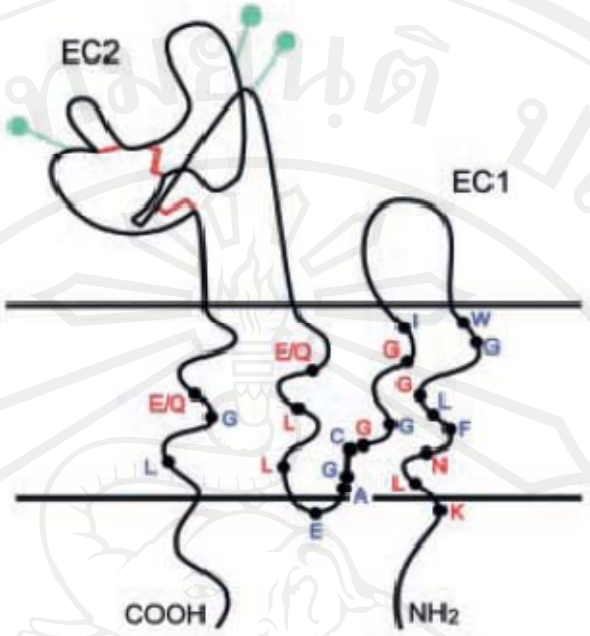

Figure 1.9 Structure of a typical tetraspanin.

The four transmembrane domains contain conserved amino acids which are indicated. Red, ≥80%, and blue, ≥60% conservation, and they flank the small and large extracellular loops , EC1 and EC2, respectively. The main localisations of potential N-glycosylation sites are shown in green The EC2 is composed of a core formed by helices (red line) is conserved among the tetraspanins. Helices are flanked by the CCG motif and further conserved cysteine residues (Boucheix and Rubinstein 2001).

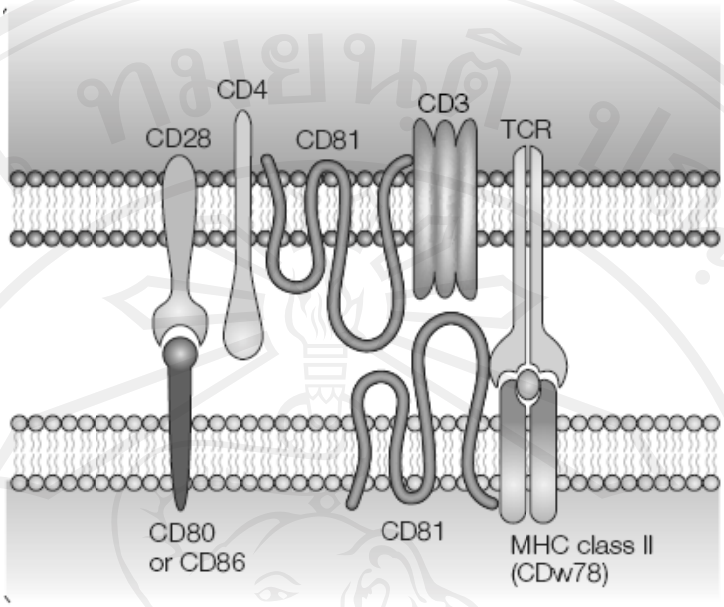

Figure 1.10 Dual role for CD81 in the antigen-presenting cell–T-cell immune synapse.

In antigen presenting cells, tetraspanin CD81 associates with MHC class II molecules, particularly the CDw78-epitope-containing subset. In T cells, CD81 associates with CD3 and CD4. Tetraspanin provide a scaffold facilitated the engagement of TCR and signaling (Schick and Levy 1993).

Table 1.1 Size and genomics of human tetraspanins

Molecule	MW (kDa)	Length a.a.	Unigene	Chromosome localization
CD9	24–26	228	Hs.1244	12p13
CD37	40–50	281	Hs.153053	19q13
CD53	32–40	219	Hs.82212	1p12-p31, 15q24 (seq)
CD63 (ME491)	30 –60	237	Hs.76294	12q13
CD81 (TAPA-1)	26	236	Hs.54457	11p15.5
CD82 (R2/IA4)	50–80	267	Hs.25409	11p12
CD151 (PETA-3)	27	253	Hs.75564	11p15
C0-029	27–34	237	Hs.84072	12q14
NAG-2	28–35	238	Hs.26518	11
Oculospanin	unk	355	AAG42857*	unk
CD231/TM4SF-2/TALLA-1	38–45	244	Hs.82749	Xq11
SAS	unk	210	Hs.50984	12q13
Uroplakin Ib	28 (bov)	260	Hs.271580	3q13.3-q21
Uroplakin Ia	27 (bov)	258	Hs.159309	19q12 (seq)
Peripherin/RDS	39	345	Hs.281564	6p21
Rom-1	33	351	Hs.1943	11q12
Tspan-1	unk	241	Hs.38972	1p32
Tspan-2	25 (rat)	224	Hs.122540	1q12 (seq)
Tspan-3	unk	253	Hs.100090	unk
Tspan-5	unk	268	Hs.20709	4
Tspan-6	unk	245	Hs.121068	Xq21.1
NET-2	unk	305	Hs.16529	7q31
NET-5	unk	239	Hs.129826	12p13 (seq)
NET-6	unk	204	Hs.284243	7p21
NET-7	unk	294	Hs.95583	10q21
TM4-B	unk	245	Hs.271943	9q37

The gene localizations resulted from genetic or cytogenetic analysis; (seq) indicates the localization that derived from the draft assembly of the human genome sequence (http://genome.ucsc.edu/goldenPath/hgTracks.html). For CD53, the two localisations are different. The GenBank accession number is given for oculospanin (*) (Boucheix and Rubinstein 2001).

1.7 Leukocyte surface molecules

Leukocytes express a large number of cell surface proteins called leukocyte surface molecules. These molecules play key roles in all aspects of leukocyte functions such as differentiation and maturation, controlling their patterns of migration, response to foreign antigen and the control of the immune response via cytokines and interactions with other cell types (Barclay *et al.* 1997; Lodish *et al.* 2008). For these functions it is necessary for cell surface antigens to interact with soluble proteins or glycoproteins as well as with the surfaces of other cells and with the extracellular matrix. In addition, many of these interactions will lead to signaling events transduced to the cell interior by proteins interacting with the cytoplasmic regions of the cell surface antigens. Most cell surface molecule are probably present as monomers at the cell surface, but there are several molecules associate with 2-7 different polypeptides to form stable complexes, e.g. TCR, BCR and Fc receptors (Barclay *et al.* 1997).

Some of leukocyte surface molecules are expressed on specific type of leukocytes, on lymphocyte subsets and differentially expressed during lymphocyte development and activation. These differentiation antigens are detected by specific monoclonal antibodies. These cell phenotype-determining antigens are assigned cluster of differentiation (CD) numbers. There are currently over 350 defined CD antigens. Updates are issued by Human Cell Differentiation Molecules (HCDM), an organization that organizes periodic Human Leukocyte Differentiation Antigen (HLDA) workshops at which newly identified cell surface molecules are defined and registered (Barclay *et al.* 1997).

1.7.1 General structure of cell surface proteins

Membrane proteins can be classified into three categories based on the nature of their association with the membrane. Integral membrane proteins are embedded directly within the lipid bilayer. Lipid-anchored membrane proteins are bound covalently to one or more lipid molecules. Peripheral membrane proteins are not inserted into the lipid bilayer but are associated with the membrane indirectly, generally by interactions with integral membrane proteins (Figure 1.10) (Lodish *et al.* 2008).

1.7.2 Integral membrane proteins

Integral membrane proteins, also called transmembrane proteins, span a phospholipid bilayer and are built of three segments. The cytosolic and exoplasmic domains have hydrophilic exterior surfaces that interact with the aqueous solutions on the cytosolic and exoplasmic faces of the membrane. In contrast, the membrane-spanning domain contains many hydrophobic amino acids whose side chains protrude outward and interact with the hydrocarbon core of the phospholipid bilayer. Some proteins include a charged residue in the membrane spanning domain, which interact with charged residue on the other proteins. In addition, most transmembrane proteins are glycosylated with a complex branched sugar group attached to one or several amino acid side chains. Invariably these sugar chains are localized to the exoplasmic domains. Protein domains on the extracellular membrane surface are generally involved in cell-cell signaling or interactions. Domains within the membrane, particularly those that form channels and pores, move molecules across the membrane. Domains localized along the cytosolic face of the membrane have a wide

range of functions, from anchoring cytoskeletal proteins to the membrane to triggering intracellular signaling pathways. Receptors often consist of two or more protein chain where one bind the ligand and other transmits the signal. In many cases, the function of a membrane protein and the topology of its polypeptide chain in the membrane can be predicted based on its homology with another, well-characterized protein (Barclay *et al.* 1997; Krauss 2003; Lodish *et al.* 2008; Gomperts *et al.* 2009).

1.7.3 **Lipid-anchored membrane proteins**

The lipid-anchored membrane proteins are bound covalently to one or more lipid molecules. The hydrophobic carbon chain of the attached lipid is embedded in one leaflet of the membrane and anchors the protein to the membrane. The cell surface proteins linked by glycosyl-phosphatidylinositol (GPI) anchors, whereas the cytoplasmic proteins are linked by lipid moieties such as myristoyl groups. The polypeptide chain itself does not enter the phospholipid bilayer. These proteins are localized to either the cytosolic or the exoplasmic face of the plasma membrane and play a role in the cellular communication (Barclay *et al.* 1997; Lodish *et al.* 2008).

1.7.4 **Peripheral membrane proteins**

The peripheral membrane proteins do not interact with the hydrophobic core of the phospholipid bilayer. Instead they are usually bound to the membrane indirectly by interactions with integral membrane proteins or directly by interactions with lipid head groups. Peripheral proteins are localized to either the cytosolic or the exoplasmic face of the plasma membrane. In addition to these proteins, which are closely associated with the bilayer, cytoskeletal filaments are more loosely associated with the cytosolic face, usually through one or more peripheral (adapter) proteins. Such

associations with the cytoskeleton provide support for various cellular membranes; they also play a role in the two-way communication between the cell interior and the cell exterior (Barclay *et al.* 1997; Lodish *et al.* 2008).

.

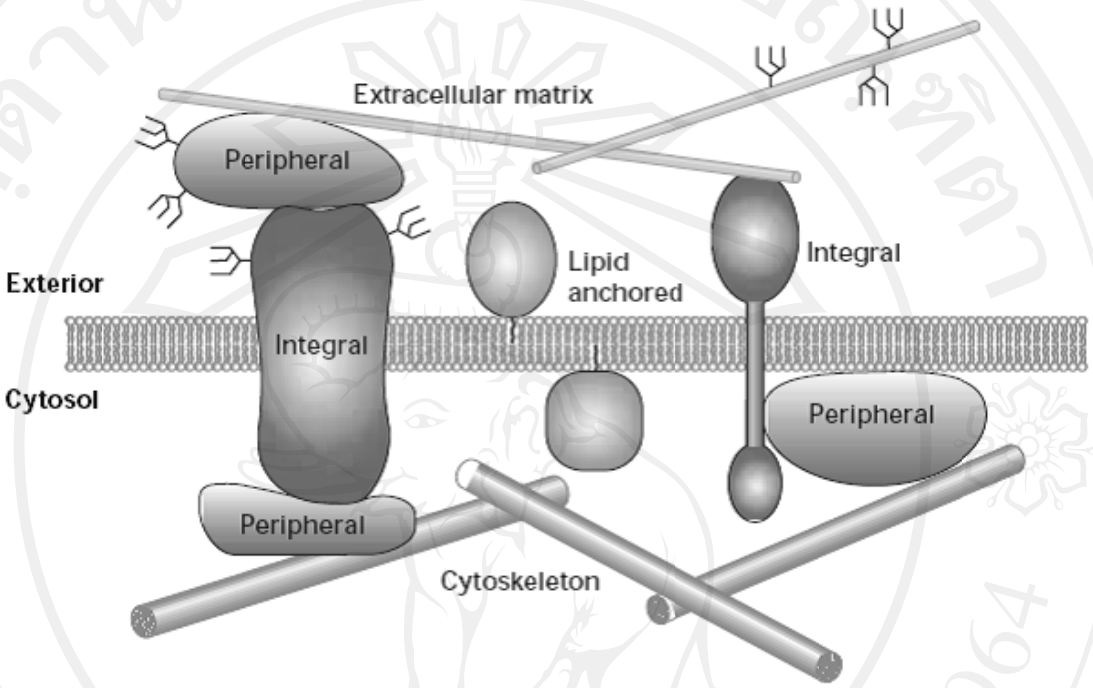

Figure 1.11 Diagram of integration of proteins associate with the lipid bilayer.

Integral (transmembrane) proteins span the bilayer. Lipid-anchored proteins are tethered to one leaflet by a long covalently attached hydrocarbon chain. Peripheral proteins associate with the membrane primarily by specific noncovalent interactions with integral proteins or membrane lipids (Lodish *et al.* 2008).

1.8 CD99

CD99 is a type 1 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 among many cell types, both hematopoietic and non-hematopoietic cells (Dworzak *et al.* 1999; Kasinrerk *et al.* 2000; Dworzak *et al.* 2004; Kovar and Bernard 2006; Khunkaewla *et al.* 2007). From a functional viewpoint, CD99 has been described as a T-cell costimulator and regulator of cytokine production (Waclavicek *et al.* 1998; Oh *et al.* 2007), and as a strong activator of the actin cytoskeleton and of integrin. Furthermore, it has been implicated in promoting cell adhesion and homotypic aggregation, immediate arrest on an inflamed vascular endothelium, and cell migration through the vascular endothelium (Waclavicek *et al.* 1998; Bernard *et al.* 2000; Kasinrerk *et al.* 2000; Schenkel *et al.* 2002; Khunkaewla *et al.* 2007). Engagement of CD99 with agonistic antibodies induced apoptosis of immune cells and tumor cells (Bernard *et al.* 1997; Scotlandi *et al.* 2000; Khunkaewla *et al.* 2007). CD99 ligation was also demonstrated to induce expression of adhesion molecules, including ELAM-1, VCAM-1 and ICAM-1, which are associated with leukocyte adhesion and transendothelial migration (Bernard *et al.* 1995; Hahn *et al.* 1997; Bernard *et al.* 2000; Kasinrerk *et al.* 2000; Schenkel *et al.* 2002; Yun *et al.* 2006; Khunkaewla *et al.* 2007; Lou *et al.* 2007;

Schneider *et al.* 2009). Furthermore, CD99 engagement has been reported to induce the expression of TCR, MHC class I and MHC class II by accelerated mobilization of these molecules from the Golgi compartment to the plasma membrane (Choi *et al.* 1998). Requirement of CD99 expression in IFN-γ induced MHC class I expression has also been observed (Bremond *et al.* 2009). Without CD99, upon IFN-γ stimulation, MHC class I molecules became accumulated within the Golgi apparatus (Bremond *et al.* 2009).

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, elevation of intracellular $Ca2^{+}$ and the tyrosine phosphorylation of cellular proteins (Waclavicek *et al.* 1998; Wingett *et al.* 1999). We have 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 has also been demonstrated (Lee *et al.* 2002).

On the cell surface, CD99 can be expressed in either a full-length unspliced form or as a short variant resulting from alternative splicing (Bernard *et al.* 1997; Hahn *et al.* 1997). The long form (type I) contains 185 amino acid residues and its mobility in SDS-PAGE corresponds to an apparent molecular weight (MW) of 32 kDa. The short form (Type II; 161 residues, apparent MW of 28 kDa) harbors a

deletion in the cytoplasmic segment. The CD99 isoforms are differentially expressed in a cell type-specific manner among hematopoietic cells and cell lines (Bernard *et al.* 1997; Hahn *et al.* 1997). The CD99 isoform expression was shown to dictate distinct functional events (Wingett *et al.* 1999; Alberti *et al.* 2002; Byun *et al.* 2006). Expression of the long form in CD99-deficient Jurkat T cell line is sufficient to promote CD99-induced cell adhesion, whereas co-expression of the two isoforms is required to trigger T cell death (Bernard *et al.* 1997). In addition, on B cells, the short form of CD99 inhibited homotypic adhesion, while the activation of the CD99 long form promoted cell-cell adhesion. The opposing effects of CD99 isoforms on homotypic B cell aggregation were shown to be due to their opposing functions in controlling the expression of the cell adhesion molecule, LFA-1 (Hahn *et al.* 1997).

The truncation of the cytoplasmic domain of CD99 short form may result in a loss of interactions 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 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 as 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 CD99 short form causes an alteration of the three-dimensional structure, leading to different binding sites for the 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 provokes

different sets of signaling pathways (Byun *et al.* 2006). Although several functional roles of CD99 are indicated, the precise mechanism is not yet clearly understood and its natural ligand has not been characterized. Role of CD99 in the regulation of immune responses is of interest to be investigated.

1.9 Objectives

1.9.1 To produce monoclonal antibodies against CD99 molecules

1.9.2 To identify the interacting partners of CD99 molecules

1.9.3 To study the function of the CD99 and its partners involving T cell activation