Chapter 10
Tetraspanins and Immunity

Mark D. Wright and Shoshana Levy

Abstract Studies of tetraspanins in cells of the immune system were the first to reveal the interactions of tetraspanins with each other and with their associated molecular partners. The extensive knowledge of immune cell subsets, the functionally distinct molecules expressed by these cells, and the availability of specific antibody reagents has had a major impact on our understanding of how tetraspanins assemble in cell membranes, and how they affect the function of their partners. Here we briefly introduce the various cell types that partake in innate and adaptive immune functions. We then highlight the role of tetraspanins in both arms of the immune system. Tetraspanins influence immune cell migration and antigen presentation. Moreover, they are present on both sides of immune synapses, namely, on antigen presenting cells and on T cells. Indeed, deficiency of specific tetraspanins in both mice and humans results in immune impairments.

Abbreviations

APC	Antigen presenting cells
BCR	B cell receptor
C	Constant region gene
CTL	Cytotoxic T cells
CVID	Variable immunodeficiency disorder
D	Diversity region gene
DCs	Dendritic cells

M.D. Wright
Department of Immunology, Monash University,
AMREP campus, Melbourne, Victoria, Australia

S. Levy (✉)
Department of Medicine, Division of Oncology,
Stanford University Medical Center, Stanford, CA, USA
e-mail: slevy@stanford.edu

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FO	Follicular
FRET	Fluorescence resonance energy transfer
ICAM-1	Intracellular adhesion molecule-1
IS	Immune synapse
J	Joining region gene
LPS	Lipopolysaccharide
MHC	Major histocompatibility molecules
MIICs	MHC class II enriched compartments
MZ	Marginal zone
NK	Natural killer cells
PAMPs	Pathogen associated molecular patterns
PRR	Pattern recognition receptors
TCR	T cell receptor
TEM	Tetraspanin-enriched microdomains
Th	T helper cells
TLR	Toll-like receptor
Treg	Regulatory T cells
V	Variable region genes
VCAM	Vascular cell adhesion molecule-1

10.1 Introduction

The tetraspanin web concept originally emerged by studying tetraspanins in immune cells (Rubinstein et al. 1996). Results of studies analyzing the associations of tetraspanins with histocompatibility molecules and integrins expressed on the surface of B cells, led Rubinstein and colleagues to postulate "the existence of a tetraspanin network which, by connecting several molecules, may organize the positioning of cell surface proteins and play a role in signal transduction, cell adhesion, and motility" (Rubinstein et al. 1996). Similarly, the concept of tetraspanin-enriched microdomains (TEM) that are distinct from lipid rafts (Kropshofer et al. 2002) was deduced following interrogation of immune cells with specific antibody reagents. The extensive knowledge of immune cell subsets, the functionally distinct molecules expressed by these cells, and the availability of specific antibody reagents has had a major impact on our understanding of how tetraspanins assemble in cell membranes, and regulate molecular function.

10.2 Immune Cells

Cells of the innate immune system initiate immune responses in a non-specific way. Innate immune phagocytic cells, such as macrophages and neutrophils, display several germline encoded pattern recognition receptors (PRR), which recognize molecular motifs in pathogens, termed pathogen associated molecular patterns (PAMPs). Upon

the recognition of PAMPs by PRR, innate immune cells initiate immune responses by becoming activated. Activated innate immune cells secrete pro-inflammatory cytokines and granules, migrate to the site of infection or to draining lymph nodes, and, ultimately play a critical role in activating the adaptive immune response.

The most important functional distinction between innate and adaptive immune cells is the acquisition of memory by adaptive immune cells. The major players in the adaptive arm of the immune system are B cells and T cells. During their development, these cells have the unique capability of rearranging their antigen receptor genes, which are comprised of a large set of variable (V) region genes and smaller sets of diversity (D), joining (J) and constant (C) region genes, in a process called somatic recombination. This process generates a vast repertoire of antibody-producing B cells and of T cells, the mediators of cellular immunity.

Mature B cells express B cell receptors (BCR), the membrane form of immunoglobulins. The BCR expressed by individual B cells not only differ by their V(D)J-C combinations, their V region genes are also subject to somatic hypermutation after activation, thereby increasing the binding affinity to a given antigen. Further maturation of B cells leads to the production of plasma cells that secrete soluble immunoglobulins. The secreted immunoglobulins (antibodies) bind directly to antigens.

By contrast to B cells, T cells need to be "presented" antigens by third party antigen presenting cells (APC). T cells express T cell receptors (TCR), which interact with peptides presented on major histocompatibility molecules (MHC) by APC thereby enabling cell-mediated recognition of non-self invaders. The mode of antigen presentation defines the two major types of T cells. CD8 T cells, also called cytotoxic T cells (CTL) recognize peptides presented by MHC class I. CD4 T cells recognize peptides presented by MHC class II. The major CD4 T subpopulations are T helper (Th) cells, which produce cytokines that influence immune cell interactions and regulatory T cells (Treg), which suppress immune responses.

The cells that best initiate T cell responses are specialized APC called dendritic cells (DC). DC have the unique ability to stimulate and activate naïve T cells. DC act as sentinels for the immune system, they are present in the skin and in tissues that contact the external environment. DC express high levels of PRR, and upon their activation also express high levels of molecules required to stimulate T cells such as MHC class I and class II molecules onto which processed peptides are "loaded" for presentation to T cells. DC also produce cytokines that influence immune cell interactions, and express cell surface molecules that are required to costimulate naïve T cells.

These major immune cell types can be subdivided into additional subsets based on functional differences and stages of development and differentiation. Immune cell subsets are well defined by exquisitely discriminating monoclonal antibody (mAb) markers. Most important, tools to monitor immune interactions both in vivo and in vitro have been developed and studied extensively. The considerable knowledge of the immune system has contributed tremendously to understanding the functional role of tetraspanins. Conversely, knowledge of tetraspanin-partner functions could shed light on interactions in the immune system. An example to illustrate the latter has been the identification of a genetic mutation in a tetraspanin gene

in a patient diagnosed with an common variable immunodeficiency disorder (CVID), as detailed in "In vivo role of Tetraspanins in Adaptive Immunity", below.

A previous review has documented the expression of at least 20 different tetraspanin family members at the mRNA level, whereas mAbs were available at the time for just a few tetraspanins (Tarrant et al. 2003). Unfortunately, this is still the case, particularly, for non-human species. Nevertheless, the available anti-tetraspanin mAbs have been used extensively to analyze their role in antigen presentation and in immune cell activation.

10.3 Tetraspanins in Innate Immunity

10.3.1 Tetraspanins in Pattern Recognition

The notion that innate immune cells, such as antigen presenting cells, do not have intrinsic activity but require activation by PRR, is comparatively recent and was first proposed by Janeway in 1989 (Janeway 1989). Convincing molecular proof that such molecules existed did not come until the functional discovery of what is now known as toll-like receptor (TLR) 4 in 1997 (Medzhitov et al. 1997). It is now appreciated that innate immune cells express a plethora of PRR that are comprised of proteins of various superfamilies. PRR can recognize PAMPS in diverse locations including the cell surface, intracellular vesicles, and alternatively in the cytoplasm. Activation by signal transduction through PRR is a critical first step in the immune response, and also plays an important role in non-infectious inflammation (Iwasaki and Medzhitov 2010).

Given the recent discovery of PRR, it is not surprising that a possible role for tetraspanins in innate immunity is only now emerging (Figdor and van Spriel 2010). There are now compelling studies that suggest that PRR are molecules whose functions can be regulated by tetraspanins, and given the impressive diversity of molecules involved in pattern recognition, it would not surprise us if more reports on tetraspanins regulating PRR will emerge in the future. Macrophages deficient in the tetraspanin CD9 have exaggerated pro-inflammatory responses to the TLR4 agonist LPS (lipopolysaccharide, a key component of gram negative bacterial cell walls) (Suzuki et al. 2009). In vitro CD9-deficient cells secreted greater TNF-α in response to LPS stimulation, and intranasal administration of LPS to CD9-deficient mice showed an increase in lung inflammation. Similarly, macrophages deficient in the tetraspanin CD37 have exaggerated pro-inflammatory responses to agonists of the C-type lectin fungal PRR Dectin-1 (Meyer-Wentrup et al. 2007). In vitro, triggering of dectin-1 leads to an exaggerated production of the pro-inflammatory cytokine IL-6. Moreover *Cd37*$^{-/-}$ mice are resistant to challenge by the fungal pathogen *Candida albicans*, although whether this is causally related to a dysregulation of Dectin-1 activity has not been determined (Figdor and van Spriel 2010).

The molecular mechanisms by which tetraspanins regulate activation in response to PAMPs have not been fully elucidated. Whether their ability to regulate the TLR4

and Dectin-1, respectively, is unique to CD9 and CD37 or a function shared by other tetraspanin has not yet been determined. CD81 has been reported as being in close proximity to TLR4 by Fluorescence Resonance Energy Transfer in LPS-stimulated human monocytes (Triantafilou et al. 2004), and it is also of note that in vitro LPS stimulation of *Cd81*$^{-/-}$ B cells leads to increase activation and proliferation, compared to their wild type counterparts (Sanyal et al. 2009). Moreover, a molecular interaction between Dectin-1 and CD63 has been observed in immature human DC (Mantegazza et al. 2004). CD9 clearly molecularly interacts with CD14, a TLR4 co-receptor, and, given the stability of this interaction after Triton X-100 solubilization, the interaction may be direct (Suzuki et al. 2009). In CD9-deficient macrophages, CD14 expression is upregulated and its association with TLR4 is enhanced. Given that no data was presented to document a molecular interaction between TLR4 and CD9, the simplest model to explain this data might be that CD9 associates with and regulates CD14, negatively controlling the interaction of CD14 with TLR4 by sequestering CD14 away from TLR4. The absence of CD9 from macrophages also affects the membrane compartmentalization of the TLR4/CD14 complex, leading to a greater incorporation of the complex into low-density membrane fractions, which some have argued enhances TLR4/CD14 signaling (Pfeiffer et al. 2001). By contrast, a different mechanism must be invoked to explain the regulation of Dectin-1 by CD37. Whilst there is evidence for a molecular interaction between CD37 and Dectin-1, CD37-deficiency leads to poor expression of Dectin-1 (in contrast to CD9-deficiency which leads to an excess of CD14). This poor Dectin-1 surface expression belies the excess IL-6 produced by Dectin-1 agonists, suggesting that CD37 plays a role in negatively regulating Dectin-1 signaling (Meyer-Wentrup et al. 2007).

10.3.2 Tetraspanins and Innate Immune Cell Migration

Leukocyte migration is of fundamental importance in almost all aspects of the immune system. It is essential to the efficient development of immune responses against microbial pathogens yet also underlies the pathophysiology of inflammation and immune-mediated diseases such as rheumatoid arthritis, multiple sclerosis and atherosclerosis. In innate immunity, leukocytes must migrate out of the circulation towards the site of infection in the periphery (Ley et al. 2007). Moreover, the innate immune system initiates adaptive immune responses as a consequence of DC capturing antigen in the periphery and migrating to lymphoid organs where antigen presentation to T cells occurs (Shortman and Liu 2002).

In non-immune cells, there is strong evidence, from multiple studies of many physiological systems, that tetraspanins regulate cell migration, primarily through the ability of tetraspanins to regulate the function of their partner integrin molecules (see Chap. 6). We would expect that the same is true for immune cells. However, whilst many tetraspanin-integrin interactions have been detected in leucocytes, it is surprising that there is currently a paucity of data suggesting that tetraspanins

regulate leukocyte migration. Monoclonal antibodies (mAbs) against CD151 can inhibit in vitro neutrophil chemo-haptotactic migration (Yauch et al. 1998). Conversely, mAbs against several tetraspanins enhanced in vitro chemotactic migration of DC (Mantegazza et al. 2004), and natural killer (NK) cells (Kramer et al. 2009). Whether this modulation of immune cell migration in any of these studies, involved a modulation of integrin function was not examined. Feigelson et al. (2003) used reverse genetics approaches and showed that in both monocyte cell lines and mouse B cells, CD81 played an important role in promoting outside-in signaling and adhesion strengthening through $\alpha_4\beta_1$ integrin, although whether this corresponded to an effect on in vivo cell migration or inflammation was not determined. There is however strong in vitro evidence that tetraspanins may indirectly play a role in leukocyte trafficking via their ability to promote the presentation of high avidity clusters of the integrin ligands vascular cell adhesion molecule-1 VCAM-1 and intercellular adhesion molecule-1 ICAM-1 on endothelial cells (Barreiro et al. 2005, 2008).

10.3.3 Tetraspanins and Antigen Presentation

There have been numerous reports documenting molecular interactions between tetraspanins and the antigenic peptide presenting MHC molecules. Tetraspanin-MHC interactions occur at the cell surface, CD9, CD37, CD53, CD81, and CD82 have all been reported to interact with MHC, and where most of the data has documented interactions between tetraspanins and MHC II (Angelisova et al. 1994; Engering et al. 2003; Kijimoto-Ochiai et al. 2004; Schick and Levy 1993; Szollosi et al. 1996; Rubinstein et al. 1996; Unternaehrer et al. 2007; Zilber et al. 2005; Hoorn et al. 2012), there are also reports of interactions with MHC I (Szollosi et al. 1996; Lagaudriere-Gesbert et al. 1997). Interactions can also occur intracellularly. For example the tetraspanin CD63 is a well-characterized marker for lysosomes and early endosomes and it has been shown to translocate to MHC class II enriched compartments (MIICs) following endocytosis of antigen (Mantegazza et al. 2004; Artavanis-Tsakonas et al. 2006; Pols and Klumperman 2009) where it has a stable direct interaction with MHC II (Hoorn et al. 2012). However, initial analyses of cells isolated from CD63-deficient mice reported no defect in lysosomal function and endocytosis, nor antigen processing and presentation (Schroder et al. 2009), although CD63 may have a role in regulating MHC II trafficking as silencing CD63 in transformed B cell lines lead to an increase in the production of immunostimulatory MHCII-expressing exosomes (Petersen et al. 2011). CD82 is another tetraspanin that shows a vesicular pattern of expression and is also found in abundance in MIICs where it can interact with not only MHC II, but also directly with the peptide editors HLA-DM (Hoorn et al. 2012) and HLA-DO (Hammond et al. 1998). The functional significance of CD82 in MIICs has not been determined, and reverse genetics analysis of CD82 in antigen presenting cells has not yet been reported. However, given the role that tetraspanins have in regulating protein trafficking (Berditchevski and Odintsova 2007), a role in MHC transport (Vyas et al. 2007) or peptide loading (Rocha and Neefjes 2008) is possible.

Cellular immune responses are initiated by the presentation of peptides by DC to naive T lymphocytes. Some molecular immunologists propose that T cell activation requires crosslinking of the T cell receptor (TCR) by peptide-MHC, as soluble MHC/peptide monomers are not capable of full T cell activation (Boniface et al. 1998; Cochran et al. 2000). Precisely how a DC can display limited amounts of a particular antigenic peptide-MHC complex, in a sea of self-peptide/MHC complexes and still induce TCR crosslinking has not been resolved. It is argued that MHC is not randomly displayed at the DC surface but organized into clusters that have been visualized microscopically (Unternaehrer et al. 2007). Biochemical evidence suggests that MHC-II molecules interact with one another to form dimers or higher order multimers (Brown et al. 1993) and even cell surface multimers with MHC-I (Jenei et al. 1997). The mechanism of MHC clustering has also not been resolved. One suggestion is that MHC is clustered via their incorporation into raft microdomains. In particular, at low peptide concentrations, the biochemical disruption of rafts abolishes efficient antigen presentation (Anderson et al. 2000). However, the relevance of rafts to membrane biology is questionable. Their definition, based on insolubility in various types and concentrations of detergent is nebulous. Moreover precisely how these detergent insoluble fractions relate to structures on native membranes is unclear. It has also been argued lipid rafts are too numerous to concentrate MHC peptide complexes and increase MHC avidity (Huby et al. 2001).

Tetraspanins represent an alternative mechanism by which high avidity peptide/MHC structures are formed at the cell surface. Kropshofer et al. identified a supramolecular complex that included the tetraspanins CD82, CD9 and CD81, MHC II, CD86, and HLA-DM (Kropshofer et al. 2002). These MHC II/tetraspanin microdomains carried a restricted peptide repertoire and were argued to be critically important in T cell activation, as their disruption diminished Ag presentation. Unternaehrer et al. also support the model that tetraspanins promote high avidity MHC clusters (Unternaehrer et al. 2007). They demonstrated that CD9 mediated complexes between I-A and I-E, and argued that the differential expression of CD9 in DC compared to B blasts may underlie the superior Ag presenting capacity of DC. However, surprisingly, there was no investigation of the antigen presenting capacity of *Cd9*$^{-/-}$ DC reported in their paper.

The model that tetraspanins are essential for antigen presentation as they promote high avidity MHC clusters is not without controversy. Firstly, Kropshofer et al. originally identified MHC/tetraspanin complexes using the CDw78 monoclonal antibody to identify tetraspanin/MHC microdomains, and the specificity of this reagent has recently been called into question (Poloso et al. 2006). Secondly, a prediction from the clustering model would suggest that dendritic cells obtained from tetraspanin-deficient mice should be poor presenters of antigen as their ability to present high avidity clusters at the cell surface would be impaired. However, to date, there have only been reports on the antigen presenting capacity of DC from two tetraspanin knockouts CD37 and CD151, and surprisingly, deficiency of either of these tetraspanins resulted in hyperstimulatory DC (Sheng et al. 2009). Here, we can put the phenotype induced by CD151 deficiency aside, as there are no reports that CD151 can interact with MHC, and the phenotype seems to be of an elevated costimulatory activity rather than enhanced MHC/antigen presentation. However,

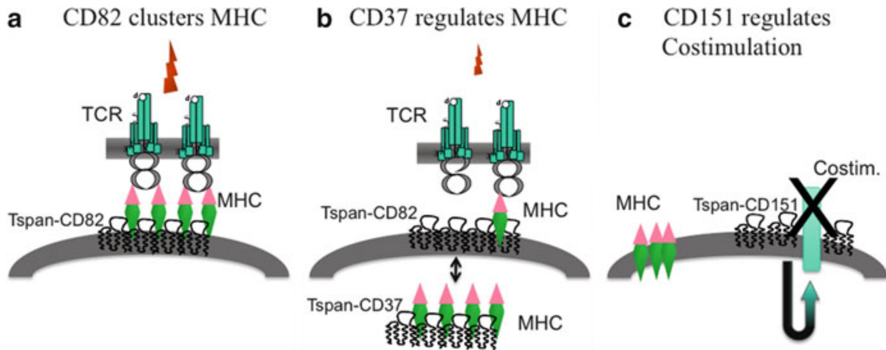

Fig. 10.1 Tetraspanins play distinct roles in antigen presenting cells. This model reconciles biochemical studies with reverse genetics approaches (**a**) CD82 (and also CD9 and CD81) clusters MHC and promotes TCR cross-linking. (**b**) CD37 regulates MHC and inhibits antigen presentation. CD37 may sequester MHC away from the cluster-promoting tetraspanin CD82. (**c**) CD151 negatively regulates co-stimulation. CD151 should not interact with MHC but may interact with co-stimulatory molecules

CD37 is a tetraspanin present in MHC complexes (Angelisova et al. 1994; Escola et al. 1998), and CD37-deficient DC are hyperstimulatory to T cell hybridomas whose activation is generally held to be dependent only on MHC/peptide and independent of costimulatory signals (Sheng et al. 2009). Clearly CD37 has an inhibitory role in antigen presentation rather than a role in promoting MHC clustering and therefore antigen presentation.

What implications then does this result have for the clustering model for tetraspanins in antigen presentation? Firstly it should be considered that the reports that support a role for tetraspanins in promoting MHC clustering studied the tetraspanins CD9, CD82 and CD81, whereas analyses of tetraspanin-deficient cells using a reverse genetics approach focused on CD37. Tetraspanins often associate with one another in the same microdomain, and can often share similar functions. However microdomains with different tetraspanin compositions do exist within the one cell (Nydegger et al. 2006), and the intracellular localization of tetraspanins can differ (Engering et al. 2003). Tetraspanins can also have opposing biological functions: CD151 and CO/029 promote, whereas CD9 and CD82 suppress cancer cell motility (Hemler 2003). Consequently, it is possible that tetraspanin function in APC also varies. For example, CD151 will not molecularly associate with MHC and will regulate costimulatory signals. Some tetraspanins, like CD82, will promote MHC clustering, others, like CD37, will regulate MHC possibly sequestering MHC away from the clustering promoting tetraspanins such as CD82. Several key experiments are required to test this hypothesis. If true, reverse genetic analyses of the tetraspanins biochemically implicated in promoting MHC clustering (e.g., CD9, CD81 and CD82) should reveal that DC deficient in these molecules are poor presenters of antigen. Biochemical and microscopic analysis might also predict different pools of tetraspanins; the MHC interacting with CD37 might be sequestered away from the MHC interacting with the clustering promoting tetraspanins such as CD82 (see model, Fig. 10.1).

Secondly, the hypothesis that T cell activation requires cross-linking of TCR by high avidity MHC is not universally accepted. Whilst soluble monomeric peptide/MHC are incapable of activating T cells, monomers incorporated into lipid bilayers are sufficient to promote T cell activation (Ma et al. 2008a). It has also been argued that the kinetics of TCR interactions with peptide/MHC are too fast to allow for adjacent TCRs to also bind to ligand (Williams and Beyers 1992). Consequently alternative models for T cell activation do exist, and whilst several of these are T lymphocyte centric and do not strongly consider the role of dendritic cells in T cells activation, the potential role of tetraspanins in antigen presentation must also be considered in context of these models. The receptor deformation model argues that TCR signaling is triggered by conformational changes in the TCR induced by mechanical stress provided by "pulling" detaching forces (Ma et al. 2008b). Here it is argued that these detaching forces originate from rearrangement of the cytoskeleton, and whilst the hypothesis most strongly considers T cell cytoskeletal rearrangements it may be possible that rearrangement of the DC cytoskeleton also provides a mechanical force. Tetraspanins are molecules that can regulate cytoskeletal rearrangement as best exemplified by the influence tetraspanins can have on phenomena such as integrin signal strengthening and spreading after cell adhesion (Feigelson et al. 2003; Goschnick et al. 2006; Lammerding et al. 2003; Delaguillaumie et al. 2004). It has also been documented that the dynamic clustering of MHC that occurs after T cell/APC contact is dependent on cytoskeletal rearrangement (de la Fuente et al. 2005). The kinetic segregation model argues that T cell signaling is initiated upon T cell/APC contact by a differential and dynamic segregation of membrane molecules (Davis and van der Merwe 2006). Large transmembrane phosphatases such as CD45 and CD148 are excluded from close contact zones that contain small kinase-associated membrane molecules such as the CD2/CD48 ligand pair and the TCR complex. This results in enhanced phosphorylation at the contact site of T cells and MHC. The model is entirely dependent on the concept of membrane organization and segregation. There are several examples where tetraspanins can regulate the membrane compartmentalization of their partner proteins (Cherukuri et al. 2004; Odintsova et al. 2003). Moreover, several of the molecules that do kinetically segregate in APC upon contact with T cells, are known to interact with tetraspanins including MHC, CD86, and ICAM-1.

10.4 Tetraspanins in Adaptive Immunity

10.4.1 Tetraspanins and T Cell Costimulation

Activation of T cells requires the engagement of the TCR complex, simultaneously with a costimulatory molecule. CD28 is the classical costimulatory molecule expressed on T cells, it binds to molecules (CD80 and CD86) expressed on APCs while MHC molecules present antigenic peptides to the TCR complex. It is possible to simulate in vitro the interaction between a T cell and an APC by antibodies that

engage both the TCR and the costimulatory molecule simultaneously, thereby inducing activation and proliferation of T cells in the absence of an APC. Interestingly, several anti-tetraspanins mAbs have been shown to be as potent as anti-CD28 antibodies in T cell costimulation.

In vitro studies have shown that engagement of CD9 or CD81 in mice was as effective as the engagement of CD28 in costimulation of T cells (Tai et al. 1996; Witherden et al. 2000). Here, the availability of *Cd28*$^{-/-}$ mice was crucial in demonstrating that engaging CD9 or CD81 on T cells led to co-stimulation by a mechanism distinct from the CD28 pathway. Subsequent studies have shown that while costimulation via CD28 led to activation of NFκB and IL-2 production, costimulation via CD9 did not activate the NFκB signaling pathway (Zhou et al. 2002).

On the other hand, studies focusing on the activation of human T cells by tetraspanin engagement have benefited from the wider availability of mAbs to family members. Thus, an anti-human CD9 mAb costimulated peripheral blood T cells, albeit, the proliferative effect measured was lower than that induced by the anti-CD28 mAb (Kobayashi et al. 2004). This difference might have been due to selective expression of CD9 on naïve CD4+ T cells, whereas CD28 is expressed on all T cells. Human T cells are also costimulated by anti-CD81 mAbs, as most recently shown (Sagi et al. 2012). Interestingly, CD28 and CD81 costimulated different T cell subsets, where a greater percentage of naïve cells responded to CD81 costimulation. This preferential activation of the naïve subset by CD81 was not due to higher expression level, as CD81 is equally expressed on both naïve and memory T cells. It was due to increased signaling of the most proximal TCR signal transduction molecules, TCRζ, SLP76 and PLCγ (Sagi et al. 2012). An additional study demonstrated that an anti-human CD63 was as effective as the anti-CD28 mAb in delivering a costimulatory signal (Pfistershammer et al. 2004). Studies aimed at understanding the role of CD81 in hepatitis C virus (HCV) infection showed that co-engagement of the T cell receptor complex with CD81 activated T cells (Wack et al. 2001; Tseng et al. 2001; Serra et al. 2008). This coengagement was shown to be mediated by lymphocyte-specific kinase (Lck) (Soldaini et al. 2003) and to induce cytoskeletal rearrangements that were also correlated with increased phosphorylation of the mitogen-activated protein (MAP) kinases Erk1 and Erk2 (Crotta et al. 2006). An anti-CD81 mAb also augmented antigen specific activation—it increased IL-4 production by CD4 T cells that were derived from an allergic individual and were presented with the allergen by the person's B cells (Secrist et al. 1996). Additional studies used super-antigens to study the role of CD81 in T cell–B cell collaboration and showed that engagement of CD81 activates lymphocyte function-associated antigen 1 (LFA-1) on T cells (VanCompernolle et al. 2001) and preferentially induces Th2 cells (Maecker 2003). Coengagement of the costimulatory molecules, CD28 and CD81 on naïve T cells (without activation of the TCR complex) also induced a strong proliferative response, similar in magnitude to CD3/CD28 costimulation. Interestingly, the transition from a naïve to an effector T cell phenotype was more evident in response to CD28/CD81 engagement, which also led to increase in Th2 type cytokines (Serra et al. 2008).

CD82 acted as a costimulatory molecule on peripheral human T cell, this was demonstrated using an anti-CD82 mAb (4F9), which bound mostly CD4+ memory T cells (Nojima et al. 1993; Iwata et al. 2002). A study comparing the costimulatory effect of anti-CD9, CD53, CD81 and CD82 mAbs in a CD4 T cell line (Jurkat) (Lagaudriere-Gesbert et al. 1997) had shown increased IL-2 production, especially by the anti-CD82 mAb. Subsequent studies in Jurkat cells demonstrated a linkage between CD82, Rho GTPases and cytoskeletal actin rearrangements (Delaguillaumie et al. 2002, 2004). Taken together, the engagement of tetraspanins on both mouse and human T cells provides a costimulatory signal by a pathway that is yet to be defined.

10.4.2 Presence of Tetraspanins in Immune Synapses

When an antigen specific T cell (cognate T cell) is presented by an APC with its cognate peptide, the two cells form an immune synapse (IS), where key cell surface and signaling molecules, of both cell types, migrate in a coordinated manner to the point of cellular contact. A tagged CD82 (YFP) was shown to colocalize with filamentous (F) actin in the IS formed between an antigen-specific mouse T cell line presented by its cognate antigen (Delaguillaumie et al. 2004). Experimentally, it is also possible to form conjugates between T cells and APC (and visualize IS formation) by the use of super-antigens, which bind to MHC class II on APC and simultaneously to certain TCR molecules on T cells. One study showed that an anti-CD9 mAb enhanced conjugate formation, as measured by flow cytometry (Zilber et al. 2005). An additional study used microscopy on IS formed between a human T and a B cell line in response to a super-antigen and demonstrated redistribution of CD81 to the interface of the interacting cells (Mittelbrunn et al. 2002). Analysis of IS formation in immune cells lacking tetraspanins has yet to be reported, nevertheless, lack of CD81 affected cognate T cell–B cell interactions (Deng et al. 2002), as detailed in genetic evidence, below.

10.4.3 In Vivo Role of Tetraspanins in Adaptive Immunity

10.4.3.1 Genetic Evidence (Human)

The recent diagnosis of an immunodeficient child, due to a mutation in CD81 (van Zelm et al. 2010) highlights the role of this tetraspanin molecule in B cell function. The offspring of first cousin parents, the patient was diagnosed because of recurrent respiratory tract infection. Further characterization revealed decreased memory-B-cell numbers, impaired specific antibody responses and absence of CD19 expression on B-cells. Unexpectedly, sequence analysis found no mutations in either allele of the CD19 gene.

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CD81
Normal   AACCTCTTCAAGGAGGACTGCCACCAGAAGATCGATGAC
         N  L  F  K  E  D  C  H  Q  K  I  D  D
Patient  AACCTCTTCAAGatgcgcgaggccgGAGGACTGCCACCAGAAGATCGATGA
         N  L  F  K  M  R  E  A  G  G  L  P  P  E  D  R  *
                     188                200
         Exon 6      Intron 6     Exon 7
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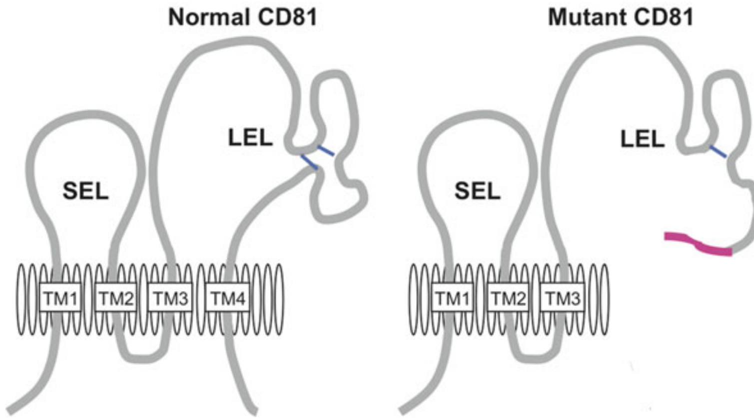

Fig. 10.2 Human CD81 mutant. Normal CD81 contains two disulfide bonds in the LEL (*blue lines*), whereas the mutant protein does not form the second disulfide bond in LEL. It contains a frameshift peptide (*magenta*) and is not anchored in the membrane by TM4

Attention was then focused on associated molecules in the CD19/CD21/CD81 signaling coreceptor complex (Bradbury et al. 1992; Fearon and Carroll 2000). Sequence analysis demonstrated a homozygous splice site mutation in CD81, suggesting that the defective CD81 gene (Fig. 10.2) caused the absence of CD19 surface expression. The human CD81 mutation occurred in a splice site located downstream of exon 6. The use of an alternative cryptic splice site generated a frameshift peptide and a stop codon. The truncated protein lacks the second disulfide bridge in the large extracellular loop (LEL) and the fourth transmembrane domain (TM4). Interestingly, mutations reported for human CD151 also occurred in the LEL (Karamatic Crew et al. 2004, 2008). Further analysis using a B cell lymphoblastoid cell line derived from the patient determined that CD19 protein was produced but sequestered intracellularly in the ER. Moreover, transduction of normal CD81 into this cell line rescued surface expression of both CD81 and CD19 (van Zelm et al. 2010).

This specific case illustrates several aspects of tetraspanin function. First, it emphasizes the function of the partnerships between tetraspanins and their associated molecules. In this particular case, the immunodeficiency is due to the loss of the normal association of CD81 with its B cell partner, CD19. Second, it

demonstrates that an association previously characterized using biochemical methods (Bradbury et al. 1992; Shoham et al. 2003, 2006) has functional significance. The third lesson learned from this case is that a mutation in CD81, a widely expressed tetraspanin molecule (Oren et al. 1990), differentially affected B cell, but not T cell function. Thus, the same tetraspanin molecule plays a different role, which is dependent on the cell type. Fourth, the mutation in the human CD81 gene resulted in a more severe phenotype than that seen in three independently derived strains of CD81 knockout mice (Miyazaki et al. 1997; Tsitsikov et al. 1997; Maecker and Levy 1997). The biochemical basis for the difference between the human and the mouse mutants is yet to be determined. It is possible that the affected patient produced a truncated protein that may act as a dominant negative mutant, whereas the mice completely lack CD81. Considering that tetraspanins can molecularly interact both with partner proteins and other tetraspanins, a dominant negative mutant has the potential to disrupt a myriad of other molecular interactions and functions

Recurrent infectious diseases were also reported in a Spanish family with CD53 deficiency (Mollinedo et al. 1997). The immunodeficiency was more severe in the mother, also a product of first cousin parents, than in her two sons, even though all three lacked CD53 (Mollinedo et al. 1997). Unfortunately, genetic information and materials are not available, as the family became "fed up with so many tests and no solutions…and became uncooperative for science" according to Dr. Lazo, the senior author of the report on this family. Thus, whether the immunodeficient phenotype and the failure to express CD53 were causally linked or merely coincident, has not been determined.

10.4.3.2 Genetic Evidence (Mice)

CD9

An attempt to identify markers on splenic marginal zone (MZ) B cells distinguishing them from splenic follicular (FO) B cells found that CD9 is highly expressed in MZ B cells, in antibody producing plasma cells, in the B-1 subset, but not in FO B cell (Won and Kearney 2002). This suggested that CD9 might play a role both in B cell development and in B cell function. However, early development of B cells in the bone marrow of *Cd9*$^{-/-}$ mice was normal, similarly, MZ and FO splenic B cells and their precursors were present in normal ratios (Cariappa et al. 2005). CD9-deficiency did not affect the peritoneal B-1 B cell population. Immunization by T-dependent and T-independent antigens showed similar antibody production in *Cd9*$^{-/-}$ and wild type mice. While, non-immunized *Cd9*$^{-/-}$ mice had a normal distribution of germinal centers, they did show a slight increase in IgM secreting cells (Cariappa et al. 2005). Thus, although CD9 is variably expressed in B cells, its absence does not affect B cell development or the B cell response to immunization.

CD37

The development of both T and B cell lineages is normal, however functional studies indicate immune dysregulation in both lymphocyte lineages.

1. Role in humoral immunity
   T cell dependent IgG antibody responses in *Cd37*$^{-/-}$ mice are poor (Knobeloch et al. 2000). The molecular mechanism for these poor B cell responses are not known as *Cd37*$^{-/-}$ B cells express normal levels of CD19 (unlike *Cd81*$^{-/-}$ B cells) and proliferate normally to B cell mitogens. Conversely, IgA responses are exaggerated, and this phenotype is of pathological relevance as the excess IgA antibodies mediate resistance to the fungal pathogen *Candida albicans* and promote IgA nephropathy in aged mice (Figdor and van Spriel 2010; Rops et al. 2010). Here, the excess production of IgA is a B cell intrinsic phenotype and the likely molecular driver of excess IgA antibody is an increased production of IL-6.

2. Role in cellular immunity
   Cd37$^{-/-}$ T cells are hyperproliferative to stimulation (van Spriel et al. 2004). The phenotype has been observed in T cells stimulated by mitogens, mixed leukocyte reactions, and monoclonal antibodies crosslinking the T cell receptor, particularly in the absence of co-stimulatory signals. The hyperproliferative phenotype, is not unique to *Cd37*$^{-/-}$ T cells and has been observed in T cells deficient in at least three other tetraspanins: CD81 (Miyazaki et al. 1997), Tssc6 (Tarrant et al. 2002) and CD151 (Lau et al. 2004). The molecular mechanisms that underlie this dysregulation in T cell proliferation are not well understood and have been most extensively studied in the *Cd37*$^{-/-}$ mice. Hyperproliferation is not due to a resistance to apoptosis, or a perturbation in TCR internalization and turnover. Cross-linking CD37 with a monoclonal antibody suggests that the molecule may transduce a signal that inhibits proliferation, and biochemical studies suggest that the autophosphorylation of the key tyrosine kinase Lck is exaggerated in the absence of CD37 (van Spriel et al. 2004).

CD63

The development of immune system cells is normal, however, functional studies have not been reported (Schroder et al. 2009).

CD81

1. Role in cell surface expression of CD19
   It has been suggested that tetraspanins' function is redundant. However, CD19 expression in human and in mouse is dependent exclusively on CD81 and not on other tetraspanins, because deficiency in other tetraspanins does not affect CD19 expression. Whereas three independently generated *Cd81*$^{-/-}$ mice display an identical B cell phenotype—reduced cell-surface expression of CD19

(Miyazaki et al. 1997; Tsitsikov et al. 1997; Maecker and Levy 1997). Unlike the homozygous human CD81 mutation, lack of CD81 in mice has a milder effect on CD19 expression.

The introduction of human CD81 into primary *Cd81*$^{-/-}$ B cells (Shoham et al. 2003) and into a B cell line derived from these mice (Shoham et al. 2006) restored CD19 expression, as shown for the human CD81 deficiency (van Zelm et al. 2010). This "add-in" approach was further used to determine whether specific CD81 domain(s) are needed for this function. Because CD81 is the only known tetraspanin required for CD19 expression, chimeric CD81/CD9 molecules were tested for restoration of surface CD19. This analysis identified specific domains of CD81 essential for the intracellular trafficking and processing of CD19 in mouse B cells. Surprisingly, the first transmembrane domain of CD81 (TM1) was sufficient to support the exit of CD19 from the endoplasmic reticulum (ER). The cytoplasmic amino-terminal tail of CD81 was required for the proper maturation of the intracellular CD19 glycoform to a mature, endo-H-resistant glycoform. In addition, CD81 LEL was shown to associate physically with CD19 during biosynthesis in the ER (Shoham et al. 2006).

2. Role in B cell function

   Despite the consistent findings of low CD19 expression in B cells in all three *Cd81*$^{-/-}$ lines, in vitro analyzes of their B cell function has generated inconsistent results (Miyazaki et al. 1997; Tsitsikov et al. 1997; Maecker and Levy 1997). Similarly, conflicting outcomes were reported on the response of these mice to antigenic stimulation (Miyazaki et al. 1997; Tsitsikov et al. 1997; Maecker and Levy 1997). A recent reanalysis of B cell activation, which included measurements of Ca^{2+} influx, phosphorylation of signaling molecules, cell proliferation and antibody secretion demonstrated a hyperactive phenotype of *Cd81*$^{-/-}$ compared to wild-type B cells responding to stimulation both in vitro and in vivo (Sanyal et al. 2009). This differs considerably from the hypo-reactive B cell phenotype observed in the human CD81 mutant (van Zelm et al. 2010). These opposing B cell phenotypes are most likely related to the difference in surface CD19 expression in CD81-deficient human and mice.

3. Role in T cell function

   In vivo studies of the immune response of *Cd81*$^{-/-}$ mice have shown impaired T helper 2 (Th2) immune responses (Maecker 2003). Subsequent studies using an allergen-induced airway hyperactivity model have demonstrated diminished hyper-reactivity (Deng et al. 2000). In vitro studies demonstrated a crucial role for CD81 in cognate T cell–B cell interactions leading to Th2 responses (Deng et al. 2002), as antigen-specific interactions involving *Cd81*$^{-/-}$ transgenic T cells produced less of the Th2-promoting interleukin 4 (IL-4) than wild-type cells, especially when antigen was presented by B cells (Deng et al. 2002). Additional studies comparing *Cd81*$^{-/-}$ and wild type T cells have demonstrated enhanced T cell proliferation in response to stimulation by CD3 and to co-stimulation by CD3 and CD28 in the absence of CD81 (Miyazaki et al. 1997). As detailed above, a similar hyper-proliferative T cell phenotype was observed in *Cd37*$^{-/-}$ mice (Knobeloch et al. 2000).

CD151

Enhanced proliferation was also seen in *Cd151*$^{-/-}$ T cell responding to in vitro stimulation of CD3 and to costimulation of CD3 and CD28 (Lau et al. 2004). The humoral response of these mice to immunization did not differ from that seen in wild type mice (Lau et al. 2004).

TSSC6 (Tspan32)

Humoral responses to immunization are indistinguishable from wild type mice, Tssc6$^{-/-}$ T cells are hyperproliferative to stimulation (Goschnick et al. 2006). Recently the phenotype of mouse lacking both CD37 and TSSC6 has been described, and the data suggests that Tssc6 can functionally cooperate with CD37 in aspects of cellular immunity. CD37$^{-/-}$Tssc6$^{-/-}$ T cells show an exaggerated hyperproliferative phenotype (relative to single knockout T cells) whilst CD37$^{-/-}$Tssc6$^{-/-}$ DC show an exaggeration hyperstimulatory phenotype. Similarly whilst cytotoxic T cell responses to influenza are impaired in single knockout mice, the response is significantly poorer in CD37$^{-/-}$Tssc6$^{-/-}$ mice (Gartlan et al. 2010).

10.4.4 Expression of Tetraspanins in Diseases of the Immune System

A survey of normal and infected human peripheral blood leukocytes (PBL) has shown reduced expression of some tetraspanin molecules in the infected patients (Tohami et al. 2004). A subsequent survey of tetraspanin expression during human B cell development noted different patterns of expression of the individual family members (Barrena et al. 2005a). The same study also noted differences in tetraspanin expression in B cell malignancies. De Bruyne et al. followed up with a study of CD9 expression in a larger number of patients diagnosed with multiple myeloma, a plasma cell malignancy. They found that patients with non-active disease expressed CD9, whereas most cases with active disease were CD9 negative (De Bruyne et al. 2008). Although the mechanisms by which CD9 expression is reduced during the course of the disease in vivo is yet to be determined, studies of human (Drucker et al. 2006) and mouse (De Bruyne et al. 2008) myeloma cell lines have implicated an epigenetic mechanism in the silencing of CD9.

An interesting anecdote reported that the pattern of tetraspanin expression could distinguish two different malignant B cell clones in a single patient where each of the two malignant cell populations showed differential expression of the tetraspanins CD37, CD53 and CD81 (Barrena et al. 2005b).

The finding that CD81 was under-expressed in precursor B cell acute lymphoblastic leukemia (pre-B ALL) (Barrena et al. 2005a) also led to an additional

subsequent study that analyzed a larger number of patients. It confirmed the original observation, it also proposed a flow cytometry approach to distinguish pre-B ALL from normal immature pre-B cells termed hematogones (Muzzafar et al. 2009).

10.5 Concluding Remarks

The function of tetraspanins is highly linked to the function of their partner proteins. The study of tetraspanins in the context of the immune system benefitted from the wealth of knowledge of proteins expressed on immune cells, as well as the understanding of their interactions in vivo and in vitro. Studies summarized within highlight the participation of tetraspanins in regulating the response of both innate and adaptive immune cells to pathogens. Tetraspanins partake in the coordination of leukocyte migration. In the immune synapse, the interface of the most important cellular interactions, tetraspanins are located. Importantly, deficiencies in tetraspanins lead to immune impairments. We believe that future studies to unravel the precise mechanism of tetraspanin action may shed light on cellular interactions in the immune system.

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