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Abstract A case of a young girl diagnosed with an antibody deficiency syndrome serves to highlight the role of CD81 in B cell biology. Moreover, this case illustrates a fundamental function of the tetraspanin family, namely their association with partner proteins. Characterization of the patient's B cells revealed lack of surface CD19 although both of her CD19 alleles were normal. Further analysis determined that her antibody deficiency syndrome was due to a mutation in the CD81 gene, which did not enable expression of CD19 on the surface of the patient's B cells. Actually, the partnership of CD81 with CD19 and the dependency of CD19 for its trafficking to the cell surface expression were first documented in CD81-deficient mice. CD81 is a widely expressed protein, yet the mutation in the antibody-deficient patient impaired mostly her B cell function. CD81 is required for multiple normal physiological functions, which have been subverted by major human pathogens, such as hepatitis C virus. However, this review will focus on the function of CD81 in cells of the adaptive immune system. Specifically, it will highlight studies focusing on the different roles of CD81 in B and T cells and on its function in B–T cell interactions.

Keywords Tetraspanins · Mutation · Trafficking · Glycosylation · Antibody deficiency

Introduction

CD81 was originally named target of an anti-proliferative antibody (TAPA-1) [1]. It was identified by an assay aimed at discovering cell surface molecules, which were still unknown at that time, and whose engagement inhibited the proliferation of human B lymphoma cell lines. Briefly, mice were immunized with human B cells, followed by a screen of hybridomas for mAbs that inhibit B cell proliferation. This screen re-identified targets that were already known to be important for B cell function, such as IgM, and MHC class I and class II, validating the screening strategy [1]. The new target, TAPA-1, was then further characterized. Interestingly, although the protein was widely expressed, B cell lines were most affected by the anti-proliferative effect of the antibody. This led to a search of coimmunoprecipitated molecules and the identification of CD19 as a CD81-associated molecule in B cells [2].

CD81 is a tetraspanin molecule, belonging to an evolutionarily conserved family of proteins. All multicellular organisms express members of this family: Mammals express 33 members, Drosophila express 37, and plants and even fungi express tetraspanins [3, 4].

Tetraspanins are embedded in the plasma membrane by four transmembrane domains that flank short amino and carboxyl cytoplasmic termini and a small and a large extracellular loop (SEL and LEL, respectively). The three-dimensional structure of human CD81–LEL has been determined [5]: It is composed of a stalk of two longer α-helices and a novel mushroom-like head structure folded with the help of two disulfide bridges (Fig. 1). Most anti-CD81 mAbs react with the LEL, as evident by reactivity with recombinant LEL proteins. CD81 is not glycosylated, unlike most tetraspanins.

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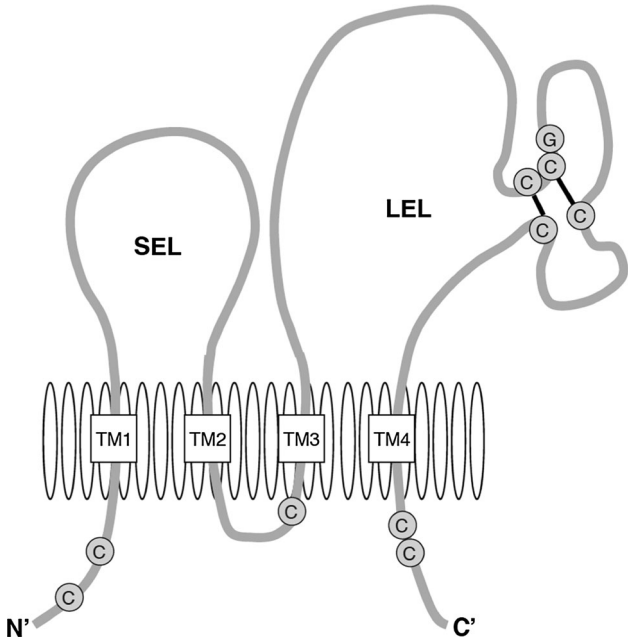

Fig. 1 Structure of CD81. Cytoplasmic cysteines (C) are sites of palmitoylation, and those in the large extracellular loop (LEL) form disulfide bonds. This structure, common to all tetraspanins, includes 4 transmembrane (TM) domains flanking a small extracellular loop (SEL) and an LEL containing a CCG motif

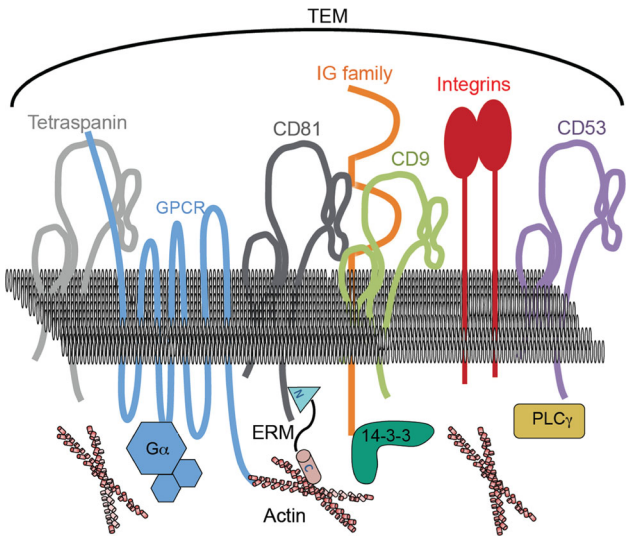

Fig. 2 Tetraspanin-enriched microdomain (TEM). The model represents assembly of the indicated tetraspanins and their partners. TEMs facilitate signal transduction and the recruitment of cytoskeletal actin via ERM proteins

Tetraspanins associate with each other in subcellular membrane microdomains, which are dynamic membrane entities that act as signaling platforms [6–8]. These tetraspanin-enriched microdomains (TEMs) include associated proteins (partner proteins). Importantly, these partnerships differ in various cell types; thus in B cells CD81 associates directly with CD19 (and indirectly with CD21) [9], whereas in T cells it associates with CD4 [10, 11] and CD8 [12]. Partnerships, which were documented using coimmunoprecipitation assays, also differ in their strength of association [13]. Some partnerships are maintained when cells are lysed in harsh detergent conditions, while others are only maintained in mild detergents. In general, tetraspanin tends to associate with integrins [14, 15] in partnerships that are cell-type specific. Thus, in a cell that expresses several integrins, only one of these integrins might be found in association with a specific tetraspanin molecule. Other partners include members of the immunoglobulin superfamily, such as CD19 and EWI-2, that are frequently associated with tetraspanin molecules [9, 16, 17]. TEMs facilitate the transmission of extracellular stimuli to intracellular signaling pathways. For example, they enable the recruitment of cytoskeletal actin by activating the ERM family proteins ezrin, radixin, and moesin [18, 19]. In addition, the association of CD81 with EWI-2 was shown to recruit α-actinin to T cell immune synapses [20]. Thus, CD81 embedded in TEMs transmits signals received at the cell membrane to downstream signaling molecules and to adaptor proteins, thereby contributing to specific immune functions (Fig. 2).

CD81 in B cells

Role of CD81 in the trafficking of CD19 to the cell surface

The CD19/CD21/CD81 molecular complex bridges the adaptive and innate immune systems [21]. When antigens engage their cognate BCR and simultaneously bind the CD19/CD81/CD21 complex, the threshold for B cell activation is lowered, enhancing downstream signaling events [22]. In this complex, CD19 is the signaling molecule, CD21 binds opsonized (complement-bound) foreign antigens, and CD81 enables assembly of the complex into TEMs [9, 23, 24]. Indeed, mutations in both CD19 and CD81 have resulted in antibody deficiency.

The partnership of CD81 with CD19 was first demonstrated using coimmunoprecipitation studies [9]. Subsequently, the generation of three independent lines of CD81-deficient mice showed reduced CD19 cell surface expression [25–27], thereby providing the first genetic evidence that CD81 is required for normal trafficking of CD19 to the cell surface. The diagnosis of antibody deficiency in a patient with a mutation in CD81 further emphasizes the dependency of CD19 on CD81 for its cell surface expression. Interestingly, surface CD19 expression is highly reduced, but not completely obliterated in *Cd81*$^{-/-}$ mice,

whereas B cells in the immunodeficient patient completely lack surface CD19 [28]. Ongoing studies are aimed at determining the reason for the difference in the two species.

It has been suggested that the function of tetraspanins is redundant. It is therefore noteworthy that only CD81 enables the trafficking of CD19, while other tetraspanin knockout mice express normal levels of CD19. Moreover, expression of CD81, but not of the tetraspanin molecule CD9, in CD81-deficient mouse and human B cells completely restored CD19 expression [28–30]. In mice, the association between CD81 and CD19 occurs in the endoplasmic reticulum (ER) early during protein biosynthesis where it enables the trafficking of only properly folded CD19 to the cell surface [29, 30]. Subsequently, a panel of chimeric CD81/CD9 molecules was tested for its ability to restore CD19 expression. Surprisingly, the first transmembrane domain of CD81 (TM1) was sufficient to support the exit of CD19 from the endoplasmic reticulum (ER) [30].

Role of CD81 in B cell signaling

An early study demonstrated that the engagement of CD81 on B cells induced the activation of multiple kinases [31]. One of these activated kinases, Syk, was subsequently identified and its phosphorylated form was shown to activate the adaptor protein ezrin, which, once activated, bound to polymerized cytoskeletal actin [19]. Mechanistically, CD81 in TEMs facilitates the connection to the actin cytoskeleton as was most recently demonstrated in a series of super-resolution microscopy studies from the laboratory of Facundo Batista. They have shown that B cell receptor (BCR) signaling can be triggered, in the absence of antigen, by disruption of the cytoskeleton [23]. They also demonstrated that the amplification of the BCR signaling pathway is dependent on the presence of the TEM partners CD19 and CD81. Their studies show that B cells derived from either CD19- or CD81-deficient mice have impaired downstream signaling due to a defect in the organization of nano-clusters needed for optimal response [23]. The results of these microscopy studies confirm a previous study showing that CD81 is necessary to cluster CD19/CD21 and BCR complexes in signaling lipid rafts [32]. The prediction from these studies is that $Cd81^{-/-}$ B cells would exhibit diminished BCR signaling, as has been observed previously [26]. However, when $Cd81^{-/-}$ B cells were activated via the BCR or by toll-like receptor (TLR) ligands, they displayed a hyperactive response to these stimuli both in vitro and in vivo, compared with wild-type B cells. The responses measured included induction of Ca^{2+} influx, phosphorylation of the downstream signaling molecules Syk and PLCγ, as well as the induction of cell proliferation and antibody secretion [33].

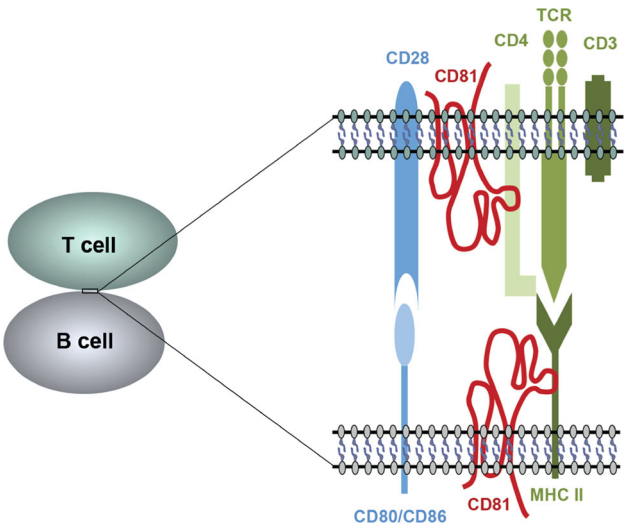

Fig. 3 A model depicting the presence of CD81 in the immune synapse. CD81 colocalizes with CD3 in c-SMAC in T cells and with MHC II in B cells

Role of CD81 in antigen presentation

The association of CD81 [34] and that of several additional tetraspanins with MHC II molecules has been documented by numerous studies using immunoprecipitation [35, 36], FRET [37], and high-resolution microscopy experimental approaches [38]. However, a functional demonstration that TEMs facilitate the presentation of antigenic peptides is still lacking. Nevertheless, when the antigenic peptide was presented to T cell by B cells, CD81 was localized in the immune synapse of both B and T cells (as detailed in CD81 in B–T cell interactions) (Fig. 3).

CD81 in T cells

Engagement of CD81 affects T cell functions

CD81 was re-identified in T cells by a functional assay that selected mAbs that inhibited syncytium formation induced by human T cell leukemia virus type 1 (HTLV-1) [39]. Interestingly, syncytium formation induced by human immunodeficiency virus (HIV) is also inhibited by different anti-CD81 mAbs, as demonstrated more recently [40]. These examples highlight the role of CD81 in membrane fusion in immune cells. In non-immune cells, CD81 is required for the fusion of egg with sperm [41], for fusion of myoblasts during muscle regeneration [42], and for promoting membrane partitioning induced by hepatitis C virus (HCV) entry [43]. In mice, an anti-CD81 antibody blocked

thymocyte maturation in fetal thymus organ cultures CD81 [44]. It is noteworthy that in mice, the protein is expressed on early $CD4^+/CD8^+$ thymocytes and on activated, but not on resting T cells [45], whereas in human, CD81 is expressed on all B and T cells.

Both human and mouse T cells respond to costimulation by anti-CD3 and anti-CD81 in magnitudes that are similar to the classic costimulation by anti-CD3 and anti-CD28 mAbs. Upon costimulation, CD81 activates different downstream signaling events than those activated by CD28, as evident by its effective costimulation of both wild-type and *Cd28*$^{-/-}$ T Cells [46]. Relatedly, in human, CD28 and CD81 costimulate different subpopulations of T cells, CD28 preferentially costimulates the effector and memory subsets, whereas CD81 costimulates naïve cells [47]. Once again, the activation of downstream signaling molecules differed—while costimulation by either CD28 or CD81 led to increased phosphorylation of PLCγ, CD3ζ, and SLP76 in all subsets, the effect of CD81 on the naïve subset was relatively greater [47]. The latter finding might have a potential application for chimeric antigen receptor (CAR) immunotherapy where naïve-derived T cells are known to persist longer than memory-derived cells, which undergo exhaustion.

In human, where CD81 is a receptor for HCV, the viral envelope protein E2 was also shown to costimulate T cells. Specifically, a series of studies from Chiron Laboratories have demonstrated costimulation of T cells and NK cells by HCV-E2 [48, 49]. This activation was correlated with increased phosphorylation of Lck [48]. As in B cells, engagement of CD81 led to cytoskeletal rearrangement in both T and NK cells [50]. Interestingly, using HCV-E2 to cross-link CD16 and CD81 on NK cells inhibited IFNγ production and reduced their cytotoxic effect [51].

T cell partner proteins

CD81 was shown to associate with several partners expressed on the surface of T cells, with CD4 [10, 11] and CD8 in human thymocytes [12]. The earlier study, which analyzed the association of CD81 with CD4, demonstrated that the interaction between the two molecules required the cytoplasmic domain of CD4 [11]. A more recent study showed that the cytoplasmic domain of CD4 was required for homodimerization of the molecule and that such dimers are associated with CD81 in TEMs (rather than in lipid rafts) [10]. An additional Ig superfamily member, PGRL/EWI-2, was shown to associate with CD81 in mouse thymocytes [52]. CD81 was also shown to associate with the cytoplasmic protein 14-3-3ε [53], an isotype of a large family known to regulate multiple cellular functions. This latter association was increased with an unpalmitoylated version of CD81 in which intracellular cysteines (Fig. 1) were mutated to alanine residues [53]. Moreover, oxidative conditions inhibited the palmitoylation of CD81, thereby increasing the association with 14-3-3ε [53].

It is noteworthy that T cell function, unlike that of B cells, was not impaired in the patient harboring a mutation in CD81 [28].

CD81 in B–T cell interactions

CD81 is localized at the central zone of the immune synapse in both B and T cells

In an early study from the laboratory of Francisco Sanchez-Madrid [54], the authors used a superantigen to induce immune synapses (IS) between the human B and T cell lines, Raji and Jurkat, respectively. First, they observed the presence of CD81 in the IS in both B and T cells. Using time-lapse confocal microscopy, they observed the localization of CD81 with CD3 at the central supramolecular activation complex (c-SMAC). Interestingly, the movement dynamics of CD81 to the IS differed in T cells and B cells. A most recent study by this research group applied several quantitative microscopy techniques to determine the contribution of CD81 to both superantigen-induced and antigen-induced IS formation between human B and T cell lines [55]. They confirmed their previous findings and, in addition, demonstrated that CD81 facilitates re-localization of CD3 and its retention in the IS using CD81 knocked-down (KD) T cells. Moreover, they observed fewer B–T cell conjugates using CD81KD compared to unmanipulated T cells. Furthermore, the activation of T cell signaling molecules, such as pCD3ζ, pZAP-70, pLAT, and pERK1/2, was downregulated in CD81KD T cells. Finally, based on analyzing the distribution of CD81 (as well as CD3 and ICAM-1) in early and late IS, they conclude that "CD81 controls sustained T cell activation signaling and defines the maturation stages of cognate immunological synapses" [55].

Engagement of CD81 on both T and B cells polarizes responses toward a Th2 phenotype

In human, coculture of $CD4^+$ T cells with B cells in the presence of an anti-human CD81 mAb correlated with the presence of large B–T cell aggregates. Importantly, the antibody enhanced IL-4 synthesis by $CD4^+$ T cells when cocultured with B cells, but not with monocytes as antigen-presenting cells (APCs) [56].

Subsequent studies in CD81 knockout (KO) mice showed impaired IL-4 production [57]. Moreover, this impairment highly affected allergen-induced airway hyperactivity (AHR). Although wild-type mice developed

severe AHR, *Cd81*$^{-/-}$ mice had normal airway reactivity upon exposure to the allergen and showed reduced airway inflammation [58]. The proliferation of T cells was similar in both groups of mice; however, Th2-type cytokine production was dramatically reduced by those derived from *Cd81*$^{-/-}$ mice [58]. A subsequent study, in which antigen-specific B and T cells were probed in vitro, revealed that expression of CD81 on T cells is critical for the induction of IL-4 by B cells [59]. The study also demonstrated that *Cd81*$^{-/-}$ T cells express lower levels of downstream signaling molecules when activated by B cells [59]. Taken together, these studies implicate CD81 expressed on T cells as a facilitator of cognate B–T cell interactions, which, in turn, augments intracellular interactions leading to Th2 polarization.

In summary

The function of CD81 and tetraspanins, in general, is highly linked to the function of their partner proteins. The study of CD81 in the adaptive arm of the immune system benefitted from the identification of a CD81-mutant patient as well as by the generation of *Cd81*$^{-/-}$ mice. Moreover, studies aimed at understanding of the function of CD81 were made possible by the wealth of knowledge of proteins expressed on B and T cells and the understanding of their interactions. Future studies aimed at unraveling the precise mechanism of CD81 action may shed light on cellular interactions in the immune system.

Conflict of interest The author declares no conflict of interest.

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