FUNCTIONAL RELEVANCE OF TETRASPANINS IN THE IMMUNE SYSTEM

EDITED BY: Carlos Cabañas, María Yáñez-Mó and Annemiek B. van Spriel
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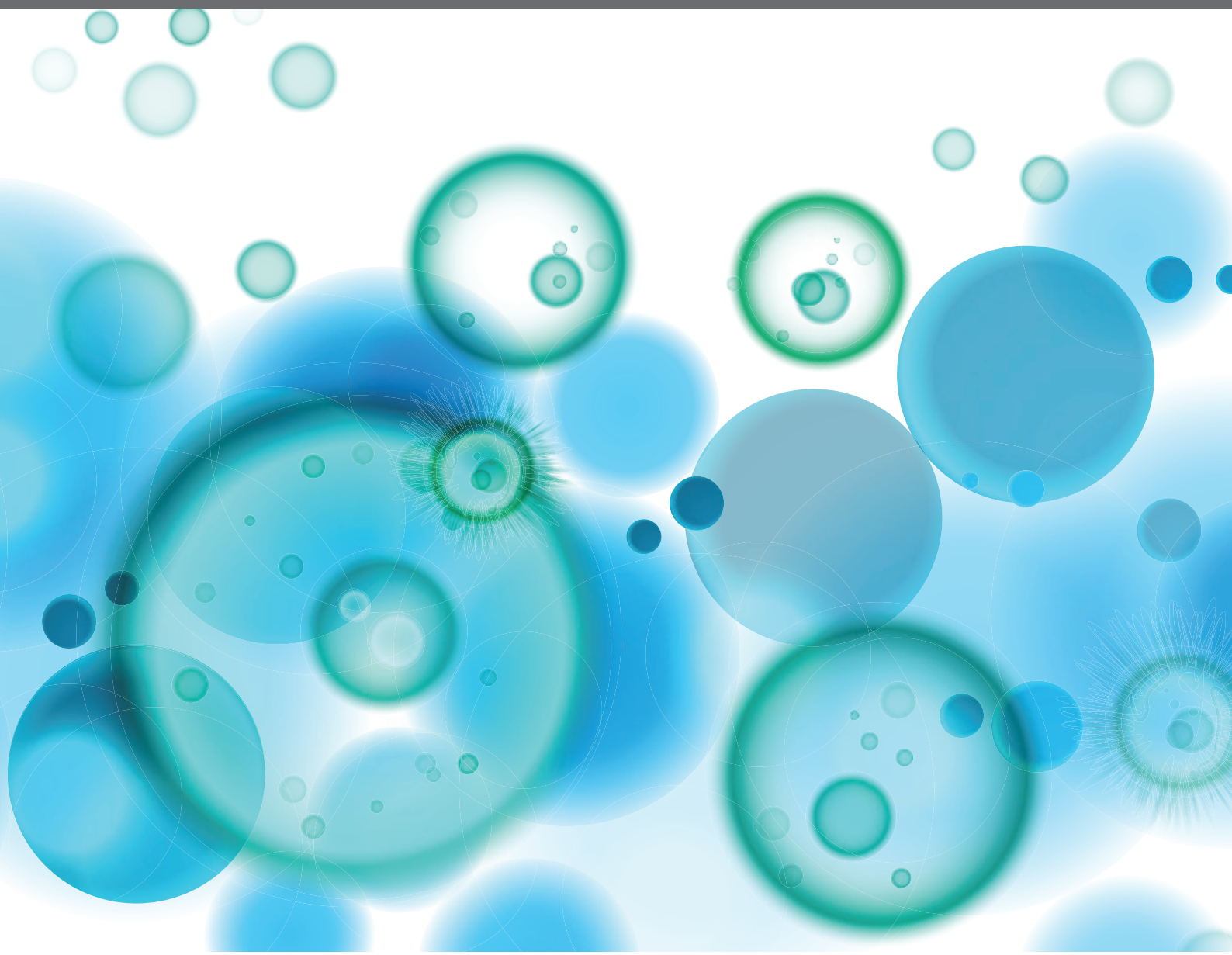

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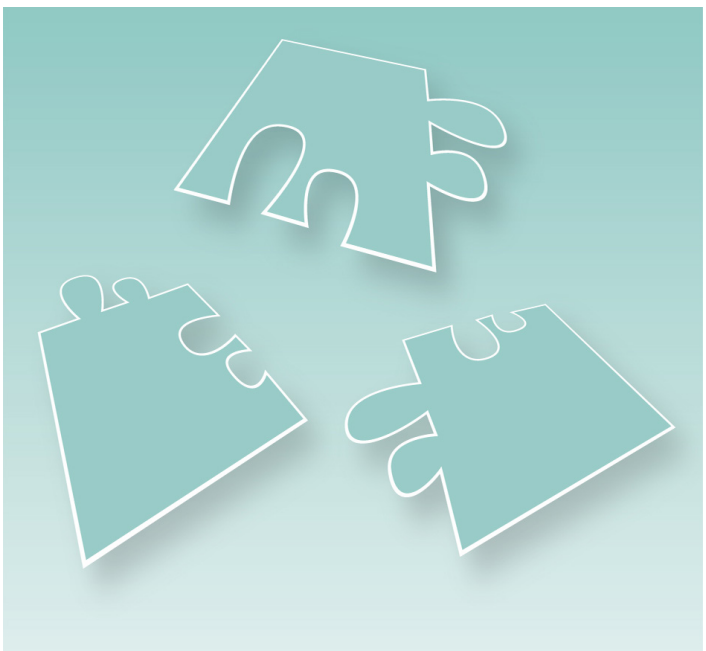
FUNCTIONAL RELEVANCE OF TETRASPANINS IN THE IMMUNE SYSTEM

Topic Editors:
Carlos Cabañas, Centro de Biología Molecular Severo Ochoa (CBMSO) (CSIC-UAM), Spain; Universidad Complutense, Spain; Instituto de Investigación Sanitaria Hospital 12 de Octubre (i+12), Spain
María Yáñez-Mó, UAM; CBM-SO/IIS-IP; Spain
Annemiek B. van Spriel, Radboud University Medical Center, Netherlands



Jigsaw pieces illustrating the building blocks of the tetraspanin web generated by Marcel Egtberts (Nijmegen, Netherlands).

Tetraspanins are small (20-50 kDa) integral membrane proteins with four transmembrane domains that have an intrinsic propensity to associate with other membrane proteins and lipids giving rise to the formation of specific tetraspanin-enriched microdomains (TEMs), also referred to as "The tetraspanin web". In mammals, the tetraspanin family comprises of 33 different members, with the majority of the members being abundantly expressed in almost all cell types, including leukocytes which are responsible for innate and adaptive immunity as well as in other cells that play pivotal roles in immune responses, such as endothelial or stromal cells. Therefore, through the wide range of specific molecular interactions in which they are engaged, tetraspanins influence many processes of up-most

relevance in the development, physiology and pathology of the immune system, including the control of immune cell morphology, signaling, adhesion, migration, invasion, fusion, infections and cancer.

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EDITORIAL
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Editorial: Functional Relevance of Tetraspanins in the Immune System

Carlos Cabañas[1,2,3]*, *María Yáñez-Mó*[4]* and *Annemiek B. van Spriel*[5]*

[1] Department of Cell Biology and Immunology, Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Madrid, Spain, [2] Department of Immunology, Ophthalmology and Otorhinolaryngology (IO2), Faculty of Medicine, Universidad Complutense, Madrid, Spain, [3] Instituto de Investigación Sanitaria Hospital 12 de Octubre (i+12), Madrid, Spain, [4] Departamento de Biología Molecular, CBM-SO, UAM/IIS-IP, Madrid, Spain, [5] Department of Tumor Immunology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, Netherlands

Keywords: tetraspanin, immune cell, infection, vaccine, cell membrane

Editorial on the Research Topic

Functional Relevance of Tetraspanins in the Immune System

Tetraspanins, members of the superfamily of four-transmembrane proteins, are evolutionary highly conserved membrane proteins that function as membrane-organizers (1–3). Immune cells express thousands of different membrane proteins (including adhesion receptors, uptake receptors, major histocompatibility molecules, enzymes, cytokine receptors, and others) that all need to be correctly localized in time and space at the cell surface. Tetraspanins specifically interact *in cis* with various immune receptors by forming multimolecular complexes (tetraspanin-enriched microdomains, TEMs) that can initiate immune cell signaling (4, 5). Thereby, tetraspanins control fundamental immune cell functions, including adhesion, pathogen uptake, immunological synapse formation, and proliferation. Tetraspanin-deficiency in mouse models and patients results in different immunological defects (6). This Review Topic provides a timely overview of the biological importance of tetraspanin-induced membrane organization in the immune system and the latest insights in targeting tetraspanins as novel drugs for infectious disease and cancer.

Immune cells are the fastest migrating cells in our body that depend on tight cooperation of different adhesion molecules (integrins, selectins, immunoglobulin superfamily members) and chemokine receptors to patrol for pathogens and reach inflammatory sites. For integrins, it has been well-established that clustering (avidity) and conformational change (affinity) both underlie activation. Tetraspanins are well-defined interaction partners for integrins (2, 7) that control adhesion and migration of lymphocytes, dendritic cells (8), and neutrophils (9). Yeung et al. discuss the functional roles of tetraspanins in leukocytes and endothelial cells during transmigration from the circulation into tissues. Most tetraspanins (CD9, CD37, CD81, and CD151) promote lymphoid and myeloid cell adhesion and migration through functional interaction with β1 and β2 integrins. In this regard, CD9 regulates the adhesive capacity of integrin α5β1 by modulating its association with the membrane protease ADAM17 on the cell surface (Machado-Pineda et al.). CD9 is also required for myeloid cell migration in a murine colitis model shown by decreased neutrophil and macrophage infiltration in colonic tissue from CD9-deficient mice (Saiz et al.). In contrast, tetraspanin CD82 in dendritic cells reduces cell motility through regulation of cytoskeletal proteins (e.g., RhoA). It is not known whether CD82 directly interacts with Rho GTPases, in line with the identified interaction between CD81 and Rac1 (10), or alternatively that CD82 regulates the cytoskeleton via interacting with ezrin/radixin/moesin (ERM) proteins. Besides directly interacting with adhesion molecules, tetraspanins have been reported to control the activity of membrane metalloproteases that can induce cleavage of adhesion receptors. For example, CD9 inhibits the shedding activity of ADAM17 and thereby supports ALCAM-dependent adhesion in antigen-presenting cells as discussed by Reyes et al. In line with this, tetraspanins of the TspanC8 subgroup, containing eight cysteine residues in their large extracellular loops, are required for ADAM10

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Edited and reviewed by:
Florent Ginhoux,
*Singapore Immunology Network (A*STAR), Singapore*

***Correspondence:**
Carlos Cabañas
ccabanas@cbm.csic.es
María Yáñez-Mó
maria.yannez@uam.es
Annemiek B. van Spriel
Annemiek.vanSpriel@radboudumc.nl

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exit from the endoplasmic reticulum and trafficking to the cell surface or other membrane compartments (11, 12). ADAM10 is well characterized as the ligand-dependent activator of Notch proteins, and Mike Tomlinson and colleagues discuss how TspanC8 members (Tspan5, Tspan10, Tspan14, Tspan15, Tspan17, and Tspan33) may control ADAM10 activity on myeloid and lymphoid cells in a specific manner (Matthews et al.). The most highly expressed TspanC8 in human and mouse T cells is Tspan14, followed by Tspan5 and Tspan17. Since both Tspan14 and Tspan5 promote Notch signaling, it is hypothesized that ADAM10 will have a major role in thymocyte development. Human and mouse B cells express high levels of Tspan33 and Tspan14, respectively, which may regulate Notch2 signaling and/or shedding of other ADAM10 ligands, such as CD23 (the IgEεRII). Some of these questions can be addressed by investigating newly generated Tspan14 and Tspan5-knockout mice. The authors also propose that targeting individual TspanC8 members may provide a novel therapeutic approach for ADAM10-associated diseases (leukemia, asthma, atherosclerosis, and Alzheimer's disease) without the toxicity of global ADAM10 inhibition.

Tetraspanins on antigen-presenting cells control multiple different functions, including cell migration, pathogen uptake, MHC trafficking, immunological synapse formation and antigen-presentation as reviewed by the group of Saiz et al. In B cells, tetraspanins (CD37, CD53, CD81) are essential for B cell receptor signaling, antibody production and cytokine secretion (Zou et al.). Interestingly, CD37-deficiency leads to spontaneous B cell lymphoma formation in mice, and patients with CD37-deficient B cell lymphomas have inferior clinical outcome than patients with CD37-positive lymphomas (13).

The pathogenesis of different infectious diseases is also influenced by different tetraspanin proteins (14). Besides adhesion and signaling, tetraspanins have been related to different membrane fusion events. The group of Peter Monk has explored the role of different tetraspanin members in membrane fusion of monocytic cells in response to *Mycobacterium tuberculosis* infection (Champion et al.). Tetraspanins can also interfere with different stages of the virus replication cycle. Florin and Lang evaluate how viruses exploit TEMs for viral entrance into cells, and subsequent budding and egress. Some viruses use specific tetraspanins as receptors (for example CD151-HPV, CD81-HCV) and by compartmentalizing host entry factors. In addition, viral envelope proteins accumulate in TEMs during morphogenesis, and induce large assemblies of tetraspanins and viral transmembrane proteins to facilitate budding. For example, tetraspanins (CD9, CD63, CD81, CD82) can be incorporated into the enveloping membrane of virions, such as HIV, feline immunodeficiency virus, influenza or hepatitis A virus, indicating that TEMs directly stimulate virus budding and exit. New evidence shows that HIV-1 replication is stimulated by CD81 through its direct interaction with the deoxynucleoside triphosphate phosphohydrolase SAMHD1 (15). Suárez et al. elaborate on the mechanisms underlying tetraspanin regulation of HIV-1 replication, which may be exploited to develop tetraspanin-based therapeutics as a novel strategy to restrict HIV-1 infection.

Given the plethora of immune functions that are controlled by tetraspanins, it is maybe not surprising that tetraspanins are important in anti-tumor immune responses. Even though this field is rather unexplored, tetraspanins expressed by immune suppressive cells (such as regulatory T cells, myeloid-derived suppressor cells) can control immune responses within the tumor microenvironment as discussed by Schaper and van Spriel. In addition, tetraspanins have been shown to modulate cancer metastasis indirectly through exosomes, and by regulating cellular interactions in the immune system as reflected on by Vences-Catalán and Levy.

Taken together, although broad in function, the underlying mechanism by which different tetraspanins accomplish their function is highly similar. Through their lateral molecular interactions with immune receptors, enzymes and/or signaling proteins, tetraspanins are in charge of organizing the protein landscape at the plasma membrane of immune cells. Evidence is now accumulating that these protein interactions are dynamic and likely change upon cell activation. Future research should provide better insight into the (1) specificity versus redundancy of individual tetraspanins on immune cell function, and (2) molecular mechanisms underlying TEM formation and coupling to signaling transduction pathways. The potency of targeting tetraspanins is currently under investigations at the (pre-)clinical level as novel therapeutics for cancer, infectious diseases and auto-immunity disorders (16, 17).

AUTHOR CONTRIBUTIONS

CC, MY-M, and AS have contributed to the writing of this editorial article. They are the three authors for correspondence of this editorial.

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REVIEW
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The Many and Varied Roles of Tetraspanins in Immune Cell Recruitment and Migration

*Louisa Yeung[1,2], Michael J. Hickey[1] and Mark D. Wright[2]**

[1] Centre for Inflammatory Diseases, Department of Medicine, Monash Medical Centre, Monash University, Clayton, VIC, Australia, [2] Department of Immunology, Monash University, Prahran, VIC, Australia

Immune cell recruitment and migration is central to the normal functioning of the immune system in health and disease. Numerous adhesion molecules on immune cells and the parenchymal cells they interact with are well recognized for their roles in facilitating the movements of immune cells throughout the body. A growing body of evidence now indicates that tetraspanins, proteins known for their capacity to organize partner molecules within the cell membrane, also have significant impacts on the ability of immune cells to migrate around the body. In this review, we examine the tetraspanins expressed by immune cells and endothelial cells that influence leukocyte recruitment and motility and describe their impacts on the function of adhesion molecules and other partner molecules that modulate the movements of leukocytes. In particular, we examine the functional roles of CD9, CD37, CD63, CD81, CD82, and CD151. This reveals the diversity of the functions of the tetraspanin family in this setting, both in the nature of adhesive and migratory interactions that they regulate, and the positive or inhibitory effects mediated by the individual tetraspanin proteins.

Keywords: tetraspanin, leukocyte migration, adhesion molecules, inflammation, integrins

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Francisco Sanchez-Madrid, Universidad Autonoma de Madrid, Spain
Martin Johannes Hoogduijn, Erasmus University Rotterdam, Netherlands
Eric Rubinstein, INSERM U935 Modèles de Cellules Souches Malignes et Thérapeutiques, France

****Correspondence:***
Mark D. Wright
mark.wright@monash.edu

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INTRODUCTION

The ability of leukocytes to migrate from the circulation to sites of inflammation is essential for effective host defense. To undertake this journey, leukocytes undergo a series of interactions in the bloodstream with endothelial cells lining the vasculature (1, 2). The critical roles of cell surface-expressed adhesion molecules on leukocytes and vascular endothelial cells in mediating these interactions are well established. Less appreciated is the emerging evidence indicating important contributions for members of the tetraspanin family of cell membrane proteins in this process. Tetraspanins work differently to classical adhesion molecules; they do not have ligands on other cells, but regulate the actions of target molecules *in cis*, i.e., expressed in the same cell. In this review, we will analyze the developing knowledge on the role of tetraspanins in controlling the movements of immune cells.

LEUKOCYTE RECRUITMENT IS A SEQUENTIAL, MULTISTEP PROCESS

Recruitment of leukocytes from the circulation is essential both to homeostatic immune surveillance and the response to infection and injury. In innate inflammation, neutrophils and monocytes undergo rapid recruitment to the affected site to mediate the appropriate response (1). Similarly, in adaptive immunity, the recirculation and trafficking of B and T lymphocytes is crucial to ongoing surveillance against potential invading pathogens (2). In both cases, leukocytes leave the bloodstream *via* a sequence of steps, collectively known as the leukocyte recruitment cascade.

This involves interactions mediated by an array of adhesion molecules that function cooperatively to arrest the cell on the endothelial surface and facilitate its transmigration into the surrounding tissue (3). The main sequential steps in leukocyte recruitment are rolling, adhesion, crawling, and transmigration (1, 3), and tetraspanin family members have been shown to contribute to each of these steps (**Figure 1**).

SELECTINS MEDIATE EARLY INTERACTIONS DURING LEUKOCYTE RECRUITMENT

The initial interactions between leukocytes and the activated vascular endothelium are mediated by the selectins. The selectin family consists of three members; L-selectin, which is constitutively expressed on leukocytes (15), and E- and P-selectin, found on activated endothelial cells (16–18). The selectins show overlapping properties and are able to interact with ligands such as P-selectin glycoprotein ligand 1 *via* recognition of the crucial SLe^x carbohydrate motif (19–21). The rapid on–off interactions mediated by selectins and their ligands allow for the initial capture of rapidly moving leukocytes in the bloodstream and their subsequent rolling along the vessel wall (22–26).

INTEGRINS MEDIATE ARREST OF LEUKOCYTES ON THE ENDOTHELIUM

Leukocyte integrins are the main adhesion molecules responsible for mediating leukocyte firm adhesion to the endothelium. G protein-coupled chemoattractant receptors expressed on the surface of rolling leukocytes are able to detect and respond to chemoattractants present within the microvasculature (27, 28). These signals rapidly (sub-second) induce integrins to undergo a conformational change from a low affinity to high affinity form, leading to integrin-dependent arrest of the leukocyte (27, 29, 30). The key integrins on circulating leukocytes are the β_2 integrins LFA-1 ($\alpha_L\beta_2$) and Mac-1 ($\alpha_M\beta_2$), which interact with their ligands on endothelial cells including ICAM-1 and ICAM-2, and the α_4 integrins VLA-4 ($\alpha_4\beta_1$) and $\alpha_4\beta_7$ which interact with VCAM-1 and MAdCAM-1, respectively (3, 31). After arrest, integrin-mediated outside-in signaling promotes the strengthening of adhesion to the endothelium (3, 32).

INTRAVASCULAR CRAWLING AND TRANSMIGRATION

Integrins also contribute to processes downstream of leukocyte adhesion, particularly intraluminal crawling and transmigration. Upon integrin binding, signal transduction results in the alteration of the internal dynamics of the cell; cytoskeletal changes allow for pseudopodia formation and intraluminal crawling along the endothelium. Crawling allows leukocytes to scan the endothelium for suitable locations for transmigration (33). Transmigration occurs predominantly at inter-endothelial cell junctions (paracellular transmigration), where leukocytes initiate transmigration *via* extension of uropods into the junction before migrating through. While paracellular migration is the predominant mode of transendothelial migration, under some circumstances leukocytes cross the endothelial barrier by

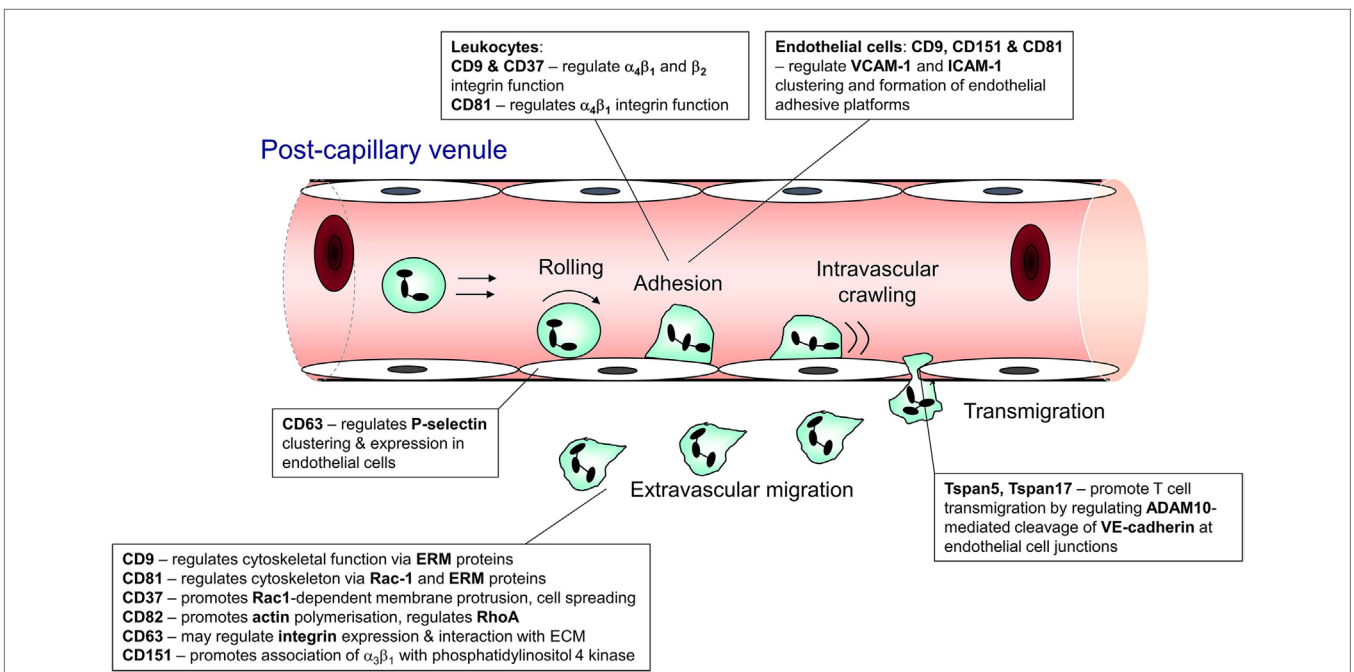

FIGURE 1 | Steps in leukocyte trafficking influenced by tetraspanins. Image shows the sequence of interactions undergone by leukocytes during their recruitment from the bloodstream and after they exit the vasculature, with the tetraspanins that influence these interactions shown adjacent to the interaction. This information is taken from the following publications: CD63 (4, 5); CD9 (6, 7); CD37 (8, 9); CD81 (10, 11); Tspan 5 and 17 (12); CD82 (9); and CD151 (13, 14).

migrating directly through endothelial cells, in what is termed transcellular migration (34, 35). Various adhesion molecules have roles in transmigration, including PECAM-1, CD99, JAM-A, β_2 and β_1 integrins, and L-selectin (36–39). Leukocytes then migrate through the interstitium by following a chemotactic gradient to the source of inflammation, a process involving further interactions of leukocyte integrins with extracellular matrix (ECM) ligands (3).

IMMUNE CELL MIGRATION AND THE CYTOSKELETON

Immune cell motility and directional migration requires the formation of lamellipodia at the leading edge with adhesion to ECM matrix proteins, while simultaneously there is a requirement for detachment at the trailing edge (40). These tightly regulated events require coordinated assembly and disassembly of actin and myosin filaments, processes heavily influenced by members of the Rho family of GTPases. Here, Rac1 regulates actin polymerization at the lamellipodia, while RhoA influences the contraction of actin at the rear of the cell, allowing for forward movement. Meanwhile, evidence indicates Cdc42 is involved in controlling the direction of migration (40).

Dendritic cell (DC) migration is essential for the initiation of the adaptive immune response and exemplifies the importance of cytoskeletal rearrangement in immune cell migration. Here, migration is driven by chemotactic gradients that guide DCs in the interstitium to the lymphatic microvasculature en route to local draining lymph nodes (41). The role of adhesion molecules in DC migration is less clear, with evidence supporting both adhesion-dependent and -independent modes of migration (42). For the latter mode, it is apparent that actin polymerization and cytoskeletal rearrangement are of critical importance (43).

TETRASPANINS: ORGANIZERS OF THE SURFACE MEMBRANE

Successful interactions between a receptor–ligand pair result in the generation of intracellular signals that alter the environmental dynamics of the cell. However, for receptors to productively interact with their ligands and efficiently transduce signals, they must be organized at the cell surface. Tetraspanins are a family of 33 membrane proteins (in humans) which are central to this membrane organization (44). Tetraspanins have the ability to interact and cluster with an array of tetraspanin and non-tetraspanin partners within the cell membrane, forming organized networks of signal transducing complexes termed tetraspanin-enriched microdomains (TEMs) (45–48).

Tetraspanins are distinguished from other four transmembrane proteins by the presence of key conserved amino acid residues located in the transmembrane regions, as well as in the large extracellular loop (LEL) (48). At least in the recently solved X-ray structure of the tetraspanin CD81, the four transmembrane domains form alpha helices that create an intramembrane cholesterol-binding pocket (49). Historically, the LEL of tetraspanins has been the predominant structure studied as it contains the sites responsible for generating protein–protein interactions (45). In addition, much attention has focused on the cytoplasmic domains which can interact with signaling molecules and contain conserved membrane-proximal cysteine residues that are palmitoylation sites (48) which aid in the stabilization of tetraspanin–tetraspanin interactions (50), and contribute to the formation of the TEMs (48).

THE DIVERSITY OF TETRASPANIN INTERACTIONS

Although tetraspanins lack conventional ligands, they interact with a diverse assortment of molecules within the TEM (51). Recent super-resolution microscopy studies indicate a considerable heterogeneity among TEMs, in that tetraspanins such as CD53 form nanoclusters in the plasma membrane, and are more likely to be directly associated with non-tetraspanin partners than with other tetraspanin family members (52). This diversity of molecular interactions and heterogeneity of microdomains explain the pleiotropic functions a single tetraspanin may play. CD81 is an excellent example: in macrophages CD81 suppresses cell growth (53), while in B cells, CD81 regulates CD19 expression, lowering the threshold for activation, and promotes adhesiveness of the $\alpha_4\beta_1$ integrin (10, 54). In T cells, CD81 interacts with CD3ζ of the TCR, regulating T cell activation in response to antigen recognition (55) as well as controlling sustained T cell activation following antigen presentation through interactions with both CD3ζ and ICAM-1 (56).

ROLE OF TETRASPANINS IN IMMUNE CELL MIGRATION AND RECRUITMENT

The role of tetraspanins in cellular migration has been examined in detail in regards to tumor cells. However, a series of more recent studies now implicate an important role of tetraspanins including CD9, CD37, CD63, CD81, CD82, CD151, and Tspan5 and Tspan17 in immune cell migration (**Figure 1**) (57). Here, there appears to be two mechanisms at play that are not mutually exclusive. First, many cell membrane-expressed adhesion molecules are tetraspanin-partner proteins, and their adhesive function and downstream intracellular signaling are regulated by tetraspanins (**Figures 2**,**3**). Second, extracellular signals stimulating migration have to be communicated to the cytoskeleton for cytoskeletal reorganization to occur. Here, tetraspanins may play a key role through their communication with Rho GTPases and other cytoskeleton-associated proteins (**Figure 2**).

The tetraspanins that influence leukocyte migration and their mechanisms of action are summarized in **Table 1**. This review will discuss how tetraspanins expressed in immune cells influence adhesion molecule function and immune cell recruitment, and also examine the functions of tetraspanins expressed in endothelial cells, which play an essential role in directing leukocytes as they migrate through the body.

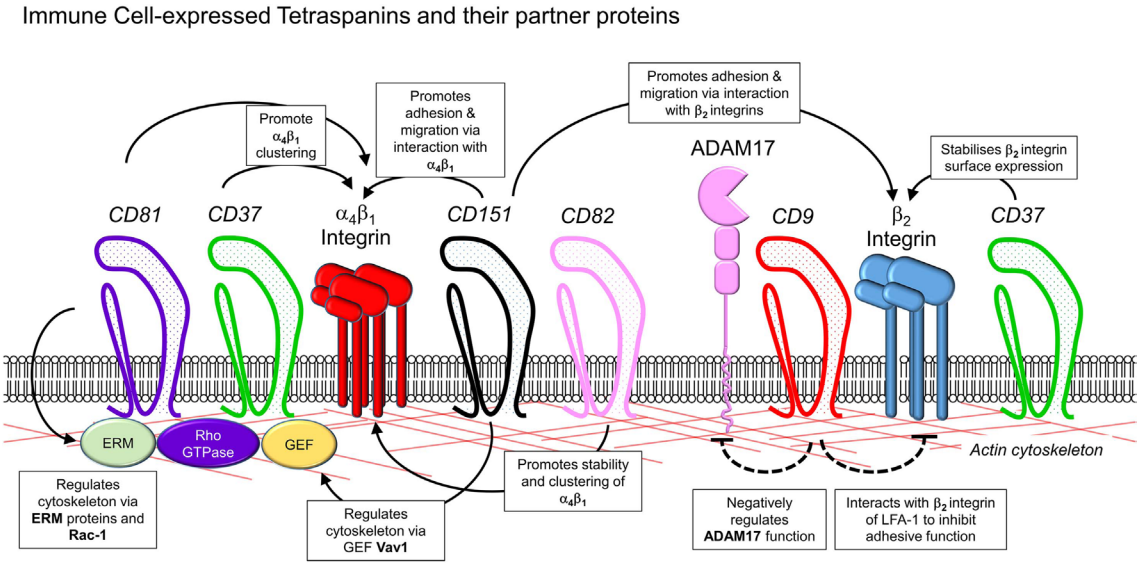

FIGURE 2 | Interactions of leukocyte-expressed tetraspanins and co-expressed molecules involved in leukocyte trafficking. Interactions of tetraspanins expressed in immune cells can occur with other tetraspanins, along with members of several other families of molecules involved in control of adhesion and cytoskeletal function. These include β_1 and β_2 integrins, metalloproteases such as ADAM17, adhesion molecules of the immunoglobulin superfamily such as ICAM-1, the actin cytoskeleton, and intracellular signaling molecules such as guanosine exchange factors (Vav1, SLP76), Rho GTPases, and ezrin/radixin/moesin proteins.

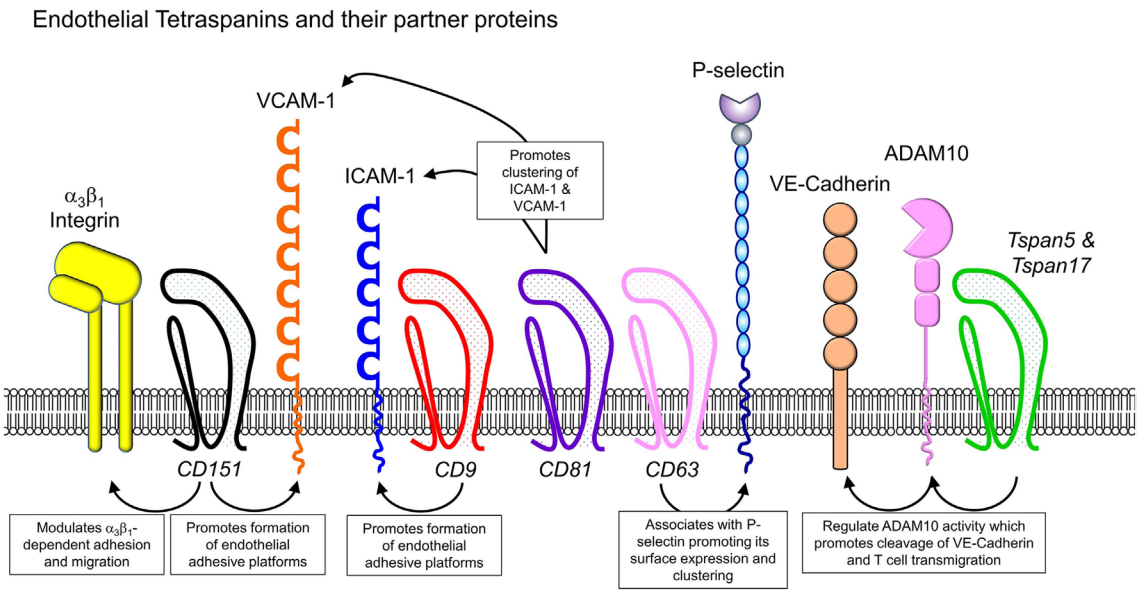

FIGURE 3 | Endothelial cell-expressed tetraspanins and co-expressed proteins relevant to leukocyte trafficking. Endothelial cells play critical roles in directing immune cells from the bloodstream into sites of inflammation or secondary lymphoid organs. Tetraspanins expressed in endothelial cells, including CD9, CD63, CD81, CD82, CD151, and Tspan5 and Tspan17, have been shown to impact on endothelial cell adhesive function, by regulating the function of various adhesion molecules (integrins, ICAM-1, VCAM-1, and P-selectin) and MMPs such as ADAM10.

TETRASPANINS AS REGULATORS OF THE LEUKOCYTE INTEGRINS

β_1 Integrins

The first publication that provided evidence of a role for tetraspanins in immune cell adhesion and migration was the report that the ectopic expression of the tetraspanin CD9 in B cell lines promoted adhesion and haptotactic migration in fibronectin-coated transwells (6). Since then, no less than four tetraspanins have been convincingly reported to regulate $\alpha_4\beta_1$ integrin function in various immune or hemopoietic cells: CD37, CD81, CD82, and CD151 (6, 73–77).

TABLE 1 | Roles of tetraspanin family members in leukocyte–endothelial cell interactions, recruitment, and migration.

Tetraspanin	Immune cell expression	Impact on recruitment	Reference
CD9	Monocytes T cells Neutrophils Endothelial cells	Promotes cell motility through regulation of adhesion molecules, e.g., LFA-1 Promotes formation of endothelial adhesive platforms (EAPs)	(7, 58–61)
CD37	B cells Dendritic cells (DCs) Neutrophils	Promotes cell adhesion through regulation of integrins, e.g., $\alpha_4\beta_1$, β_2. Facilitates chemokine-directed migration Promotes cell spreading through regulation of integrin–cytoskeleton cross-talk, and/or integrin stability	(8, 62, 63)
CD63	Endothelial cells	Promotes leukocyte rolling on human umbilical vein endothelial cells through expression and clustering of P-selectin	(5)
CD81	NK cells B cells T cells Monocytes DCs Endothelial cells	Promotes cell adhesion through regulation of adhesion molecules, e.g., LFA-1, VLA-4 Promotes cell adhesion through regulation of actin-associated proteins, e.g., Rac1, Ezrin Required for DC migration	(10, 11, 64–68)
CD82	DCs T cells	Reduces cell motility through regulation of cytoskeletal proteins, e.g., RhoA Promotes cell adhesion through regulation of adhesion molecules, e.g., LFA-1, $\alpha_4\beta_1$	(9, 69–72)
CD151	T cells Neutrophils Endothelial cells	Promotes cell migration through regulation of extracellular matrix binding Promotes cell adhesion through regulation of actin remodeling and formation of EAPs	(13, 14, 58, 59, 68)
Tspan5 and Tspan17 (TspanC8 family members)	Endothelial cells	Promote T cell transmigration *via* regulation of endothelial MMP ADAM10	(12)

In B cells, there are similarities in the way CD37 and CD81 regulate this key integrin. Tetraspanin CD37 is expressed on most lymphoid cells and has particularly high expression on B cells. Under shear flow conditions *in vitro*, CD37 was required for optimal B cell rolling and adhesion on fibronectin and VCAM-1. CD37 was found to colocalize with $\alpha_4\beta_1$ in clusters within the B cell membrane and to be essential in the formation of high avidity $\alpha_4\beta_1$ clusters upon ligand binding to VCAM-1 and the subsequent transduction of survival signals through the Akt pathway (62). Similarly, the ubiquitously expressed CD81, which has been shown using biochemical approaches to preferentially interact with α_4 integrins, is also essential for $\alpha_4\beta_1$ function (69). CD81 strengthened $\alpha_4\beta_1$-dependent adhesion of B cells and monocytes to VCAM-1 under flow and promoted multivalent integrin interactions (10). Despite these studies, the *in vivo* implications of CD81-mediated regulation of $\alpha_4\beta_1$ adhesiveness are yet to be determined—neither CD81 nor CD37 has been reported to have a role in B cell migration. By contrast, the many B cell impairments caused by CD81 deficiency have been attributed to impaired CD19 expression (78, 79).

Tetraspanin CD151 is best known for its regulation of the laminin and fibrinogen-binding integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ in non-immune cells [reviewed in Ref. (73)]. Nonetheless, there is some evidence for a role of CD151 within immune cells, where the principal β_1 integrin is $\alpha_4\beta_1$ which mediates leukocyte adhesion to the ECM protein fibronectin and the endothelial cell adhesion molecule VCAM-1. Early studies of the role of CD151 in the immune system focused on CD151 expressed by neutrophils. Inhibition of CD151 *via* function-blocking antibodies abolished neutrophil migration on the ECM protein fibronectin (13). However, as tetraspanins exist in supramolecular complexes, it is not clear what physiological processes monoclonal antibody (mAb) cross-linking may be mimicking. As such, we have long urged caution in attributing functions to tetraspanins based solely on the use of mAbs (80).

Nonetheless, recent molecular analyses of CD151 function in T cells suggest that CD151 does indeed regulate α_4 integrin adhesiveness and immune cell migration (14). This paper, by Zelman-Toister and colleagues, eloquently described CD151-integrin interactions in T cells and showed that low-dose CCL2 modulated CD151 expression and cell migration (14). Immunoprecipitation analyses of T cells exposed to CCL2 revealed CCL2-dependent dissociation of CD151/$\alpha_4\beta_1$ complexes. In addition, ligation of CD151 on the surface of mouse T cells induced actin polymerization through Vav1 phosphorylation and elevated CCL21-induced T cell migration (14). Finally, CD151 ablation on T cells was shown to protect mice from experimental colitis, a result confirmed by interruption of CD151:CD151 associations using an antagonistic peptide to the CD151 LEL (14). The latter reagent resulted in impaired T cell actin remodeling and chemotactic migration *in vitro*. As CD151 has been shown to directly interact with integrin α but not β chains (81), and as $\alpha_4\beta_7$ is known to be critical for immune cell recruitment to the gut, it is tempting to interpret these data as an effect of CD151 on $\alpha_4\beta_7$ rather than $\alpha_4\beta_1$ function. However, a biochemical interaction between any tetraspanin and $\alpha_4\beta_7$ integrin has not been reported. Nonetheless, these findings illustrate that the CD151 tetraspanin directly affects leukocyte migration and importantly that this role extends to the capacity to influence inflammatory responses.

Finally, the tetraspanin CD82 has also been reported to interact with several integrins including $\alpha_4\beta_1$ (69, 82). In hemopoietic stem cells (HSC), the CD82/$\alpha_4\beta_1$ axis has functional relevance. These

two molecules colocalize to a polarized membrane domain that has been implicated in mediating HSC adhesion to osteoblasts. Treating HSCs with mAbs against CD82 impairs both their homing to the bone marrow and adhesion to osteoblasts (83). Elegant analyses by super-resolution microscopy in a transfection system using a leukemic progenitor cell line demonstrated that CD82 expression promoted adhesion to fibronectin by promoting both the stability of $\alpha_4\beta_1$ at the cell surface and the formation of high avidity $\alpha_4\beta_1$ clusters (84).

Certainly, CD37, CD81, CD82, and to a lesser extent, CD151 are co-expressed in many immune cell types. Why there appears to be functional overlap where all four tetraspanins can promote $\alpha_4\beta_1$ clustering and adhesiveness and whether there is interplay between the tetraspanins in regulating this integrin is not known. It will be interesting to determine whether these tetraspanins exist within the same microdomain together with $\alpha_4\beta_1$, or whether distinct tetraspanin/$\alpha_4\beta_1$ complexes exist.

β_2 Integrins

In contrast to the well-described interactions between tetraspanins and the β_1 integrins, the literature implicating molecular and functional interaction between tetraspanins and β_2 integrins is less extensive. Nonetheless, CD63, CD82, and CD9 have all been reported to interact with β_2 integrins (4, 70, 85–87). The functional consequences of the interactions of CD63 and CD82 with β_2 integrins remain to be defined, although CD82 overexpression has been implicated in LFA-1-dependent homotypic and heterotypic cell–cell adhesion (88). However, the CD9/LFA-1 interaction on monocyte and T cell lines has been confirmed using various techniques including co-precipitation with chemical cross-linking and proximity ligation assays. This association is mediated *via* interaction of the β_2 subunit of LFA-1 with the LEL of CD9 and is of functional significance, as CD9 negatively regulates LFA-1 adhesive function. However, the mechanism is not fully elucidated, as CD9 did not affect inside-out signaling or display of high affinity integrin, although it was suggested that CD9 modulates LFA-1 clustering (7). Whether this interaction has a functional impact on leukocyte migration *in vivo* remains unknown. Deletion of CD9 was observed to restrict neutrophil and macrophage migration in experimental colitis, an effect consistent with dysregulated LFA-1 function. However, further analyses using bone marrow chimeric mice demonstrated that CD9 expressed on non-hematopoietic cells, rather than leukocytes, was required for disease attenuation (89). Given this observation, the importance of leukocyte-expressed CD9 in immune cell migration remains to be determined.

Perhaps the best evidence for the functional regulation of β_2 integrins comes from analysis of the CD37$^{-/-}$ mouse. CD37$^{-/-}$ neutrophils display reduced capacity to adhere to β_2 integrin ligands *in vitro*. *In vivo,* CD37$^{-/-}$ neutrophils also displayed reduced chemokine-induced adhesion in postcapillary venules, as well as dysregulated directional migration in response to chemotactic stimuli. Deletion of CD37 reduced the stability of integrin expression on the surface of activated neutrophils, by promoting an increase in the rate of β_2 integrin internalization (8). Thus, CD37 constitutively acts to retain β_2 integrins on the cell surface, a function that would act to stabilize leukocyte adhesion. However, despite these findings of a functional link between CD37 and the β_2 integrins, super-resolution microscopy analyses failed to reveal significant co-clustering of CD37 and the β_2 integrin. Furthermore, the absence of CD37 did not affect β_2 integrin clustering (8). Further experiments will be required to understand the molecular basis of this functional interaction.

TETRASPANINS AS REGULATORS OF THE CYTOSKELETON AND DC MIGRATION

How then can a tetraspanin like CD37, which does not colocalize with the β_2 integrin and does not regulate β_2 integrin clustering, control integrin adhesiveness, and internalization? A recurring theme in the literature is the concept that the cytoskeletal rearrangements required for cellular polarization, spreading, adhesion, and migration are under the influence of CD37. Indeed, both neutrophils (8) and DCs (63) from CD37$^{-/-}$ mice have been found to be impaired in their capacity to spread and form membrane protrusions on adhesive substrates, processes which are actin-dependent. CD37$^{-/-}$ DCs also displayed impaired adhesion to fibronectin, as well as impairments in migratory function (63). Together, these findings raise the possibility that CD37 functions as a molecular link between integrins and the cytoskeleton, possibly by regulating signaling through the Rho GTPase Rac1 (9). However, the observation that CD37$^{-/-}$ DCs display reduced migration to lymph nodes (63), behavior that can occur in the absence of integrins (43), indicates that CD37 may also regulate integrin-independent migration. The CD81$^{-/-}$ DC phenotype is strikingly similar to that of CD37$^{-/-}$ DCs, in that CD81$^{-/-}$ DCs are also unable to form Rac1-dependent membrane protrusions and show impaired motility and reduced Rac1 activation (11).

However, while migration of CD81$^{-/-}$ DCs on two-dimensional substrates was impeded, their migration in three-dimensional collagen gels was equivalent to that of wild-type DCs (11), indicating that the contribution of CD81 to DC migration is variable, according to the migratory field being encountered. Moreover, these observations are reminiscent of studies showing that DC migration in three-dimensional substrates is unimpeded in the absence of integrins, being dependent instead on actin-mediated cellular contraction and protrusion (43). One possible explanation for this unexpected finding is that in the absence of integrins, actin polymerization and retrograde flow are increased, compensating for reduced capacity to attach to the ECM (90). Interestingly, Quast et al. also observed increased retrograde actin flow in CD81$^{-/-}$ DCs (11). Together, these similar observations seen in the absence of CD81 and integrins provide further evidence of an intimate functional association between these molecular pathways.

By contrast, DCs lacking expression of CD82 are hypermigratory, as shown in both *in vitro* chemotactic assays and *in vivo* lymph node homing assays (9). Like CD37$^{-/-}$ and CD81$^{-/-}$ DCs, CD82$^{-/-}$ DCs lack membrane protrusions, but in contrast, spread to a greater extent than wild-type DCs upon adhesion to fibronectin. Thus, the CD82$^{-/-}$ phenotype is associated with a defect in actin polymerization, likely brought about by dysregulation of signaling through another Rho GTPase, RhoA (9). This integrated relationship between CD82 and the cytoskeleton is reminiscent of previous investigations in T cells. Here, CD82 was found to

colocalize with F-actin in lipid rafts (71), and cross-linking CD82 induced dynamic morphological changes such as pseudopodia formation, that are dependent on Rho GTPase activity (72, 91).

The precise molecular interactions by which tetraspanins regulate the cytoskeleton are not understood for CD37 and CD82. On the other hand, in T cells, CD81, *via* its cytoplasmic tail, can interact with Rac1 (92). A further key mechanism bridging membrane proteins to cytoskeletal actin is the ezrin/radixin/moesin (ERM) proteins (93, 94). Tetraspanins CD9 and CD81 have been shown to interact directly with ERM proteins in immune cells (95). In NK cells, cross-linking of CD81 induces phosphorylation of ERM proteins and colocalization of CD81 with phosphorylated ERM at uropods. This is associated with increased cell polarization and migration toward chemoattractants (64). Similarly in B cells and human PBMCs, CD81 cross-linking induces Syk-dependent ezrin phosphorylation and CD81 colocalization with ezrin and polymerized actin (65). Deletion of the C-terminal tail of CD81 resulted in reduced ezrin phosphorylation, providing clear evidence that the association between these molecules impacts on ERM function.

INFLUENCE OF TETRASPANINS ON ENDOTHELIAL CELL ADHESIVE FUNCTION

As leukocytes are required to undergo extensive interactions with, and eventually transmigrate through, the endothelium during the process of recruitment, adhesive function of endothelial cells is equally as important as that of leukocytes in facilitating an appropriate inflammatory response. In addition to their effects in leukocytes, multiple members of the tetraspanin family have been shown to act in endothelial cells to modulate their capacity to support interactions with leukocytes (**Figure 3**). For instance, CD9 silencing in human umbilical vein endothelial cells (HUVECs) led to decreased ICAM-1 expression and abrogated leukocyte adhesion and transendothelial migration under flow conditions (58). Subsequent analysis of this phenomenon revealed that both CD9 and CD151 are integral to the formation of membrane structures termed endothelial adhesive platforms (EAPs) in which ICAM-1 and VCAM-1 cluster in the endothelial cell membrane at contact sites with adherent leukocytes (59). This leads to increased avidity for leukocyte integrin ligands and tetraspanin-dependent promotion of leukocyte adhesion and transmigration. The role of CD9 in promoting endothelial cell adhesive function was further examined in a study that used atomic force microscopy to examine the morphology of the endothelial surface at high resolution (60). In response to TNF stimulation, F-actin-containing microvilli decorated with ICAM-1 formed on the endothelium. While these structures developed in the absence of adherent leukocytes, they were thought to serve as precursors for EAPs that form around adherent immune cells (58). CD9 was also incorporated in these structures, and CD9-siRNA studies demonstrated that their formation required CD9. These studies provide further evidence for a role for CD9 in endothelial cells in supporting leukocyte adhesion and recruitment (60).

Endothelial cell-expressed CD81 has also been shown to contribute to leukocyte–endothelial cell interactions. In early atherosclerotic lesions, where monocyte–endothelial interactions are increased, endothelial cells express CD81 at elevated levels (66). *Via* confocal microscopy of TNF-treated endothelial cells, CD81 was found to colocalize with both VCAM-1 and ICAM-1 at contact sites with monocytes. Furthermore, forced expression of CD81 in endothelial cells was sufficient to increase monocyte adhesion to endothelial monolayers, notably without a requirement for stimulation of the endothelium with inflammatory mediators. This increased adhesion was dependent on endothelial ICAM-1 and VCAM-1 but occurred in the absence of upregulation of these adhesion molecules (66). By contrast, overexpression of CD81 increased clustering of ICAM-1 and VCAM-1, with this increased avidity likely to facilitate monocyte adhesion. These findings indicate a role for CD81 in the redistribution of ICAM-1 and VCAM-1 into adhesion-supporting clusters within the endothelial cell membrane (66). This is paralleled by studies in T cells which demonstrate that CD81 influences leukocyte recruitment by promoting integrin avidity (10, 67).

Weibel–Palade bodies in endothelial cells are secretory vesicles that contain von Willebrand factor and P-selectin. These vesicles are released upon endothelial cell activation and aid in promotion of leukocyte rolling in response to acute inflammatory stimuli and hemostasis (96, 97). It has been long established that the tetraspanin CD63 is an additional major component of these structures, although its function was not clear. This was addressed in studies in which CD63 expression was silenced in HUVECs using siRNA (5). CD63-deficient HUVECs showed a reduced capacity to support leukocyte rolling, findings supported by *in vivo* analyses of leukocyte rolling in postcapillary venules of $CD63^{-/-}$ mice. The nature of the association between CD63 and P-selectin was examined using immunogold scanning electron microscopy in HUVECs, revealing that CD63 clustered with P-selectin on the endothelial cell surface. In addition, proximity ligation assays in HEK293 cells showed that surface CD63 and P-selectin colocalized within 20–30 nm of each other (5). Finally, in the absence of CD63, both P-selectin clustering and the level of P-selectin surface expression were reduced relative to non-silenced cells. This indicated that CD63 is a molecular partner of P-selectin and supports its clustering with this being essential for the capacity of P-selectin to mediate rolling.

Metalloproteases (MP) are also tetraspanin-partner proteins. For example, CD9 is a molecular partner and negative regulator of MP ADAM17 (61), substrates of which include ICAM-1 and L-selectin (98). Similarly, the TspanC8 subfamily of tetraspanins (consisting of six members: Tspan5, 10, 14, 15, 17, and 33) have been reported to regulate ADAM10 (99), the targets of which include Notch proteins, amyloid precursor protein associated with Alzheimer's disease (100), and adhesion molecules. In regards to leukocyte recruitment, TspanC8 members Tspan5 and Tspan17 promote transmigration of T cells by regulating cleavage of VE-cadherin on endothelial cells (12). VE-cadherin is an ADAM10 substrate, and its cleavage is a necessary step toward the completion of T cell transmigration (101). Reyat et al. demonstrated that endothelial ADAM10 function is regulated by Tspan5 and Tspan17, and that silencing of these tetraspanins in HUVECs resulted in inhibition of T lymphocyte transmigration (12). The mechanisms whereby TspanC8 subgroup tetraspanins regulate ADAM10 activity are thought to include effects on intracellular

trafficking, promoting ADAM10 exit from the endoplasmic reticulum and enzymatic processing, promoting cleavage of ADAM10 into its mature form (102).

Tetraspanins are also known to be highly expressed on extracellular vesicles (EVs) (103). EVs are membrane-bound subcellular particles released by a wide range of cells, including immune cells such as DCs, macrophages, B cells and endothelial cells (104, 105). Exosomes, EVs in the ~50–100 nm diameter range, are enriched in CD9, CD37, CD53 CD63, CD81, and CD82, with the relative abundance of different tetraspanins varying according to the cell of origin. In many cases, these particles also carry adhesion molecules (103). Furthermore, in some circumstances, EVs have been shown capable of modulating immune cell migration (106–110). However, whether the tetraspanins contained within EVs contribute to this effect on immune cell migration requires further investigation.

FUTURE DIRECTIONS

Other tetraspanins may also be worthy of investigation for their actions in leukocyte recruitment. For example, a small case study of individuals deficient in the leukocyte-restricted tetraspanin CD53 revealed that they were affected by recurrent bacterial infections (111, 112). This observation is consistent with this genetic defect resulting in a form of immune deficiency. As effective combat of bacteria by neutrophils is heavily reliant upon their migratory capacity, these observations raise the yet to be investigated possibility of a role for CD53 in these activities.

There is no question that the tetraspanin family of transmembrane molecules is instrumental in ensuring the correct functioning of proteins and processes involved during immune cell migration. Of particular interest is the ability of tetraspanins to functionally associate with the cytoskeleton and influence the remodeling of actin filaments to produce extensions such as lamellipodia and filopodia, structures which are required for leukocyte recruitment and migration. As detailed, some tetraspanins, such as CD82, can elicit inhibitory effects on cell migration, while others, including CD37 and CD151, are able to enhance recruitment events. Though research in this field has documented numerous tetraspanin partners and downstream signaling pathways modulated by these interactions, there is still much to be learnt about the significance of these interactions during immune cell migration. Further investigation into tetraspanin regulation of localization and clustering of integrins and other adhesion molecules in the cell membrane are warranted. In addition, an important distinction that needs to be made is how applicable these mechanisms of regulation are to different immune cell subsets, as tetraspanins have been repeatedly demonstrated to mediate different functions in different cell types. Finally, and most importantly, the influence of these functions on leukocyte recruitment and behavior *in vivo* during inflammatory responses must be examined, to determine if these molecules have potential as therapeutic targets in inflammatory disease.

AUTHOR CONTRIBUTIONS

LY wrote the initial draft of the manuscript and was involved in its editing. MH is an expert in leukocyte recruitment and had particular responsibility for the parts of the manuscript dealing with this topic. He had a major role in the editing of the manuscript and made the figures. MW is an expert in tetraspanin immunology and had particular responsibility for the parts of the manuscript dealing with this topic. He also made suggestions to amend the figures and edited the manuscript.

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CD9 Controls Integrin α5β1-Mediated Cell Adhesion by Modulating Its Association With the Metalloproteinase ADAM17

Yesenia Machado-Pineda[1†], Beatriz Cardeñes[1†], Raquel Reyes[1], Soraya López-Martín[1,2], Víctor Toribio[1,2], Paula Sánchez-Organero[1], Henar Suarez[1,2], Joachim Grötzinger[3], Inken Lorenzen[4], María Yáñez-Mó[1,2,5] and Carlos Cabañas[1,6]*

[1] Departamento de Biología Celular e Inmunología, Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Madrid, Spain, [2] Departamento de Biología Molecular, Facultad de Ciencias, Universidad Autónoma de Madrid, Madrid, Spain, [3] Institute of Biochemistry, Christian-Albrechts University, Kiel, Germany, [4] Department of Structural Biology, Institute of Zoology, Kiel, Germany, [5] Centro de Biología Molecular Severo Ochoa, Instituto de Investigación Sanitaria la Princesa (IIS-IP), Madrid, Spain, [6] Departamento de Inmunología, Oftalmología y OTR, Facultad de Medicina, Universidad Complutense, Madrid, Spain

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***Correspondence:**
Carlos Cabañas
ccabanas@cbm.csic.es

[†] These authors have contributed equally to this work

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Integrin α5β1 is a crucial adhesion molecule that mediates the adherence of many cell types to the extracellular matrix through recognition of its classic ligand fibronectin as well as to other cells through binding to an alternative counter-receptor, the metalloproteinase ADAM17/TACE. Interactions between integrin α5β1 and ADAM17 may take place both in *trans* (between molecules expressed on different cells) or in *cis* (between molecules expressed on the same cell) configurations. It has been recently reported that the *cis* association between α5β1 and ADAM17 keeps both molecules inactive, whereas their dissociation results in activation of their adhesive and metalloproteinase activities. Here we show that the tetraspanin CD9 negatively regulates integrin α5β1-mediated cell adhesion by enhancing the *cis* interaction of this integrin with ADAM17 on the cell surface. Additionally we show that, similarly to CD9, the monoclonal antibody 2A10 directed to the disintegrin domain of ADAM17 specifically inhibits integrin α5β1-mediated cell adhesion to its ligands fibronectin and ADAM17.

Keywords: CD9, ADAM17, α5β1, integrin, tetraspanin, cell adhesion, fibronectin, metalloproteinase

INTRODUCTION

Integrins constitute an important family of heterodimeric (αβ) cellular receptors which, upon recognition and binding to specific ligands, mediate the adhesion of cells to components of the extracellular matrix (such as fibronectin, laminin, collagens) as well as cell-cell adhesion phenomena with crucial relevance in a variety of physiological and pathophysiological processes [reviewed in (1–3)]. Integrin α5β1 (also termed CD49e/CD29 or VLA5) binds to its canonical ligand fibronectin (Fn) through recognition of the Arg-Gly-Asp (RGD) motif in Fn-type-III module 10 and of a synergy site in Fn-type-III module 9, contributing to fibronectin assembly into fibrils (4). In addition to mediating cell adhesion through binding to its canonical Fn ligand, integrin α5β1 also has been reported to specifically recognize and bind to an alternative ligand, the disintegrin domain of ADAM17 (5–7).

ADAMs (A Disintegrin And Metalloproteinase) are a family of type-I transmembrane proteins with a modular structure that comprises the following domains (from N- to C-termini): a pro-, a

catalytic-, a disintegrin-, a cysteine-rich-, and an EGF-like-domain, followed by a transmembrane- and a cytoplasmic region. 40 ADAMs have been identified in the mammalian genome from various species with the human genome containing 21 functional ADAMs, of which only 13 are proteolytically active while the rest lack the Zn-binding motif in the catalytic domain which is required for the proteolytic activity [reviewed in (8–10)]. Two closely related members of this family, ADAM10 and ADAM17, stand out among the catalytically active ADAMs as they are the two main cellular enzymes responsible for the cleavage and release of ectodomains from many cell surface proteins, a process known as "shedding" which plays an essential role in the development of tissues and organisms and in many other physiological as well as pathophysiological processes (11). ADAM10 and ADAM17 are also considered atypical members of the ADAM family since the extracellular cysteine-rich and EGF-like domains found in the rest of ADAMs are replaced in these two enzymes by a unique membrane proximal domain (MPD), which is involved in substrate recognition and binding as well as in regulation of their shedding activity [reviewed in (12, 13)].

All ADAMs contain a disintegrin domain in their extracellular region, which is structurally related to snake venom disintegrins. The disintegrin domains of ADAMs can potentially act as ligands for integrin binding, thus influencing cell adhesion and cell-cell interactions, with some degree of selectivity existing for these interactions between specific members of integrin and ADAM families (12–14).

Interactions of α5β1 with ADAM17 may occur among molecules expressed on the same cell (*cis*) or on different cells (*trans*), with the latter reported to support cell-cell adhesion events (6, 13). Interestingly, the interaction between integrin α5β1 and the disintegrin domain of ADAM17 has been shown to cause the inhibition of both the adhesive capacity of the integrin (i.e., its ability to bind its ligands) as well as that of ADAM17 metalloproteinase activity due to steric hindrance leading to decreased accessibility of its catalytic site for the substrates (6, 13). In contrast, stimuli that promote the dissociation of the α5β1-ADAM17 complex, such as an excess of soluble ADAM17 disintegrin domain, induce the activation of ADAM17 sheddase activity and enhance integrin adhesive capacity (6, 13).

The tetraspanin CD9, within the context of tetraspanin-enriched microdomains (TEMs), has been reported to associate on the cell surface with different adhesion receptors of the immunoglobulin and integrin families (15), including the integrin α5β1 (16–18). Through these interactions, CD9 exerts different regulatory effects on the function of associated adhesion molecules (19–23). On the other hand, CD9 also has been reported to associate directly with ADAM17 on the surface of different types of cells, thus exerting an inhibitory effect on ADAM17 sheddase activity against a variety of its substrates (19, 24–27).

Here we report that integrin α5β1 mediates the specific adhesion of different tumoral and leukocytic cells to immobilized recombinant ADAM17-Fc protein, which can be efficiently abrogated with blocking mAbs directed against the α5 or the β1 subunits of the integrin. Interestingly, the expression of CD9 on the cell surface or preincubation with mAb 2A10, which is directed to the disintegrin domain of human ADAM17, also abrogated the α5β1-mediated adhesion both to its canonical ligand Fn and to ADAM17-Fc. *In situ* proximity ligation assays (PLA) and biochemical experiments based on co-immunoprecipitation collectively revealed that the mechanism by which CD9 and mAb 2A10 inhibit α5β1-mediated cell adhesion is related to the reinforcement of *cis* interactions between ADAM17 and α5β1 on the cell surface, which takes place without alteration in α5β1 integrin affinity but is rather evidenced by changes in the organization of integrin molecules at the plasma membrane.

MATERIALS AND METHODS

Generation of mAB 2A10 Against the Disintegrin Domain of Human ADAM17

The mAb 2A10 was generated after mice immunization with the recombinant chimeric protein ADAM17-Fc, which encompasses the whole extracellular region of human ADAM17 fused to the Fc fragment of human IgG_1, by employing the standard murine hybridoma technology. The experimental protocol followed was in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and was approved by the Animal Ethics Committee of the "Centro de Biología Molecular Severo Ochoa" (Madrid, Spain). The 2A10 mAb was selected from among the several hundred hybridomas generated based on its high and specific reactivity against ADAM17-Fc in ELISA assays. Assessment of the reactivity of 2A10 mAb against purified disintegrin (Dis) and membrane-proximal (MP) domains of human ADAM17, revealed that the epitope recognized by this mAb maps to the disintegrin domain.

Cells and Antibodies

Raji (Burkitt's lymphoma-derived B lymphoblastoid), JY (EBV-immortalized B lymphoblastoid), K562 (erythroblastic cell line), HSB2 (T lymphoblastic), Jurkat (T lymphoblastic), and Colo320 (colorectal adenocarcinoma) human cell lines were cultured in RPMI-1640. SKOV-3 (ovarian carcinoma) human cell line was grown in DMEM. LoVo (colorectal adenocarcinoma) human cell line was cultured in DMEM supplemented with F-12 nutrient mixture. All culture media were supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 50 μg/ml streptomycin and 50 U/ml penicillin.

2A10 (anti-ADAM17); P1D6 (anti-α5 integrin) (28); TS2/16 (anti-β1 integrin), Lia1/2 (anti-β1 integrin) (29, 30), and HUTS21 (anti-β1 integrin) (31); TS1/18 (anti-β2 integrin) (32); PAINS-10 (anti-CD9) (33) and MEM-111 (anti-ICAM1/CD54) (34) mAbs were purified by protein A- or protein G-affinity chromatography. The A300D (specific for the disintegrin domain of human ADAM17) and A300E (specific for the membrane proximal domain of human ADAM17) mAbs have been described previously (35). When necessary, purified mAbs were biotinylated as previously described (33).

Expression DNA Constructs and CRISPR/Cas9-Mediated Gen Knock Out

For stable transfection experiments, Colo320 and HSB2 cells were incubated in 2.5% FCS–RPMI-1640 with the cDNA (20 μg) coding for human CD9 (in the pcDNA3 expression vector).

Colo320 cells were electroporated at 412 V/cm and HSB2 cells at 200 V/cm (2 × 10 ms pulses in a 0.4 cm electroporation cuvette) in the ElectroSquarePorator ECM830 (BTX, Holliston,MA), positive clones were selected with G418 (0.8 mg/ml) in the culture medium (20).

To generate "Colo320-CRISPR ADAM17" and "Jurkat-CRISPR CD9" cell lines, cells were transfected with the CRISPR/Cas9 knockout plasmid pX461 encoding GFP and Cas9 nickase and the following sequences to generate the specific single guide RNAs: 5′-CACCGATCTAATATCCAGCAGCATT-3′ and 5′-CACCGTTTTTCTTACCGAATGCTGC-3′ for ADAM17 and 5′-CACCGTTCTTGCTCGAAGATGCTCT-3′ and 5′-CACCGGAATCGGAGCCATAGTCCAA-3′ for CD9. Transfected cells were sorted by flow cytometry based on their GFP transient fluorescence and then expanded and checked for suppression of ADAM17 or CD9 expression.

Flow Cytometry Analysis

For flow cytometry analysis of protein surface expression cells were washed three times in RPMI-1640, incubated with primary antibodies at 4°C for 30 min followed by Alexa Fluor®647-conjugated anti-mousse IgG and fixed in 2% formaldehyde in PBS. Changes in integrin affinity were probed with the anti-β_1 integrin activation reporter HUTS21 mAb. Cells were washed in cation-free medium (Hepes 20 mM, NaCl 149 mM, 2 mg/ml glucose) and incubated for 20 min at 37°C with Mn^{2+} (400 μM) or with Ca^{2+}/Mg^{2+} (0.5 mM/1 mM, respectively) in the presence of biotinylated HUTS21 mAb. Cells were then washed and stained with Alexa Fluor®488-conjugated streptavidin. Fluorescence was measured using a FACScan™ flow cytometer (Beckton-Dickinson).

Immunofluorescence, Proximity Ligation Assays and Confocal Microscopy

For double immunofluorescence studies, cells were seeded on 12-mm diameter glass coverslips coated with poly-L-lysine (100 μg/ml). Cells were fixed in 2% formaldehyde (8 min at room temperature), blocked in 1% BSA in TBS (30 min at room temperature), and incubated for 1h with mAb 2A10 (10 μg/ml), followed by washes and incubation with the secondary antibody Alexa Fluor™-647-conjugated anti-mousse IgG (Thermo-Fisher Scientific). After incubation for 1 h with mouse serum (1/100) to block any free Fab binding sites of the secondary antibody, samples were incubated for 1 h with biotinylated-TS2/16 mAb (10 μg/ml), then with the secondary reagent streptavidin-Alexa Fluor™-488 (Life Technologies) and finally mounted on microscope slides with Fluoromount™/DAPI (Sigma-Aldrich).

In situ proximity ligation assays (PLA; Duolink kit, Sigma Aldrich) allows detection of direct protein–protein interactions in cell samples by fluorescence microscopy (36). Colo320 cells were seeded, fixed and blocked as described above. Next, samples were incubated simultaneously with 2A10 (anti-ADAM17) mouse mAb and with an anti-α5 rabbit polyclonal antibody, followed by specific oligonucleotide-labeled secondary antibodies (anti-mouse plus and anti-rabbit minus probes). Only if the two target proteins are in close proximity (≤40 nm), the oligonucleotides of the two probes will hybridize and after a rolling-circle amplification reaction and detection with a different fluorescently labeled oligonucleotide, fluorescent dot signals can be visualized by microscopy. Samples were mounted with ProLong® anti-fade reagent and images were obtained with a Leica LSM510 inverted confocal microscope. Fiji/Image-J software was used for detection and analysis of fluorescent dots.

Co-immunoprecipitation

Co-immunoprecipitation experiments were performed using intact cells, in order to detect only surface protein–protein interactions. Cells were incubated for 1 h at RT with the anti-β1 mAb Lia1/2 or the anti-CD9 mAb PAINS-10 in the presence of Ca^{2+}+Mg^{2+} (500 μM + 500 μM) or Mn^{2+} (200 μM), followed by washing the non-bound antibody excess. Cells were then lysed for 15 min at 4°C in TBS containing 1% Brij-97 in the presence of corresponding extracellular cations and protease inhibitors and, after removal of insoluble material, incubated for 4 h at 4 °C with protein A-sepharose. Beads were then washed with 1:5 diluted lysis buffer, boiled in nonreducing (for detection of CD9 and β1 integrin) or reducing (for detection of ADAM17) Laemmli buffer, resolved by 8% or 12% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with 3% BSA and developed either with biotinylated anti-CD9 mAb PAINS10 or biotinylated anti-β1 mAb TS2/16 or anti-ADAM17 mAb A300D, followed by incubation respectively with streptavidin-HRP (Thermo-Fisher Scientific) or anti-mouse IgG-HRP (Sigma-Aldrich) secondary reagents and ECL-chemiluminescence detection on an ImageQuant LAS4000-mini system.

Cell Adhesion Assays

Static cell adhesion to ADAM17-Fc, ICAM1-Fc or fibronectin-coated wells was performed as described previously (19, 37). 96-well flat-bottom plates were pre-coated overnight at 4°C with ADAM17-Fc (20 μg/ml), ICAM1-Fc (20 μg/ml) or fibronectin (7.5 μg/ml) and blocked for 2 h with 1% BSA in PBS. For PMA-stimulated cell adhesion, cells were incubated with PMA (200 ng/ml) in RMPI-1640 for 2h at 37°C. Cells (2×10^5 cells/well) were loaded with the fluorescent probe BCECF-AM for 20 min at 37°C in PBS, washed, resupended in adhesion buffer (Hepes 20 mM, NaCl 149 mM, 2 mg/ml glucose) containing 200 μM Mn^{2+}, added to the wells in the presence of the appropriate mAbs (10 μg/ml) and allowed to adhere for 60 min at 37°C. When indicated in **Figure 1D**, only the cells or the plates precoated with immobilized ligands ADAM17-Fc or Fn were incubated first with 10 μg/ml of anti-ADAM17 (2A10), anti-β_1 (Lia1/2), anti-α_5 (PID6), or the control anti-ICAM1 (MEM-111) mAbs for 60 min at 4°C, then the excess non-bound antibody was washed and the cells were subsequently allowed to adhere onto the immobilized ligand by transferring the plates to 37°C for 60 min. After gently washing the wells several times with PBS at 37°C to remove non-adherent cells, the percentage of adherent cells in each well was calculated by determining their fluorescence in a microplate reader (TecanGENios) before and after having removed the non-adherent cells.

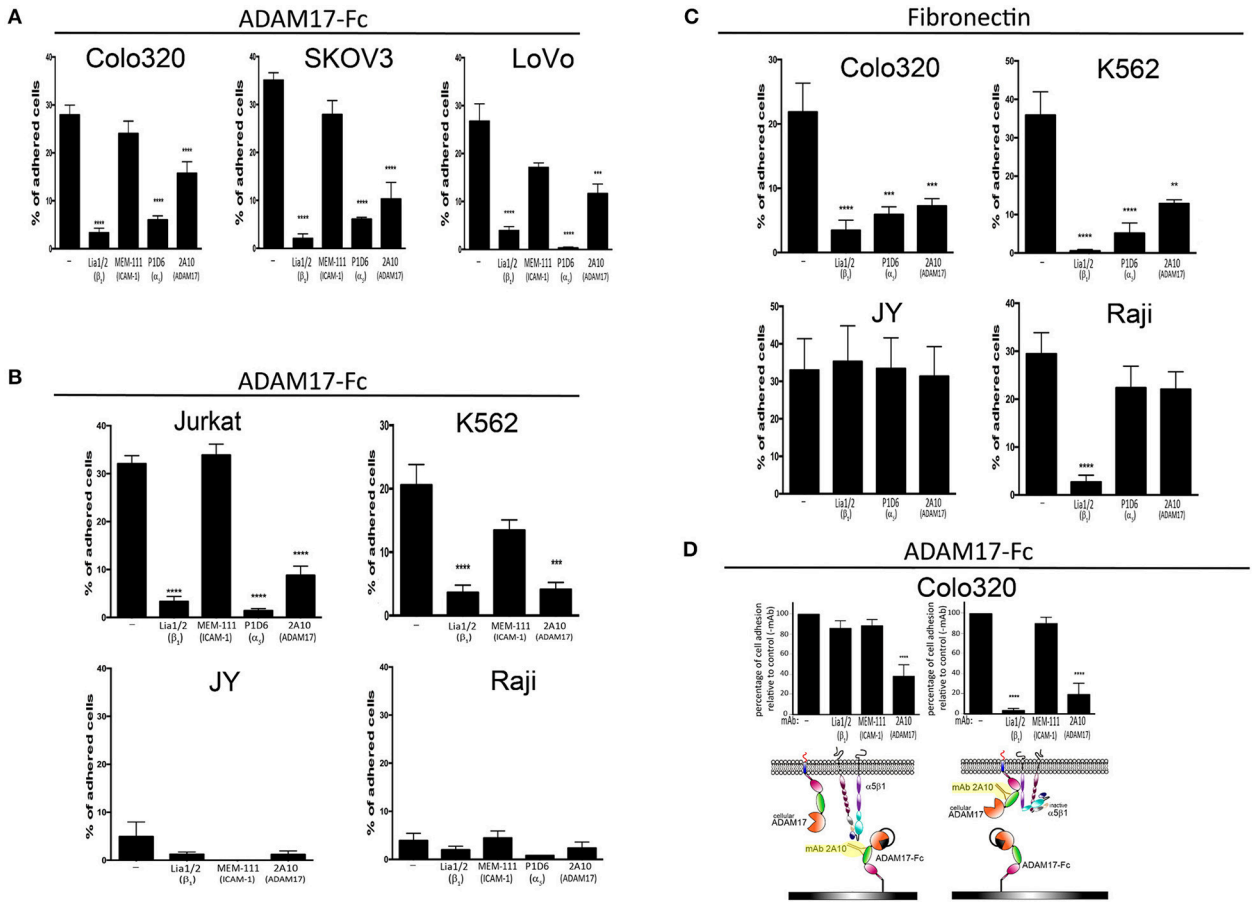

FIGURE 1 | The adhesion of different tumoral and leukocytic cells to Fn and ADAM17-Fc is specifically mediated by integrin α5β1. The adhesion of different human cell lines derived from solid tumors **(A)** or hematopoietic malignancies **(B)** to immobilized ADAM17-Fc or to fibronectin **(C)** is represented. The effect of mAbs Lia1/2 (blocking anti-β1 integrin subunit), P1D6 (blocking anti-α5 integrin subunit) and mAb 2A10 (specific for the disintegrin domain of human ADAM17) on cell adhesion is shown. The anti-ICAM1/CD54 mAb MEM 111 was included as a negative control. Data show the percentages of adherent cells (means ± SEM of at least three different experiments, each performed in triplicates). **(D)** Effect of preincubating plastic-immobilized ADAM17-Fc (left graph) or Colo320 cells (right panel) with the indicated mAbs (the excess non-bound antibody was subsequently washed away) prior to adding the cells to the ADAM-17-coated wells and allowing their adhesion at 37°C. In all cases, cells were stimulated with PMA (200 ng/ml) for 2 h, loaded with the fluorescent probe BCECF-AM and then allowed to adhere to plastic-immobilized ligands ADAM17-Fc (20 μg/ml) or Fn (7.5 μg/ml) for 60 min at 37°C in the presence of Mn^{2+} (200 μM). Data show the percentages of adherent cells (means ± SEM of three experiments, performed in triplicates) relative to the 100% cell adhesion considered in the absence of antibody treatment. Data were analyzed by one-way ANOVA with Dunnett's post-test analysis $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ values denote the statistical significance of differences between a specific condition and the control condition in the absence of antibody (–).

Statistical Analysis

As indicated in the individual figure legends, different statistical analyses of data were performed depending on the quantitative and qualitative nature of the variables being considered. These analyses include the two-tailed paired *T*-test and the one-way ANOVA coupled with Dunnet's, Tukey's, or Šidák's multiple comparison tests.

RESULTS

Integrin α5β1 Mediates the Adhesion of Tumoral and Leukocytic Cells to Immobilized ADAM17

Recombinant ADAM17 has been reported to support integrin α5β1-dependent fibroblast and kidney mesangial cell adhesion, and such adhesion was demonstrated to occur through integrin binding to the disintegrin domain of ADAM17 (5–7). We decided to build on these findings by assessing whether the adhesion of several other human cell lines derived either from solid tumors ("cancer cell lines") or hematological malignancies ("leukocytic cell lines") to immobilized recombinant ADAM17-Fc was also mediated by integrin α5β1. **Figure 1A** shows that, when stimulated with phorbol ester PMA and divalent cation Mn^{2+} (a potent activating agent that induces integrin high affinity state), Colo320 (colorectal adenocarcinoma), LoVo (colorectal adenocarcinoma) and SKOV-3 (ovarian carcinoma) cancer cells readily and specifically adhered to a substrate coated with ADAM17-Fc. In all cases, adhesion of these cells to ADAM17-Fc was specifically mediated by integrin α5β1, as demonstrated by the potent inhibition achieved

either with a blocking anti-α5 mAb (P1D6) or anti-β1 mAb (Lia1/2) antibodies. Likewise, the PMA/Mn^{2+}-stimulated adhesion of leukocytic Jurkat (T lymphoblastic) and K562 (erythroblastic) cells to ADAM17-Fc was also shown to be fundamentally dependent on integrin α5β1 (**Figure 1B**). In contrast, the leukocytic JY (EBV-immortalized B lymphoblastoid cell line) cells, which do not express any β1-containing integrins (including α5β1) due to the lack of expression of this integrin chain, and Raji (Burkitt's lymphoma-derived B lymphoblastoid cell line) cells, which do not express integrin α5β1 although they express abundant α4β1 (another important Fn receptor), only displayed a negligible level of adhesion to ADAM17-Fc (<5%), even after strong stimulation with PMA/Mn^{2+}.

Colo320 and K562 cells, which selectively express abundant levels of the integrin α5β1 on their surface, also adhered efficiently to Fn after stimulation with Mn^{2+} and this adhesion was blocked by either P1D6 or Lia1/2 mAbs. However, although JY and Raji cells also adhered very efficiently to Fn, such adhesion was not inhibited by the blocking anti-α5 (P1D6) mAb as it was not mediated by integrin α5β1, but rather through integrins α4β7 and α4β1, respectively (**Figure 1C**).

Taken together, these results concur with previous reports (5–7) and confirm that integrin α5β1, in addition to binding to its canonical ligand Fn, also mediates the adhesion of a wide variety of cells to immobilized ADAM17-Fc.

The mAb 2A10 was generated in our laboratory and shown by ELISA assays to be specific against an epitope located on the disintegrin domain of human ADAM17 mAb (**Figure 2**).

We then assessed the effects of mAb 2A10 on cell adhesion to both α5β1 ligands, ADAM17-Fc and Fn. As shown in **Figure 1**, in all cases where cell adhesion was predominantly mediated by integrin α5β1, mAb 2A10 exerted a potent inhibitory effect on cell adhesion. In contrast, mAb 2A10 had no effect on the adhesion of JY or Raji cells to Fn which, as indicated above, is not mediated by α5β1. Furthermore, mAb 2A10 had no effect on the cell adhesion mediated by integrin LFA-1 (αLβ2), clearly showing that its effects are specifically mediated through integrin α5β1 (**Supplementary Figure 1**).

The observed blockade of cell adhesion to Fn with mAb 2A10 suggested that this antibody must exert its effects on α5β1-mediated adhesion through a *cis*-type mechanism after binding to cell surface ADAM17. In any case, and to rule out a possible steric hindrance of cell adhesion caused by binding of mAb 2A10 to the disintegrin domain of the immobilized ligand ADAM17-Fc, we first incubated Colo320 cells with 2A10 and washed away the excess of unbound antibody before allowing cells to adhere onto immobilized ADAM17-Fc (**Figure 1D**, right panel). These

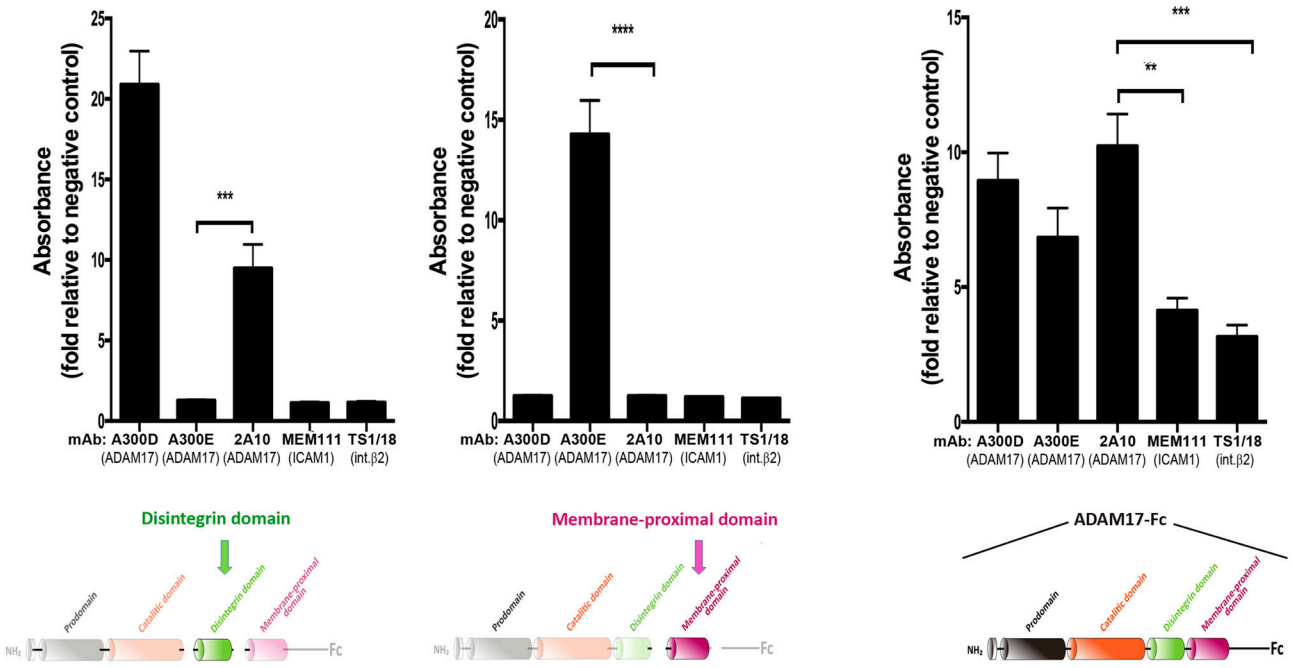

FIGURE 2 | Monoclonal antibody 2A10 is specific for the disintegrin domain of human ADAM17 (hADAM17). Assessment by ELISA of the reactivity of mAb 2A10 against the purified disintegrin domain of hADAM17 **(left)**, the membrane-proximal domain of hADAM17 **(middle)** or the recombinant chimeric protein ADAM17-Fc **(right)**. The mAb A300D, which recognizes an epitope mapped to the disintegrin domain of human ADAM17 and the mAb A300E, which recognizes an epitope mapped to the membrane-proximal domain of human ADAM17, were used respectively as positive controls for each of these domains. The mAb TS1/18 (anti-human β_2 integrin) and mAb MEM111 (anti-ICAM1/CD54) were used as negative controls. Graphs show the absorbance, expressed in arbitrary units, relative to the unspecific absorbance background in the absence of antibody. Data correspond to the mean ± SEM from three independent experiments, each performed in triplicate and analyzed by one-way ANOVA with Dunnett's post-test analysis $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. The colored schematics underneath each panel highlight the specific domains of hADAM17 immobilized on plastic for each ELISA assay.

analyses confirmed that the adhesion blocking effect exerted by mAb 2A10 was due to a regulation in *cis* of α5β1-mediated adhesion.

Expression of CD9 Inhibits α5β1-Mediated Cell Adhesion to ADAM17 and Fibronectin

We have previously shown that CD9 on the cell surface is engaged in direct interactions with ADAM17 and through such association inhibits ADAM17 metalloproteinase activity (19, 24, 25). Therefore, we decided to assess whether the presence or absence of CD9 on the cell surface could influence not only ADAM17 sheddase activity but also its regulatory effect exerted on the adhesive activity of integrin α5β1. For this purpose, we generated a series of paired variants derived from Jurkat, HSB2 and Colo320 cell lines, that differed in the presence/absence of CD9 following either the neoexpression of this tetraspanin (by stable transfection of CD9 cDNA in $CD9^-$ Colo-320 and HSB2 cells) or its supression by CRISPR/Cas9 gene knockout (in $CD9^+$ Jurkat cells; **Figure 3A**). For all these three cell lines, the variants expressing CD9 (Colo320-CD9, HSB2-CD9, Jurkat) displayed a greatly reduced capacity to adhere to ADAM17-Fc, even in the presence of the potent integrin stimulus Mn^{2+}, compared to their respective $CD9^-$ (Colo320, HSB2, Jurkat-CRISPR-CD9) counterparts **(Figure 3B**). We also assessed the adhesion of these paired cell variants to Fn and again, the presence of CD9 resulted in reduced cell adhesion **(Figure 3C**), except for the case of Jurkat cells that, in addition to α5β1, also express very high levels of the fibronectin-binding integrin α4β1 **(Figure 3A).**

CD9-Induced Inhibition of α5β1-Mediated Cell Adhesion Cannot be Attributed to Decreased Integrin Conformational Change to the High Affinity State

The observed abrogation of integrin α5β1-mediated cell adhesion caused by the cell surface expression of CD9 could be due either to diminished integrin affinity or to an alteration in the organization of α5β1 integrin molecules on the cell surface resulting in lower multivalent avidity for ligand. First we investigated whether changes in the affinity of α5β1 were being induced by the presence of CD9, and for this purpose we compared in Colo320 and Colo320-CD9 cells the expression of the epitope HUTS21, which reports the high affinity state—characterized by an extended and open headpiece conformation—of integrins that contain the β1 subunit (31), and particularly of integrin α5β1 (38, 39). Colo320/Colo320-CD9 cells represent a very clean cellular system to assess α5β1 integrin-mediated adhesion, since they selectively express high levels of α5β integrin, but neither α4β1 integrin nor any other β2-associated integrins, and only very low levels -if at all- of other major integrins (not shown). As shown in **Figures 4A,B**, no significant differences in the expression of the HUTS21 epitope between Colo320 and Colo320-CD9 cells were detected, neither in the presence of extracellular Ca^{2+} and Mg^{2+}, which resemble resting physiological conditions in terms of integrin activation, or under conditions which fully induce the acquisition of integrin high affinity such as the presence of Mn^{2+} or the combination of Mn^{2+} and phorbol ester PMA. Moreover, pretreatment with mAb 2A10 did not change the expression of HUTS21 epitope relative to cells not preincubated with 2A10 under any of the conditions tested (Ca^{2+}/Mg^{2+}, Mn^{2+}, or Mn^{2+}/PMA). Taken together, these results indicate that the inhibitory effects caused by the presence of CD9 or by preincubation with mAb 2A10 on the adhesive activity of integrin α5β1 are not primarily mediated by alterations in the ability of integrin molecules to switch to the high affinity conformation.

We also investigated whether the observed inhibitory effects on α5β1-mediated cell adhesion induced by the presence of CD9 are caused by changes in the organization of α5β1 molecules on the cell surface. Double immunofluorescence staining of integrin α5β1 and ADAM17 molecules in CD9-negative (Colo320) or CD9-positive (Colo320-CD9) cells using mAbs specific for integrin α5β1 (anti-β1 subunit mAb TS2/16) or ADAM17 (mAb 2A10) and analysis by confocal microscopy revealed a certain degree of colocalization between α5β1 and ADAM17 in both Colo320 and Colo320-CD9 cells, with Pearson's coefficients over 0.6 (not shown). However, a different pattern of integrin and ADAM17 distribution was observed depending on whether cells expressed or not CD9 on the cell surface **(Figure 4C),** displaying a dotty distribution in Colo320 cells and a more homogeneous appearance on the surface of Colo320-CD9 cells. Quantitation of the number of clusters of α5, β1 and ADAM17 molecules revealed that on cells expressing CD9 (Colo320-CD9) the number of clusters was significantly reduced, indicating that the presence of CD9 favors the formation of larger clusters containing both α5β1 and ADAM17 molecules (**Figure 4D**).

The association between α5β1 and ADAM17 has been reported to result in inhibition of the activity of both molecules. *Vice versa*, the induction of the activation of either integrin α5β1 or ADAM17 causes their dissociation from the complex (6, 13). We therefore postulated that the inhibitory effect on α5β1-mediated cell adhesion exerted by the presence of CD9 could be due to a reinforcement of the *cis* interaction between α5β1 and ADAM17 caused by this tetraspanin. If this was the case, the inhibition of α5β1-mediated adhesion by CD9 expression, should not be observed in the absence of ADAM17. To directly assess this hypothesis, ADAM17 expression was knocked-out using the CRISPR/Cas9 technique both in Colo320 and Colo320-CD9 cells **(Figure 5A)**. Interestingly, when ADAM17 was absent in Colo320-CD9 cells, these cells recovered their full capacity to adhere efficiently to both ADAM17-Fc and Fn through integrin α5β1 **(Figure 5B).** In addition, the distribution of α5β1 molecules was also restored showing again an organization in smaller and more dispersed clusters, similar in size to those on Colo320 cells **(Figure 5C**). Taken together, our results indicate that CD9 exerts an important control on both the distribution and the adhesive capacity of α5β1 integrin molecules.

However, clustering analysis by confocal microscopy provide insight only about the high-order organization and subcellular localization of molecules in the cell. To explore the organization α5β1 integrin and ADAM17 at the plasma membrane closer to the molecular level, we chose to use *in situ* Proximity Ligation Assays (PLA), that have been previously employed

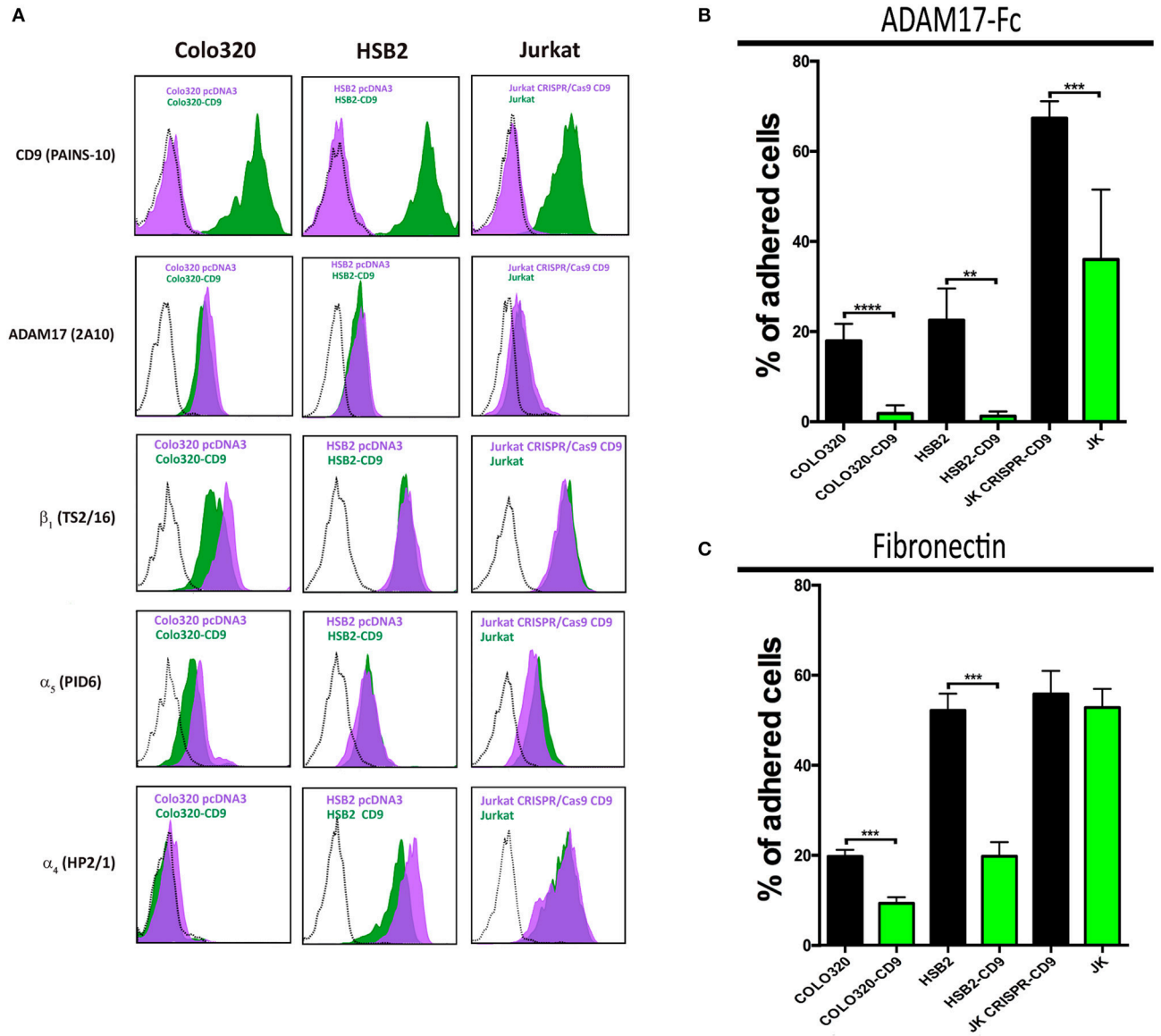

FIGURE 3 | Ectopic expression or knocking-out of CD9 regulates α5β1-mediated cell adhesion to ADAM17-Fc and Fn. Flow cytometric detection of cell surface CD9 (mAb PAINS-10), ADAM17 (mAb 2A10), β_1 (mAb TS2/16), α5 (mAb P1D6), and α4 (mAb HP2/1) on Colo320, HSB2 and Jurkat cells **(A)**. The black dotted line histograms correspond to negative controls, green filled histograms correspond to the expression of the indicated molecules on CD9 expressing cells (Colo320-CD9, HSB2-CD9, Jurkat) and purple filled histograms correspond to the expression of the indicated molecules on their respective CD9-lacking counterparts (Colo320, HSB2, Jurkat-CRISPR-CD9). The percentages of adhesion of paired variants of Colo320, HSB2 and Jurkat cells, either expressing CD9 (Colo320-CD9, HSB2-CD9, Jurkat; green bars) or lacking expression of this tetraspanin (Colo320, HSB2, Jurkat CRISPR-CD9; black bars) on plastic-immobilized ADAM17-Fc **(B)** or on fibronectin **(C)**. In all cases, cells were stimulated with PMA (200 ng/ml) for 2 h, loaded with the fluorescent probe BCECF-AM and then allowed to adhere to plastic-immobilized ligands ADAM17-Fc (20 μg/ml) or Fn (7.5 μg/ml) for 60 min at 37°C under conditions of integrin activation in the presence of Mn^{2+} (200 μM). Data are presented as the percentage of adhered cells (means ± SEM of three experiments each performed in triplicates). Statistical analysis was carried out using the two-tailed paired *T*-test. **, *** and **** denote $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively.

to demonstrate the cis interaction between ADAM17 and integrin α5β1 (6). PLA provide positive signals ("PLA fluorescent dots") only if the two proteins under analysis are in close proximity (typically <40 nm) that is compatible with a direct interaction between them. To quantitatively analyze the effect of CD9 expression on the α5β1/ADAM17 interactions, PLA were performed to detect the interactions between ADAM17 and α5β1 on both Colo320 and Colo320-CD9 cells. As shown in the images of **Figure 6A**, the number of PLA dots was clearly higher on Colo320-CD9 cells and a detailed quantitative analysis revealed a 5-fold increment in the number of dots/cell on CD9-positive cells (Colo320-CD9) compared to CD9-negative

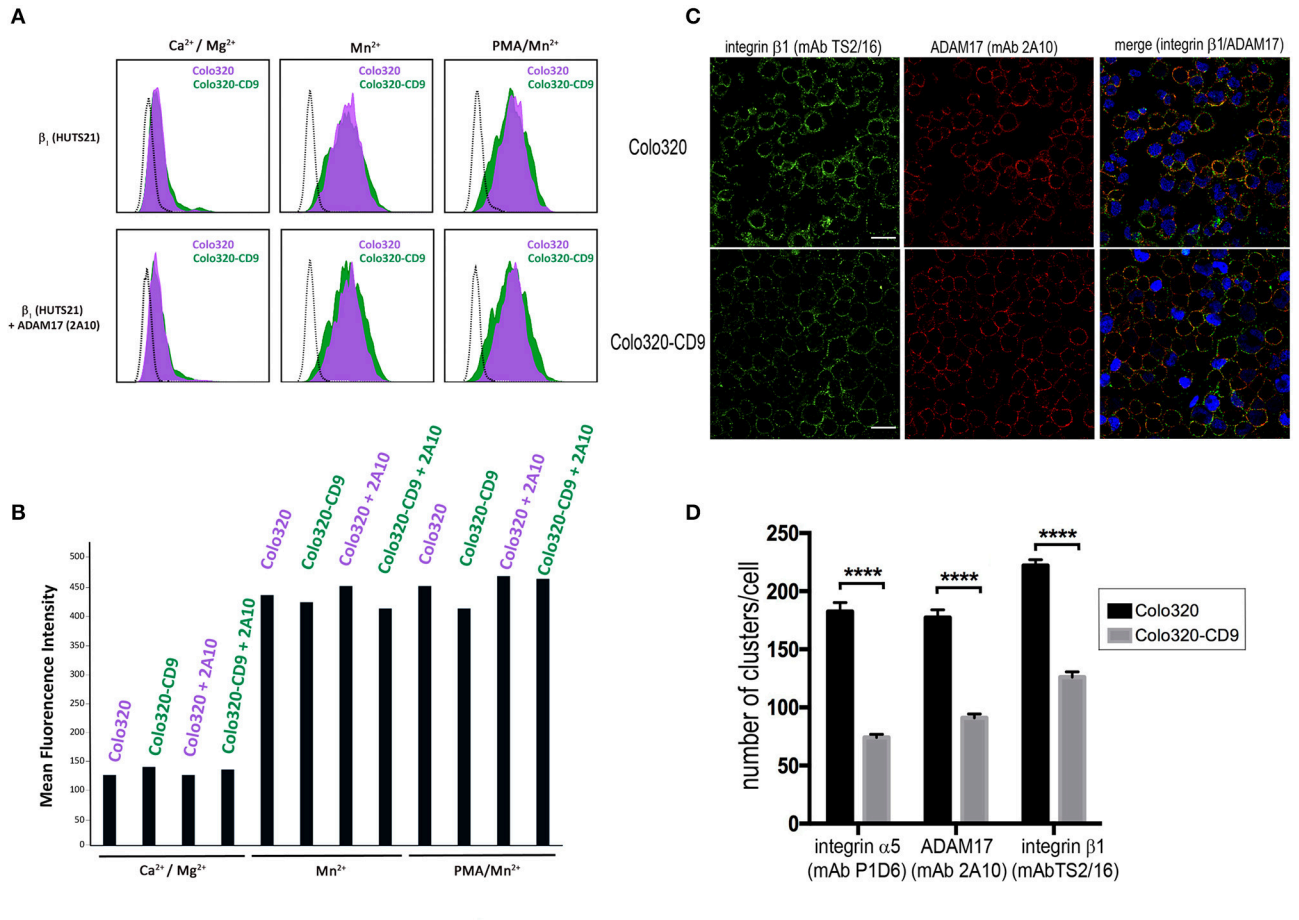

FIGURE 4 | CD9-induced inhibition of integrin α5β1-mediated cell adhesion does not involve alterations of integrin affinity. **(A)** Flow cytometric analysis of the expression of epitope HUTS21 in different integrin activation conditions: Ca^{2+}/Mg^{2+} (0.5 mM/1mM, respectively), Mn^{2+} (200 μM) or PMA/Mn^{2+} (200 ng/ml/200 μM, respectively) in Colo320 (purple-filled histograms) and Colo320-CD9 (green-filled histograms). The lower row of histograms represents the effect of cell pre-incubation with the mAb 2A10 on the expression of HUTS21 epitope in Colo320 and Colo320-CD9 cells. The black dotted line histograms correspond to negative controls. The fluorescence of 2,000 cells was acquired in the flow cytometer for each histogram. **(B)** Expression of HUTS21 epitope represented as the mean florescence intensities from the flow cytometry histograms shown in **(A)**. **(C)** Double immunofluorescence staining and confocal microscopy analysis showing the effects of CD9 expression on the organization of integrin α5β1 (green) and ADAM17 (red) molecules on the surface of Colo320 cells. Colo320 and Colo320-CD9 cells were stained with the anti-β1 (TS2/16) or anti-ADAM17 (2A10) mAbs. Representative images of confocal sections are shown. Scale bar = 20 μm. **(D)** Quantitation of the number of clusters/cell of integrin α5, β1, and ADAM17 molecules on the surface of Colo320 and Colo320-CD9. Graph shows the means ± SEM of the number of clusters/cell, calculated from at least 350 different cells for each condition using the Image-J thresholding and particle analyses.****denotes $p < 0.0001$ in a one-way ANOVA with Tukey's post-test.

cells (Colo320; **Figure 6B**). As a relevant negative control for these experiments, when ADAM17 was knocked-out in Colo320-CD9 cells (Colo320-CD9 CRISPR-ADAM17), no PLA dots were observed. These results provide strongly support for the *cis* α5β1-ADAM17 association being enhanced on the cell surface by the presence of CD9, probably through the formation of ternary complexes among these molecules (α5β1:CD9:ADAM17).

To biochemically confirm these data, we immunoprecipitated (IP) α5β1 integrin from Colo320 and Colo320-CD9 cells using mAb Lia1/2, which recognizes an extracellular epitope on the β1 subunit of the integrin, and analyzed by immunobloting the amount of co-immunoprecipitated ADAM17. Importantly, to detect only those interactions of α5β1 and ADAM17 taking place at the plasma membrane, we selectively precipitated only the subset of α5β1 molecules expressed on the surface of cells by incubating intact living cells with the anti-β1 mAb followed by washing the excess unbound antibody prior to cell lysis and IP. As shown in **Figure 7A**, mature ADAM17 (mADAM17) was significantly more efficiently co-immunoprecipitated with α5β1 from Colo320-CD9 than from Colo320 cells, evidencing that α5β1-ADAM17 association on the cell surface is enhanced by the presence of CD9.

In order to directly probe the presence of CD9, ADAM17 and α5β1 in TEMs, as well as their association, we selectively immunoprecipitated cell surface CD9 (TEM fraction) from

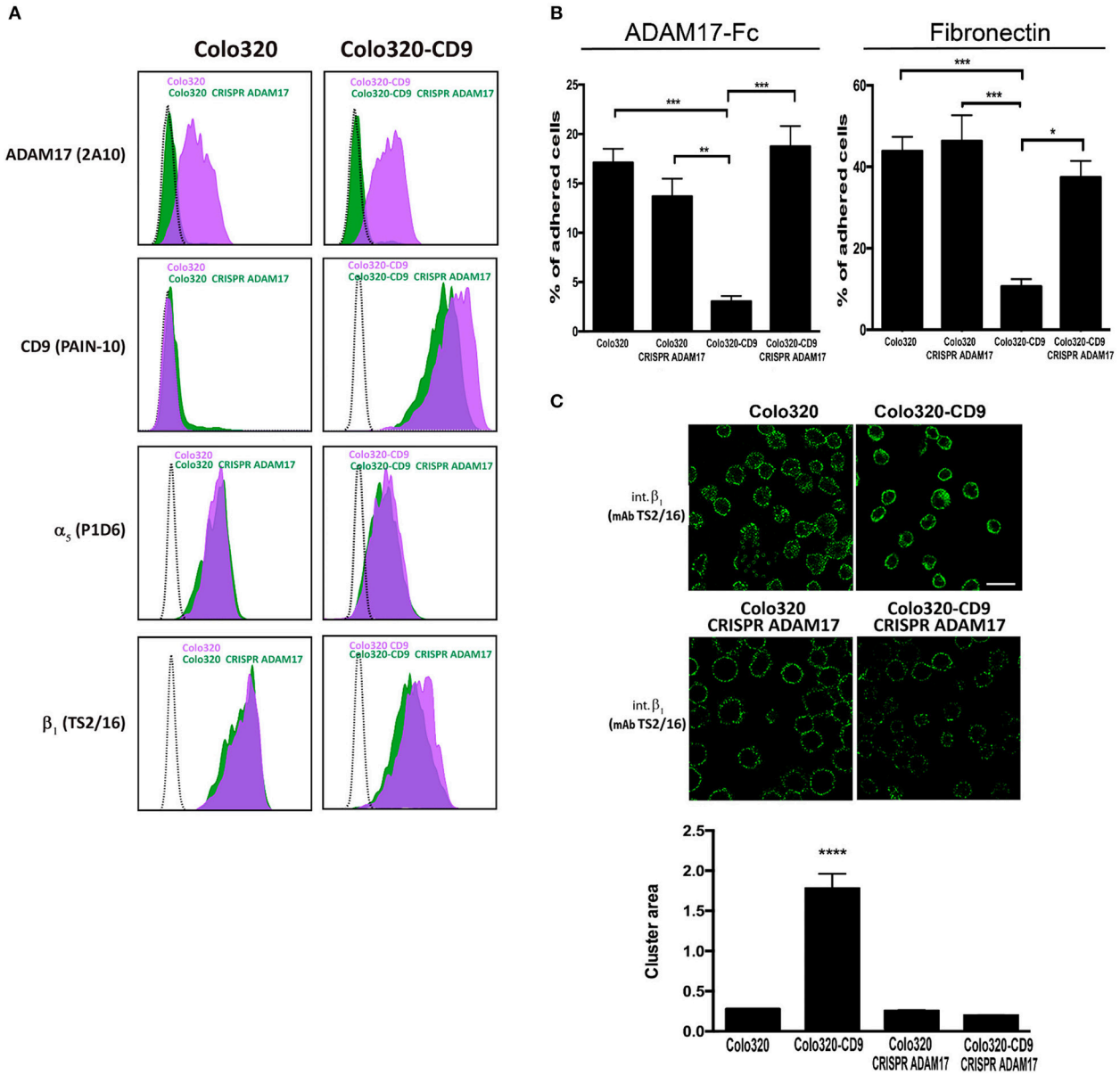

FIGURE 5 | Knocking out ADAM17 reverts the CD9-mediated abrogation of α5β1-dependent cell adhesion and the alteration of α5β1 distribution on the cell surface. **(A)** Flow cytometric detection of ADAM17 (mAb 2A10), CD9 (mAb PAINS-10), α5 (mAb P1D6) and β1 (mAb TS2/16) on the surface of Colo320 and Colo320-CD9 cells either expressing or lacking ADAM17 expression. The dotted black line histograms correspond to negative controls, green-filled histograms show the expression of the indicated molecules on ADAM17-lacking cells (Colo320 CRISPR ADAM17 and Colo320-CD9 CRISPR ADAM17) and purple-filled histograms show to the expression of the indicated molecules on Colo320 and Colo320-CD9 (ADAM17-positive cells). For each histogram the fluorescence signal of 2000 cells was acquired in the flow cytometer. **(B)** Cell adhesion of ADAM17-positive and ADAM17-negative Colo320 and Colo320-CD9 cells. Cells were stimulated with PMA (200 ng/ml) for 2 h, loaded with the fluorescent probe BCECF-AM and then allowed to adhere to immobilized ligands ADAM17-Fc (20 μg/ml) or Fn (7.5 μg/ml) for 60 min at 37°C under conditions of integrin activation in the presence of Mn^{2+} (200 μM). The percentage of cells that remained adhered is indicated as mean ± SEM. Statistical analysis performed was one-way ANOVA and Tukey's multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **(C)** Immunofluorescence staining and confocal microscopy analysis of $\alpha_5\beta_1$ integrin molecules organization on the surface of ADAM17-positive and ADAM17-negative Colo320 and Colo320-CD9 cells. α5β1 molecules were stained with the anti-β1 (TS2/16) mAb. Representative images of confocal microscopy sections are shown. Scale bar = 20 μm. Lower panel: Quantitation of the area of integrin $\alpha_5\beta_1$ clusters in square micrometers on the surface of the different types of cells. Graph depicts the means ± SEM of cluster areas calculated from 800-3000 different cells using the Image J software. ****denotes $p < 0.0001$ in a one-way ANOVA with Šidák's post-test.

intact Colo320-CD9 cells and detected the amount of co-immunoprecipitated α5β1 and ADAM17. **Figure 7B** shows that when IP of cell surface CD9 was performed in the presence of extracellular Ca^{2+} and Mg^{2+}, α5β1 and ADAM17 were efficiently co-immunoprecipitated. Interestingly, induction of conformational changes in α5β1 by the presence of Mn^{2+}

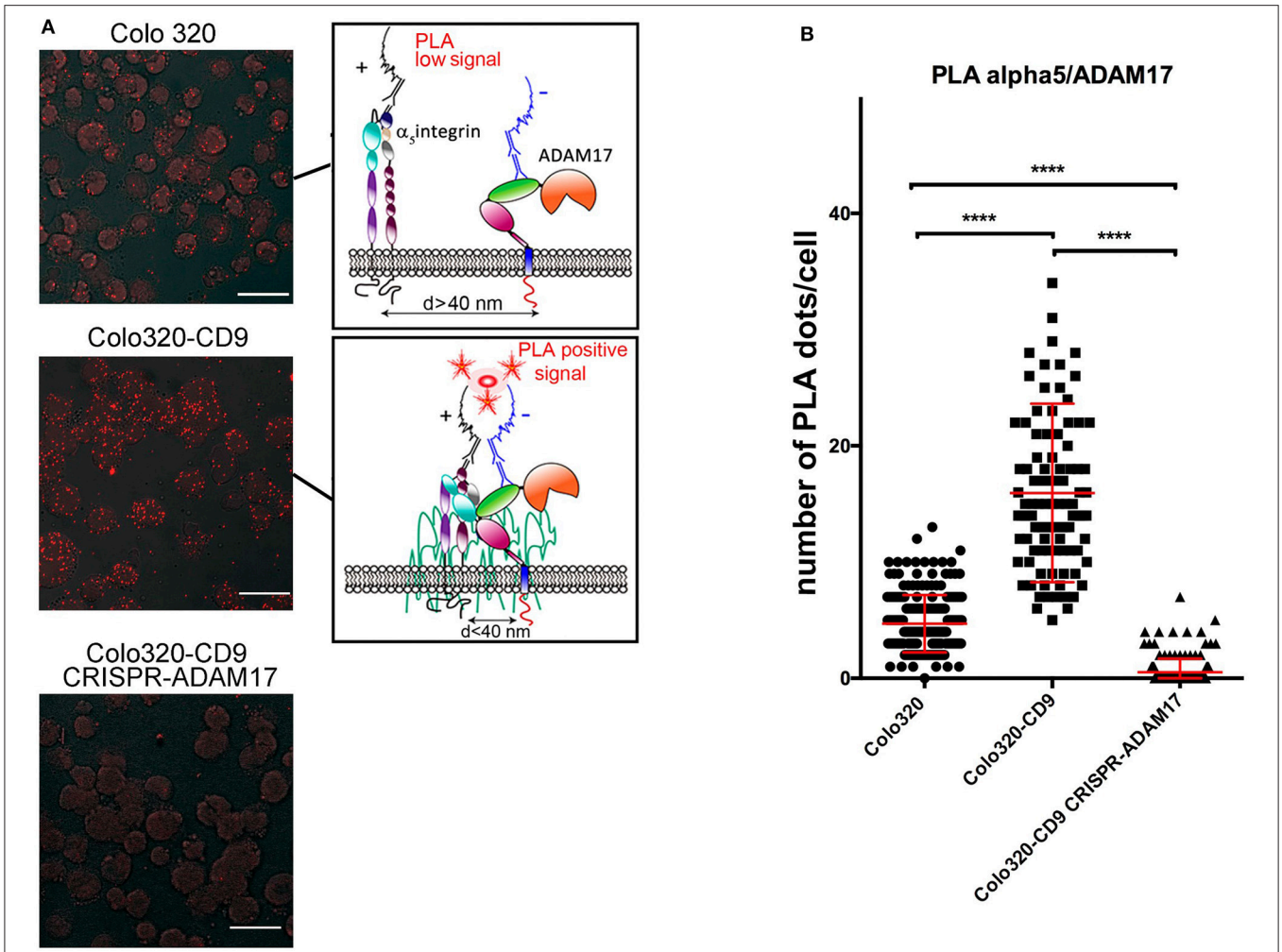

FIGURE 6 | Effects of the expression of CD9 on the *cis* interactions between integrin $\alpha_5\beta_1$ and ADAM17. **(A)** Analysis by *in situ* proximity ligation assays (PLA) of the molecular association of ADAM17 with integrin α5β1 on cells expressing CD9 (Colo320-CD9, middle image) or not (Colo320, top image). Cells expressing CD9 but lacking ADAM17 (Colo320-CD9/CRISPR-ADAM17, bottom image) were included as an internal negative control for ADAM17-α5 association. Scale bars = 20 μm. Sketches on the right summarize the principle of PLA. **(B)** The graph represents the mean ± SEM of the number of PLA dots/cell calculated from at least 100 cells (for each cell line) from different micrographic fields in two different experiments and analyzed by one-way ANOVA with Tukey's post-test analysis. ****$p < 0.0001$.

resulted in lower amounts of co-immunoprecipitated α5β1 and ADAM17 indicating that, in even in the presence of CD9, some degree of dissociation of these molecules takes place when the integrin conformation is altered.

DISCUSSION

Integrin α5β1, also named VLA5 or CD49e/CD29, is a major cellular receptor for the extracellular matrix protein fibronectin (Fn) [reviewed in (40)]. As such, this integrin is crucial for cell adhesion to the extracellular matrix and is also critically involved in many cell signaling and migration phenomena, with important implication in tumor invasion and progression (41, 42). Integrin α5β1 binds to its canonical ligand Fn through recognition of the Arg-Gly-Asp (RGD) motif in Fn-type-III module 10 as well as a synergy site in Fn-type-III module 9, contributing to the assembly of Fn molecules into fibrils (4, 43). In addition to binding to its canonical ligand Fn, integrin α5β1 has more recently been reported by different groups to bind specifically to the disintegrin domain of the transmembrane metalloproteinase ADAM17 (5–7, 13). These interactions of α5β1 with ADAM17 can occur between molecules expressed on the same cell (*cis* interactions) or on different cells (*trans* interactions). The α5β1-ADAM17 interactions have been investigated in some detail, both in *in vitro* cell free assays through employment of recombinant proteins (5, 6) and also in cellular assays which collectively confirmed that these interactions support intercellular adhesion, such as the one taking place between tumor and fibroblastic cells (7, 13). Interestingly, in those cell free studies it was observed that the direct interaction between ADAM17 and integrin α5β1 molecules resulted in inhibition of ADAM17 proteolytic activity (6). In cellular experiments with kidney mesangial cells it was established that β1 integrin silencing caused an increase in ADAM17

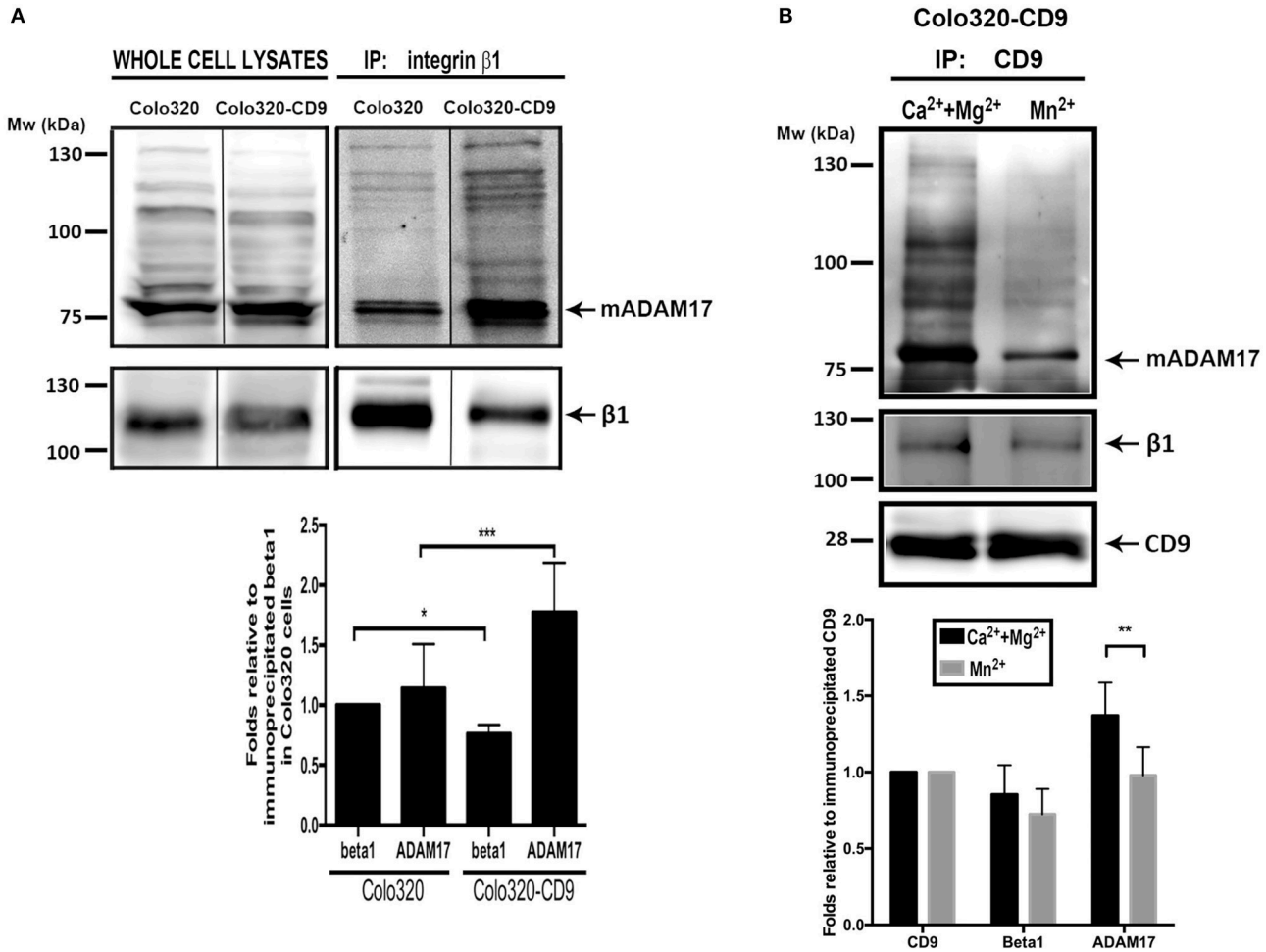

FIGURE 7 | Cell surface ADAM-17 is more efficiently co-immunoprecipitated with integrin α5β1 from Colo320-CD9 cells than from Colo320 cells. **(A)** Only cell surface integrin $\alpha_5\beta_1$ molecules were selectively immunoprecipitated by incubating the cells with mAb Lia1/2 (anti-β_1) in the presence of Ca^{2+}+Mg^{2+} (500 μM each) and washing the excess non-bound antibody prior to cell lysis and immunoprecipitation. Immunoprecipitated integrin $\alpha_5\beta_1$ and co-immunoprecipitated ADAM17 were detected by immunoblotting with the anti-β1 (TS2/16) and anti ADAM17 (A300D) mAbs, respectively. The gel shown is representative of five different experiments. The graph below shows the densitometric quantitation of the amount of precipitated integrin and co-immunoprecipitated ADAM17 (means ± SEM) from five different experiments, normalized to β_1 precipitated in Colo320 cells in each experiment. **(B)** Integrin α5β1 and ADAM17 are coimmunoprecipitated with CD9 from cell surface TEMs. CD9 was immunoprecipitated with mAb PAINS10 as described for integrin β1 in **(A)** but under two different extracellular cation conditions: in the presence of Ca^{2+}+Mg^{2+} (500 μM each) or Mn^{2+} (200 μM). Immunoprecipitated CD9 and co-immunoprecipitated β_1 and ADAM17 were detected by immunoblotting with mAbs PAINS10 (anti-CD9), TS2/16 (anti-β1), and A300D (anti ADAM17), respectively. The gel shown is representative of four different experiments. The graph below represents the densitometric quantitation of the amount of precipitated CD9 and co-immunoprecipitated integrin β_1 and ADAM17 (means ± SEM) normalized to the immunoprecipitated CD9 in each of the four independent experiments. Statistical analysis was carried out using two-tailed paired *T*-test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

sheddase activity whereas β1 integrin overexpression resulted in reduced ADAM17 activity (6), confirming the inhibitory role of integrin α5β1 on ADAM17 activity in living cells. Noteworthy, in these studies stimulation of integrin α5β1 with divalent cation Mn^{2+}, a potent activator of integrins which induces their extended and open headpiece high-affinity conformation, brought about the dissociation of the α5β1-ADAM17 complex with a concomitant increase in ADAM17 sheddase activity. Thus, it was concluded that it is the inactive form of the integrin α5β1 which becomes selectively engaged in direct interactions with ADAM17, thus keeping low the metalloproteinase activity of this enzyme.

While the functional consequences on ADAM17 shedding activity derived from its association with α5β1 have been investigated, no reports have addressed so far the effects of those interactions on integrin α5β1 adhesive activity. Thus, in the present study we have focused on the functional outcome brought about by treatment with mAb 2A10 (which is directed to the disintegrin domain of human ADAM17) or expression of the tetraspanin CD9, on the adhesive capacity the integrin

α5β1. We have demonstrated that treatment with mAb 2A10 or expression of CD9 on the cell surface specifically abrogates the α5β1-mediated adhesion of different types of tumor and leukocytic cells both to its canonical ligand Fn and also to its alternative ligand ADAM17-Fc, a recombinant protein which encompasses all the domains of the extracellular region of human ADAM17 (pro-, catalytic-, disintegrin- and membrane proximal-domain) fused to the Fc constant region of human IgG. Previous reports have shown that α5β1-dependent cell adhesion to its ligand ADAM17 is specifically supported by the disintegrin domain of this recombinant protein (5, 7, 13). The fact that mAb 2A10, specific for the disintegrin domain of ADAM17, not only inhibits the α5β1-mediated adhesion of Colo320 and K562 cells to ADAM17-Fc, but also to Fn, suggests that 2A10 mAb, similarly to CD9 expression, could enhance the *cis* ADAM17-α5β1 interactions on the cell surface.

Integrin adhesive capacity is regulated mainly by two alternative and often complementary mechanisms involving, on the one hand, alterations in the conformation of individual integrin molecules that are reflected by changes in affinity, and on the other hand, modifications in the aggregation and organization of integrin molecules which affect their multivalent avidity for ligands (2, 44, 45). Thus, one possibility to explain the inhibition of α5β1-mediated cell adhesion that is brought about by the expression of CD9 could be that this tetraspanin prevents the acquisition of the extended and open headpiece (i.e., the high affinity) conformation of this integrin. Our results showing that the expression of the HUTS21 epitope is not affected by the presence of CD9 under any of the stimulation conditions tested seem to rule out this possibility. An alternative possibility to account for the observed inhibition of α5β1-mediated cell adhesion is that expression of CD9 produces changes in the organization of integrin molecules on the cell surface that would account for a reduction in integrin avidity. Our confocal microscopy analyses of immunofluorescently-stained integrin α5β1 and ADAM17 show that the pattern of distribution of this integrin is affected both by the expression of CD9 and of ADAM17. Thus, on CD9-negative Colo320 cells integrin α5β1 and ADAM17 molecules are distributed in a high number of small discrete clusters, while upon CD9-expression the localization is somewhat more continuous and the number of clusters per individual cell is reduced. Furthermore, when ADAM17 was knocked-out in Colo320-CD9, α5β1 regained the dotted pattern, correlating with a recovery of the adhesion capacity. However, although interesting, the analyses of the overall distribution of α5β1 and ADAM17 at the plasma membrane by confocal microscopy are limited by the resolution limit of the technique and thus, provide limited insight of the detailed molecular environment of each receptor.

Using a combination of different approaches, including *in situ* proximity ligation assays (PLA), immunofluorescence staining followed by confocal microscopy and biochemical co-immunoprecipitation experiments, we have established that tetraspanin CD9 inhibits α5β1-mediated cell adhesion by reinforcing the *cis* association between ADAM17 and integrin α5β1 on the cell surface. *In situ* PLA have been previously employed by Gooz et al. to confirm and quantitatively assess the strength of the interaction between ADAM17 and integrin α5β1 (6). This technique provides positive signals only when two molecules are in close proximity, typically <40 nm, implying a direct molecular interaction. We have employed PLA to quantitatively analyze the effect of CD9 expression on the association between α5β1 and ADAM17 and our results clearly show that the number of PLA fluorescent dots was significantly higher in CD9-positive than in CD9-negative cells, indicating that the *cis* α5β1-ADAM17 association on the cell surface is enhanced by the presence of CD9. Interestingly, when expression of ADAM17 is knocked-out, CD9 is no longer capable to abrogate integrin α5β1-mediated cell adhesion, strongly pointing out to the formation of trimolecular α5β1:CD9:ADAM17 complexes as the mechanism behind the regulation of both the adhesive activity of α5β1 and the shedding function of ADAM17 by tetraspanin CD9.

Gooz et al. also performed co-immunoprecipitation experiments to confirm the association of integrin α5β1 and ADAM17 (6) and showed that stimulation with extracellular manganese ions (Mn^{2+}), a potent inducer of integrin activation (2, 31, 44, 46), decreased the association of these two molecules. We performed co-immunoprecipitation experiments to assess both the influence of CD9 expression and that of integrin activation (under different divalent cations conditions) on the association of α5β1 with ADAM17 molecules selectively on the cell surface. Our immunoprecipitation data clearly show that expression of CD9 greatly enhances the association between α5β1 and ADAM17. Upon integrin activation with Mn^{2+} the amount of α5β1 and ADAM17 that co-immunoprecipitates with CD9 is reduced, but not completely abolished. These biochemical results, together with the PLA data, confirm that expression of CD9 reinforces *cis* interactions of α5β1 and ADAM17 on the cell surface highlighting a dominant role for tetraspanin CD9 in regulating the adhesive activity of integrin α5β1 through a reinforcement of the α5β1-ADAM17 association on the cell surface.

AUTHOR CONTRIBUTIONS

YM-P and BC performed a large part of experimental work, contributed to the analysis and interpretation of data and made the figures. RR, SL-M, VT, HS and PS-O performed experimental work, analyzed data and discussed results. JG and IL contributed new reagents and aided in interpretation of results. MY-M contributed to design research, analysis of data and interpretation of results. CC planned research, analyzed and interpreted data, discussed results, contributed to the design and final form of figures and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02474/full#supplementary-material

Supplementary Figure 1 | The adhesion of HSB2 and Jurkat lymphocytic cell lines to plastic-immobilized ICAM-1-Fc is not affected by the presence of mAb 2A10. Cell adhesion to ICAM-Fc is mediated specifically by the integrin LFA-1 (αLβ2), which is only expressed on some leukocytic cells, including HSB2 and Jurkat cells, but not on K562 or Colo320 cells. In all cases, cells were stimulated with PMA (200 ng/ml) for 2 h, loaded with the fluorescent probe BCECF-AM and then allowed to adhere to plastic-immobilized ligand ICAM1-Fc (20 μg/ml) for 60 min at 37°C in the presence of Mn^{2+} (200 μM). Data show the percentage of adhered cells (means ± SEM of three experiments, performed in triplicates). mAb 2A10 did not exert any statistically significant effect on the LFA-1 mediated cell adhesion to ICAM1-Fc for any of the cell lines as analyzed by two-tailed paired *T*-tests.

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Tetraspanin CD9 Limits Mucosal Healing in Experimental Colitis

*María Laura Saiz[1,2], Danay Cibrian[1,2,3], Marta Ramírez-Huesca[2], Daniel Torralba[1,2], Olga Moreno-Gonzalo[1,2] and Francisco Sánchez-Madrid[1,2,3]**

[1] Immunology Service, Hospital de la Princesa, Universidad Autónoma de Madrid, Instituto de Investigación Sanitaria del Hospital Universitario de La Princesa, Madrid, Spain, [2] Department of Vascular Biology and Inflammation, Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain, [3] CIBER Cardiovascular, Madrid, Spain

Tetraspanins are a family of proteins with four transmembrane domains that associate between themselves and cluster with other partner proteins, conforming a distinct class of membrane domains, the tetraspanin-enriched microdomains (TEMs). These TEMs constitute macromolecular signaling platforms that regulate key processes in several cellular settings controlling signaling thresholds and avidity of receptors. In this study, we investigated the role of CD9, a tetraspanin that regulates major biological processes such as cell migration and immunological responses, in two mouse models of colitis that have been used to study the pathogenesis of inflammatory bowel disease (IBD). Previous *in vitro* studies revealed an important role in the interaction of leukocytes with inflamed endothelium, but *in vivo* evidence of the involvement of CD9 in inflammatory diseases is scarce. Here, we studied the role of CD9 in the pathogenesis of colitis *in vivo*. Colitis was induced by administration of dextran sodium sulfate (DSS), a chemical colitogen that causes epithelial disruption and intestinal inflammation. $CD9^{-/-}$ mice showed less severe colitis than wild-type counterparts upon exposure to DSS (2% solution) and enhanced survival in response to a lethal DSS dose (4%). Decreased neutrophil and macrophage cell infiltration was observed in colonic tissue from $CD9^{-/-}$ animals, in accordance with their lower serum levels of TNF-α, IL-6, and other proinflammatory cytokines in the colon. The specific role of CD9 in IBD was further dissected by transfer of $CD4^{+}$ $CD45RB^{hi}$ naive T cells into the $Rag1^{-/-}$ mouse colitis model. However, no significant differences were observed in these settings between both groups, ruling out a role for CD9 in IBD in the lymphoid compartment. Experiments with bone marrow chimeras revealed that CD9 in the non-hematopoietic compartment is involved in colon injury and limits the proliferation of epithelial cells. Our data indicate that CD9 in non-hematopoietic cells plays an important role in colitis by limiting epithelial cell proliferation. Future strategies to repress CD9 expression may be of therapeutic benefit in the treatment of IBD.

Keywords: tetraspanins, CD9, mucosal healing, dextran sodium sulfate, colitis

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***Correspondence:**
Francisco Sánchez-Madrid
fsmadrid@salud.madrid.org

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INTRODUCTION

Inflammatory bowel disease (IBD) defines a group of intestinal disorders, principally, ulcerative colitis (UC) and Crohn's disease (CD). Both diseases are characterized by chronic inflammation of the gastrointestinal tract interspersed with relapsing phases (1). Much progress has been made in understanding UC and CD disease mechanisms, for example, through genome-wide association

studies in patients; however, these diseases remain incompletely understood. Identified genetic risk loci have revealed defects in IBD patients affecting genes crucial for intestinal homeostasis, including epithelial barrier function, restitution, and wounding (2). Moreover, recent clinical studies have revealed mucosal healing (MH) as the major prognostic predictor of long-term remission in IBD patients (3, 4), suggesting that epithelial regeneration is critical to improving IBD therapy (5).

Tetraspanins are proteins that span the cell membrane four times and play an important role in plasma membrane organization through the formation of tetraspanin-enriched microdomains, which enable them to associate with multiple proteins, including other tetraspanins (6). The tetraspanin CD9 is broadly expressed on the surface of several cell types, including many malignant tumor cells, as well as normal hematopoietic, endothelial, and epithelial cells (7, 8). Soon after its identification, CD9 was found to associate with several integrins (9), enabling CD9 to exert pro- or anti-migratory effects (10). CD9 can also interact with the immunoglobulin superfamily members EWI-2 and EWI-F (11), DDR1 (12), other tetraspanins (e.g., CD81 and CD151) (13), claudin-1 (14), ADAM10 (15), and ADAM17 (16) metalloproteases, epidermal growth factor receptor (EGFR) (17), and membrane-bound EGFR ligands (18, 19). Moreover, CD9 has been reported to regulate endothelial nanoscopic organization and expression levels of ICAM-1 and VCAM-1 upon TNF-α activation, enabling formation of the docking structure required for leukocyte extravasation (20, 21). Anti-CD9 agonistic antibodies or ectopic expression of CD9 both exert an antiproliferative effect on human colon carcinoma cell lines (22). However, the role of CD9 in IBD has not been previously addressed *in vivo*. Here, we show that CD9 acts as a limiting factor for epithelial regeneration and colonic MH in dextran sodium sulfate (DSS)-induced colitis.

MATERIALS AND METHODS

Mice

Experiments were performed with sex and age matched (8- to 12-week old) $CD9^{-/-}$ and WT mice on the C57BL/6 background. $CD9^{-/-}$ mice have been described previously (23). $Rag1^{-/-}$ mice (24) used in the adoptive transfer colitis model were kindly provided by Dr. J. M. Fernández-Granado (CNIC). For chimeric reconstitution experiments, B6SJL CD45.1 mice (Jackson Laboratories) were used. All animals were housed in pathogen-free conditions at the CNIC animal facility. Experimental procedures were approved by the local research ethics committee and conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC, enforced in Spanish law under Real Decreto 53/2013.

Induction and Assessment of DSS-Induced Colitis

Dextran sulfate sodium salt (DSS, MP Biomedicals; $M_W = 36{,}000$–$50{,}000$) was dissolved at 2 or 4% (w/v) in sterile drinking water provided to mice *ad libitum*. Mice were checked daily for development of colitis by monitoring body weight, fecal occult blood (Hemoccult II Sensa; Beckman Coulter) or gross rectal bleeding, and stool consistency. Overall disease severity was assessed by a clinical scoring system defined as follows: weight loss: 0 (no loss), 1 (1–5%), 2 (5–10%), 3 (10–20%), and 4 (>20%); stool consistency: 0 (normal), 2 (loose stool), and 4 (diarrhea); and bleeding: 0 (no blood), 1 (Hemoccult positive), 2 (Hemoccult positive and visual pellet bleeding), and 4 (gross bleeding, blood around anus). At the end of the experiment, tissues were fixed in 10% neutral buffered formalin (Bio Optica) for 24 h and transferred to 70% ethanol. After embedding in paraffin, transverse sections (4–5 μm) of proximal and distal colon were stained with H&E for histological studies. Images were digitized using Hamamatsu Nanozoomer 2.0 RS scan and NDP.scan 2.5 digitization software. Three images of two serial sections cut at a separation of 100 μm (six sections in total) were evaluated for each mouse for each part of the colon (proximal and distal). Histological scoring evaluated inflammation severity, crypt damage, and ulceration. Inflammation severity was scored as follows: 0, rare inflammatory cells in the lamina propria; 1, increased numbers of granulocytes in the lamina propria; 2, confluence of inflammatory cells extending into the submucosa; 3, transmural extension of the inflammatory infiltrate. Crypt damage was scored as follows: 0, intact crypts; 1, loss of the basal one-third; 2, loss of the basal two-thirds; 3, entire crypt loss; 4, change of epithelial surface with erosion; 5, confluent erosion. Ulceration was scored as follows: 0, absence of ulcers; 1, 1–2 ulceration foci; 2, 3–4 ulceration foci; 3, confluent or extensive ulceration. Scores for each parameter were summed to give a maximum histological score of 11.

T Cell-Mediated Colitis

Naive $CD4^+$ T cells were sorted (FACSaria sorter, BD) from single-cell spleen suspensions of $CD9^{-/-}$ or WT mice. Live cells were isolated after labeling with antibodies to CD4, CD62, CD25, and CD45RB (eBiosciences) and hoescht 33258. Cells were transferred to recipient mice ($4–5 \times 10^5$ cells per mouse) by intraperitoneal injection.

Bone Marrow Chimeras

Bone marrow transfer was used to create chimeric mice in which genetic deficiency for CD9 was confined to either circulating cells ($CD9^{-/-}$ > WT) or nonhematopoietic tissue (WT > $CD9^{-/-}$). Briefly, bone marrows were collected from femur and tibia of congenic WT donor mice (expressing CD45.1 leukocyte antigen) or $CD9^{-/-}$ and WT donor mice (expressing CD45.2 leukocyte antigen) by flushing with PBS. Erythrocytes were lysed (ACK lysis buffer, Lonza) for 1 min on ice. After a washing step, cells were resuspended in PBS at 1×10^8/ml. This cell suspension (100 μl) was injected intravenously into 13 Gy-irradiated recipient mice 48 h postirradiation. Four chimera groups were generated: WT > WT (WT cells expressing CD45.1 into WT mice expressing CD45.2); WT > $CD9^{-/-}$ (WT cells expressing CD45.1 into $CD9^{-/-}$ mice expressing CD45.2); WT > WT (WT cells expressing CD45.2 into WT mice expressing CD45.1); $CD9^{-/-}$ > WT ($CD9^{-/-}$ cells expressing CD45.2 into WT mice expressing CD45.1). Bone marrow reconstitution was verified after 8 weeks by staining for CD45.1 or CD45.2 in blood cells with anti-CD45.1 or anti-CD45.2 specific antibodies (BD Biosciences).

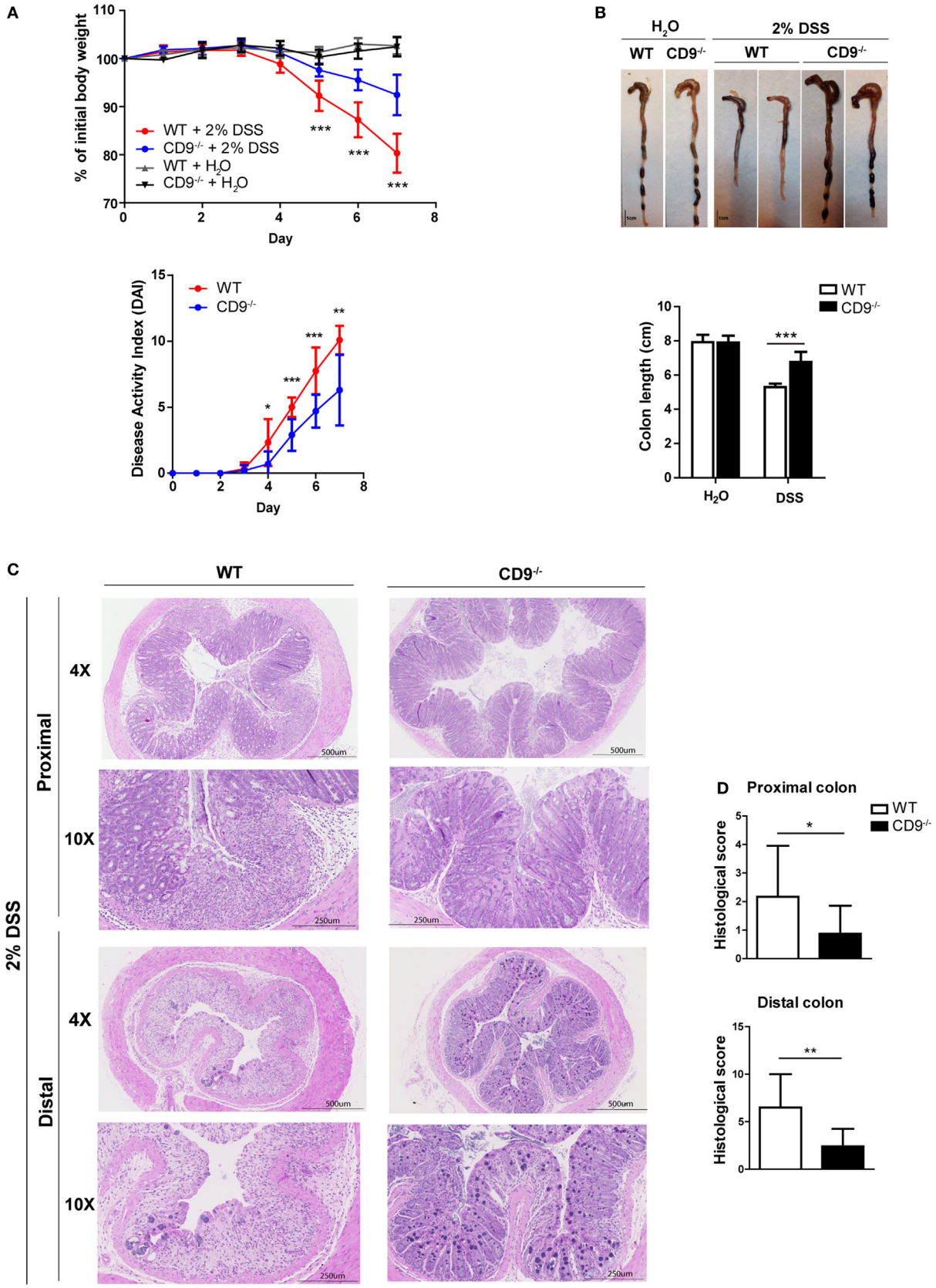

FIGURE 1 | Continued

FIGURE 1 | CD9-deficiency reduces sensitivity to dextran sodium sulfate (DSS)-induced colitis. **(A)** *Top,* body-weight loss in WT and CD9$^{-/-}$ mice after administration of 2% DSS in drinking water for 7 days. Controls for each genotype were administered with unadulterated drinking water. *Bottom*, disease activity index (DAI) score in WT and CD9$^{-/-}$ mice after administration of 2% DSS for 7 days. n = 10–12 per group; $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$, unpaired t-test. **(B)** Macroscopic colon damage in DSS-treated WT and CD9$^{-/-}$ mice. *Top*, Colon shrinkage. *Bottom*, changes in colon length. Representative colons are shown of n = 10–12 mice per group. **(C)** Representative photomicrographs of proximal colon (near the cecum) and distal colon (near the anus) from WT and CD9$^{-/-}$ mice at day 7 of DSS administration (H&E; magnifications: 4× and 10×). **(D)** Histological scores obtained from H&E-stained proximal and distal colon tissue sections from DSS-treated WT and CD9$^{-/-}$ mice. Data are pooled from two independent experiments (n = 4). Values represent mean ± SD of the mean: $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$, unpaired t-test.

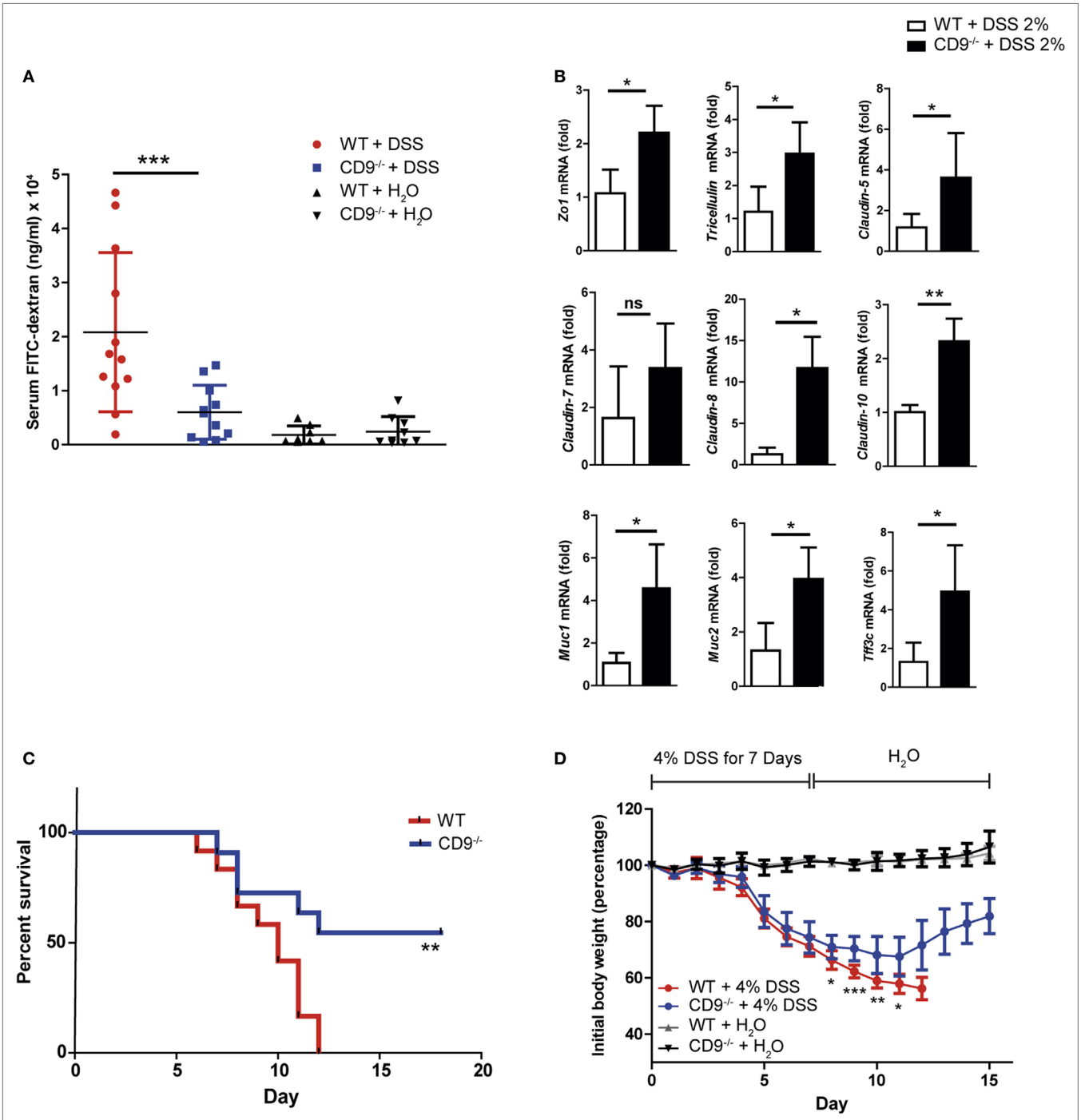

FIGURE 2 | Continued

FIGURE 2 | Enhanced epithelial barrier integrity and survival in CD9$^{-/-}$ mice after dextran sodium sulfate (DSS) challenge **(A)** *In vivo* colon permeability, indexed from the serum level of 4 kDa FITC-dextran 4 h after feeding by gavage. Data are pooled from two independent experiments, $n = 5$–6 animals per group. Data were analyzed by one-way ANOVA and the Newman–Keuls multiple comparison test; $^{***}P < 0.001$. **(B)** qPCR analysis of tight junction and mucin gene expression in colon samples after 7 days of DSS exposure. Data are from one experiment repeated two times with similar results. **(C)** Kaplan–Meier survival for WT and CD9$^{-/-}$ mice given 4% DSS in drinking water. $^{**}P < 0.01$, Log-rank (Mantel–Cox) test. **(D)** Percentage of initial body weight of WT and CD9$^{-/-}$ mice after 7-day intake of 4% DSS chased by unadulterated water. $n = 11$–12 per group: $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$; unpaired *t*-test for WT and CD9$^{-/-}$ groups in **(B,C)**.

In Vivo Permeability Assay

Food was withdrawn overnight and mice were gavaged with the permeability tracer FITC-dextran (MW 4,000; Sigma-Aldrich) at 60 mg/100 g body weight. After 4 h, blood was collected by heart puncture and serum FITC-dextran was measured with a fluorescence spectrophotometer (Fluoroskan Ascent; Thermo Labsystems) using emission and excitation wavelengths of 490 and 520 nm, respectively. FITC-dextran concentration was determined from a standard curve generated by serialdilution.

Isolation and Flow Cytometry Analysis of Colonic Leukocytes

Colons were dissected longitudinally, washed several times with PBS to remove feces, and cut into small pieces. Samples were digested with 0.25 mg/ml Liberase TM (Roche), 50 µg/ml DNAseI (Roche), and 1 mM DTT diluted in Hank's Balanced Solution for 30 min at 37°C. At the end of the incubation period, enzyme activity was blocked by adding 50 ml PBS supplemented with 0.5% BSA and 0.05 mM EDTA (PBS–BSA–EDTA), and the sample was mechanically disrupted by passing through a 70-µm cell strainer to obtain a cell suspension. When only epithelial cells were required, samples were incubated in 5 mM EDTA, 1 mM DTT for 20 min before enzyme digestion. Before all staining procedures, colon cell suspensions were incubated with anti-mouse FcRII/III (clone 2.4G2, TONBO Biosciences) for 10 min at 4°C in PBS-BSA-EDTA. Flow cytometry analysis of DSS-induced inflammation was performed with anti-mouse antibodies to the following antigens: CD45 (BD Horizon) and CD11b, CD64, and Ly6G (BD Pharmingen). For epithelial cell proliferation analysis, antibodies were used targeting EpCAM (Biolegend) and Ki67 (BD Pharmingen). Absolute cell numbers were obtained using TruCount Tubes (BD Biosciences). Cell samples were acquired in a FACSCanto Flow Cytometer (BD Biosciences), and the data were analyzed with FlowJo (Tree Star) or FACSDiva (BD Biosciences) software.

Flow Cytometric Bead Array (CBA)

Serum TNF-α, IL-6, and IFNγ were determined using the mouse Th1/Th2/Th17 BD CBA.

RNA Extraction and Real-time Quantitative PCR

RNA was isolated by disrupting colon tissue samples with TRIzol Reagent (1 ml per 50–100 mg tissue, Qiagen) and homogenizing in a tissue disruptor (Ika ultra-turrax T10 homogenizer). DSS traces were removed by the LiCl method (Ambion). Residual DNA contamination was eliminated with the Turbo DNA-free Kit (Ambion). Total RNA (1 µg) was reverse transcribed to cDNA with a Reverse Trancription Kit (Applied Biosystems). Quantitative PCR was then performed in an AB7900_384 (Applied Biosystems) using SYBR Green (Applied Biosystems) as the reporter. Gene-specific primers used are listed in Table S1 in Supplementary Material. Expression of each gene of interest was normalized to housekeeping gene GAPDH. Data are presented as relative fold differences calculated by the $2^{-\Delta\Delta Ct}$ method.

In Vitro T Cell Differentiation

Naive CD4$^+$ T cells were obtained by incubating single-cell suspensions of spleen and lymph nodes with biotinylated antibodies to CD8, CD16, CD19, F4/80, Gr-1, MHC class II (I-Ab), CD11b, CD11c, and DX5 followed by incubation with Streptavidin Microbeads (MACS, Miltenyi Biotec). CD4$^+$ T cells were isolated by negative selection in an auto-MACSTM Pro Separator (Miltenyi Biotec). Next, cells were activated with plate-bound anti-CD3 (5 µg/ml) and anti-CD28 (2 µg/ml) in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FCS, 2×10^{-3} M L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol, and the corresponding cytokine cocktail: for Th0, anti-IFNγ (4 µg/ml), anti-IL-4 (4 µg/ml), and IL-2 (10 ng/ml); for Th1 anti-IL-4 (4 µg/ml), IL-12 (10 ng/ml), and IL-2 (10 ng/ml); for Th17 anti-IFNγ (4 µg/ml), anti-IL-4 (4 µg/ml), IL-6 (20 ng/ml), IL-23 (10 ng/ml), and TGF-β1 (5 ng/ml); and for Treg anti-IFNγ (4 µg/ml), anti-IL-4 (4 µg/ml), and TGF-β1 (10 ng/ml). After 72 h of culture, IFNγ, IL-17, or IL-10 in the supernatant were measured by ELISA (Ready-SET-Go, eBiosciences). For FACS analysis, intracellular cytokine staining was preceded by restimulation for 4 h with 50 ng/ml phorbol dibutyrate (PMA) and 500 ng/ml ionomycin in the presence of brefeldin A (1 µg/ml) (BD Biosciences).

Immunofluorescence and Immunohistochemical Analysis

For IF and IHC staining, colon sections were deparaffinized, boiled in antigen retrieval solution (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0 for Ki67 and 10 mM sodium citrate, 0.05% Tween 20, pH = 6 for caspase-3), and incubated with the rabbit monoclonal anti-mouse Ki67 primary antibody (Master Diagnostica, clon SP6) for IF or anti active caspase-3 rabbit polyclonal antibody for IHC (R&D system, catalog AF835). Bound antibodies were detected with a goat anti-rabbit 647 (ThermoFischer Scientific) for IF or the anti-rabbit EnVision FLEX-HRP detection system (Agilent) for IHC. Staining was developed with DAB substrate (Dako K3468), and slides were counterstained with Mayers Hematoxylin. Ki67 and

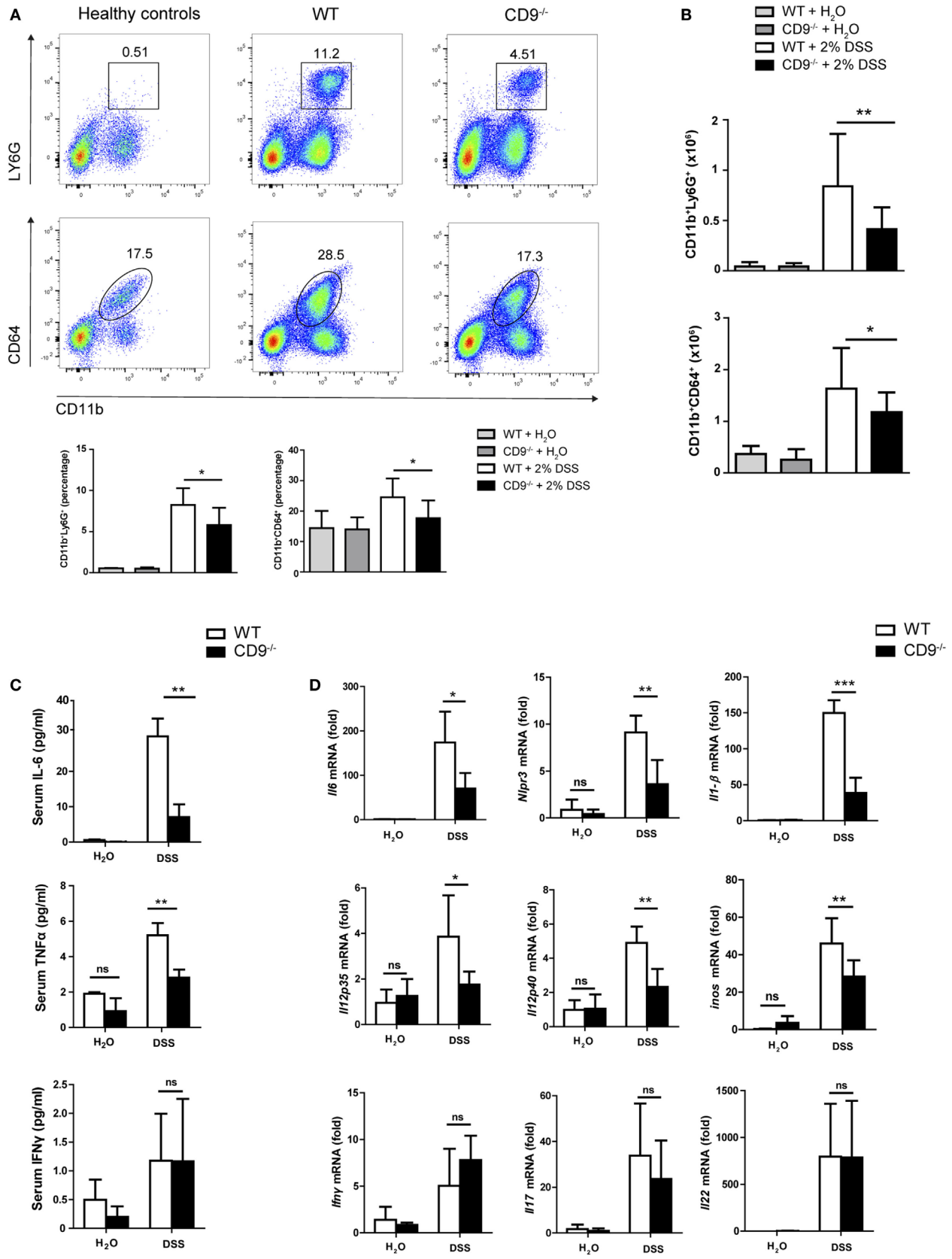

FIGURE 3 | Continued

FIGURE 3 | CD9$^{-/-}$ mice exhibit lower leukocyte infiltration and proinflammatory cytokines in serum and colon after 2% dextran sodium sulfate (DSS) administration. **(A)** Flow cytometry analysis of whole colon from WT and CD9$^{-/-}$ mice after 7-day 2% DSS intake. Representative dot plots and percentage quantification of CD45^{+} populations show similar DSS-induced infiltration by neutrophils (Ly6G^{+}) and macrophages (CD64^{+}) in both genotypes. **(B)** Total neutrophil and macrophage numbers in the CD45^{+}-gated population, determined by TruCount Tubes. Data are pooled from two independent experiments. For **(A,B)**, n = 6–7 mice per group with two repeats and analysis by one-way ANOVA and the Newman–Keuls multiple comparison test. *P < 0.05; **P < 0.005; ***P < 0.001. **(C)** Serum levels of TNF-α, IL-6, and IFNγ measured by cytometric bead array assay. **(D)** qPCR analysis of colonic proinflammatory cytokine mRNA expression. Bars denote the mean ± SD of n = 4–5 mice per genotype. Data from **(C,D)** were analyzed by two-way ANOVA and the Bonferroni multiple comparison test.

active caspase-3 staining in epithelial cells were quantified in the whole colon sections from each DSS-treated mouse (4–5 mice per group). Image J (1.46r) was used to measure Ki67 positive individual nuclei and to measure caspase-3 intensity relative to the total area corresponding to the complete epithelial layer in each image.

Statistical Analysis

Data are presented as mean ± SD. Normal data distribution was assessed with the Kolmogorov Smirnov test, and the statistical significance of between-group differences was assessed by one-tailed unpaired Student's *t*-test or one-way ANOVA with Newman–Keuls multiple comparison *t*-test, as required. All statistical analyses were performed with GraphPad Prism (GraphPad Software Inc.).

RESULTS

CD9$^{-/-}$ Mice Are Protected against DSS-Induced Colonic Injury

To explore the function of CD9 in colitis development, we challenged CD9$^{-/-}$ and WT mice with the toxic compound DSS (2% solution) in drinking water for 7 days. CD9$^{-/-}$ animals lost less than 10% of their initial body weight, whereas WT counterparts lost around 20% (**Figure 1A**, top). To monitor disease activity, we recorded a daily disease activity index (DAI) combining weight loss, stool consistency, and bleeding. From day 4, DAI values were lower in CD9$^{-/-}$ mice than in WT counterparts (**Figure 1A**, bottom). Autopsy revealed that DSS-treated CD9$^{-/-}$ mice had significantly larger colons than WT counterparts (**Figure 1B**). Histology revealed a better preservation of tissue architecture in CD9$^{-/-}$ mice, in both the proximal and the distal colon. DSS-treated WT animals showed more pronounced epithelial denudation, crypt distortion, leukocyte infiltration of the lamina propria, and submucosal swelling (**Figure 1C**). Histological sections were scored for the severity of DSS-induced inflammation as described in Section "Materials and Methods." In both proximal and distal colon, histological scores were lower in CD9$^{-/-}$ mice than in WT mice, with the difference more pronounced in the distal colon (**Figure 1D**).

CD9 Exacerbates Tissue Injury and Decreases Mouse Survival after a Lethal DSS Dose

Intestinal epithelial integrity is necessary for efficient defense against intraluminal toxins, antigens, and enteric bacteria. Cells are tightly joined in a healthy epithelium, and transepithelial permeability can thus be determined as an index of epithelial integrity. To monitor gut barrier function *in vivo*, we treated CD9$^{-/-}$ and WT animals with 2% DSS for 7 days and then orally administered 4KDa FITC-Dextran. Fluorescence spectrophotometry detection of serum FITC after 4 h revealed markedly lower gastrointestinal permeability in CD9$^{-/-}$ mice than in WT mice (**Figure 2A**). Serum FITC levels in non-treated animals showed no significant between-genotype differences and remained below 5 μg/ml, consistent with an intact intestinal barrier function in the steady state (**Figure 2A**). Consistent with the FITC-Dextran data, qPCR of colon samples from DSS-treated CD9$^{-/-}$ mice revealed elevated expression of genes encoding epithelial tight junction proteins, such as ZO-1, tricellulin, and claudin family members (**Figure 2B**). CD9$^{-/-}$ colon also showed elevated expression of genes encoding epithelial globet cell proteins, such as the secretory mucin glycoproteins MUC1, MUC2, and trefoil factor 3, indicating normal intestinal function (**Figure 2B**). In a further approach, we exposed mice to a lethal DSS dose (4%) for 7 days followed by unadulterated drinking water for a further 8 days. At the end of the experiment all WT mice had died, whereas only 45% of the CD9$^{-/-}$ group were dead (**Figure 2C**). Moreover, the surviving CD9$^{-/-}$ mice showed a recovery in body weight (**Figure 2D**). These results show that CD9 impedes epithelial repair and contributes to colon injury at both sublethal and lethal DSS doses.

Reduced Myeloid Cell Infiltration and Proinflammatory Cytokine Expression in the Colon of CD9$^{-/-}$ Mice

To characterize the immune mechanisms of colonic mucosa damage, we analyzed CD9$^{-/-}$ and WT colon cells by flow cytometry. After 7-day exposure to 2% DSS, CD9$^{-/-}$ colon showed markedly lower neutrophil and macrophage infiltration than WT colon (**Figures 3A,B**). In contrast, in non-treated mice, gut populations of these immune cell subsets were comparable between genotypes (**Figures 3A,B**), as were mesenteric lymph nodes, intraepithelial lymphocyte and lamina propria populations (Figure S1 in Supplementary Material). DSS-treated CD9$^{-/-}$ mice also had lower serum levels of IL-6 and TNFα than WT mice, whereas IFNγ was similarly increased in response to DSS in both genotypes (**Figure 3C**). Analysis of colon samples by qPCR revealed lower DSS-induced levels of IL-6, IL-1β, NLPR3, iNOS, IL-12p35, and IL-12p40 mRNA in CD9$^{-/-}$animals, whereas IL-17, IL-22, and IFNγ showed no significant between-genotype differences (**Figure 3D**).

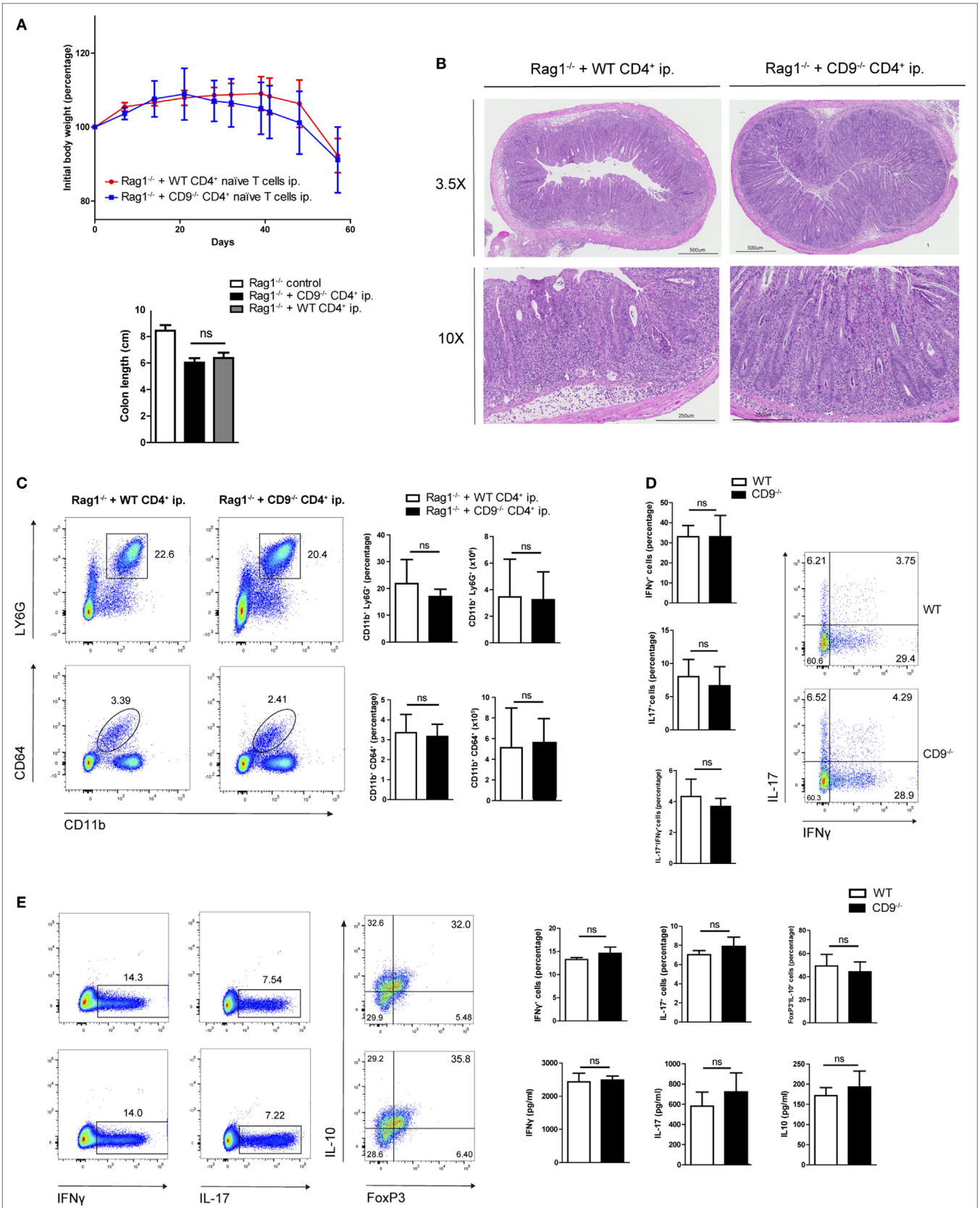

FIGURE 4 | Continued

FIGURE 4 | $CD4^+$ T cell-expressed CD9 does not contribute to adoptive transfer-mediated colitis or T cell differential subset skewing. **(A)** *Top,* body weight after intraperitoneal adoptive transfer of $CD4^+CD45RB^{hi}$ $CD62L^+CD25^-$ T cells from WT and $CD9^{-/-}$ donors into $Rag1^{-/-}$ recipients. *Bottom*, colon length at sacrifice on day 57. Data are from a representative experiment repeated three times with similar results. $n = 5$–6 mice per group; unpaired *t*-test. ns, non significant. **(B)** Representative 3.5× and 10× magnification H&E-stained colonic sections from $Rag1^{-/-}$ mice injected with WT and $CD9^{-/-}$ $CD4^+$ cells, showing transmural infiltration affecting all colon layers in both settings. **(C)** FACS analysis of myeloid cell infiltration. Representative dot plots are shown on the left; quantification of $CD45^+$-gated cell percentages and total numbers is shown on the right. **(D)** Flow cytometry analysis of intracellular staining for IFNγ and IL-17 in T cells from the mesenteric lymph nodes (MLNs) of $RAG1^{-/-}$ mice 2 months after $CD4^+$ T cell transfer. Cells were cultured for 72 h on an anti-CD3/CD28-coated plate and brefeldin A was added for the last 4 h. Representative dot plots and bar quantifications are shown of $CD4^+CD25^+$-gated cells. $n = 5$–6 mice per group; unpaired *t*-test. **(E)** *In vitro* T cell differentiation toward Th1, Th17, and Treg $CD4^+$ T cell subsets. Representative dots plots are shown of intracellular IFNγ, IL-17, and IL-10 in sorted populations, with quantification on the right (top row). Cytokine release was quantified by ELISA (bottom). Data are from a representative independent experiment of three performed and are presented as mean ± SD. $n = 5$ per genotype; unpaired *t*-test.

CD9 Expressed on $CD4^+$ T Cells Does Not Contribute to Immune-Cell Adoptive Transfer-Mediated Colitis

To further explore possible CD9-mediated immune mechanisms in IBD, we used an alternative model of colitis induced by intraperitoneal transfer into $Rag1^{-/-}$ mice of $CD4^+CD62L^+$ $CD25^-CD45RB^{hi}$ naive T cells sorted from WT or $CD9^{-/-}$ mice. Body weight was recorded over 2 months, showing no between-group differences (**Figure 4A**, top). Colon shortening was also similar in both genotypes (**Figure 4A**, bottom). Consistent with these findings, histological analysis revealed a similar extent of transmural inflammation in injected animals (**Figure 4B**), and flow cytometry showed similar increases in neutrophil and macrophage infiltration (**Figure 4C**). Restimulation of mesenteric lymph node $CD4^+$ cells with CD3/CD28 revealed no significant differences in Th1 and Th17 effector cell populations or cytokine production (**Figure 4D**). Likewise, no between-genotype differences were observed in the percentages of Th1, Th17, and Treg cells upon *in vitro* polyclonal differentiation of $CD4^+$ naive T cells from $CD9^{-/-}$ and WT mice (**Figure 4E**).

$CD9^{-/-}$ Bone Marrow Cells Transplanted into WT Mice Do Not Provide Protection against Colonic Injury

We next investigated the possible role of CD9 in myeloid cell populations or the resident non-hematopoietic cell compartment (mainly endothelial and epithelial cells). Two groups of chimeric mice were generated using the CD45.1 and CD45.2 haplotypes. Flow cytometry showed reconstitution levels of 95–99% (data not shown). Reconstitution experiments were carried out with WT CD45.1 mice and $CD9^{-/-}$ or WT CD45.2 mice, with irradiation and transplantation in either direction. Protection against DSS-induced colitis was observed only when irradiated $CD9^{-/-}$ mice were used as recipients of WT bone marrow (**Figures 5A,B**). Histology revealed typical DSS-induced changes in the distal and proximal colon of WT recipients and less pronounced alterations in $CD9^{-/-}$ recipients reconstituted with CD45.1 WT bone marrow (**Figures 5C,D**). Only $CD9^{-/-}$ recipients had lower DSS-induced levels of serum IL-6 measured by ELISA (**Figure 5E**), and colon samples from $CD9^{-/-}$ recipients also had lower induced transcript expression of proinflammatory cytokines measured by qPCR (**Figure 5F**). These results underscore the conclusion that susceptibility to DSS-induced colitis is increased by CD9 expression in the non-hematopoietic compartment.

Enhanced Colonocyte Proliferation after DSS-Induced Injury in $CD9^{-/-}$ Mice

After DSS-induced epithelial cell damage, the colonic epithelium actively proliferates to restore intestinal barrier integrity. Flow cytometry analysis of the proliferation marker Ki67 in colonic $EpCAM^+$ intestinal epithelial cells (IECs) from mice revealed that CD9 deficiency supports elevated colonic epithelial cell proliferation after DSS exposure (**Figure 6A**, top). However, no differences were detected in the proliferation of epithelial cells extracted from non-treated animals (Figure S2 in Supplementary Material). Remarkably, although the percentage of $Ki67^+$ cells was slightly higher in $CD9^{-/-}$ colon after 2 and 4 days of DSS exposure, the significant difference was observed at day 6. This is coincident with significant lower body weight loss and higher colon length in $CD9^{-/-}$ mice (**Figure 6A**, bottom). Moreover, $CD9^{-/-}$ colon showed higher mRNA expression of c-myc, c-fos, and cyclin D1 (**Figure 6B**). Analysis of the apoptosis marker caspase-3 was carried out by IHC in DSS-treated chimeric mice, with no differences between genotypes (Figure S3 in Supplementary Material). Proliferation in DSS-exposed colon of these animals was determined by counting immunostained $Ki67^+$ cells in colon crypts on histological sections. The percentage of $Ki67^+$ colonic cells was higher after DSS exposure in $CD9^{-/-}$ recipients than in WT recipients (**Figures 6C,D**). Taken together, these results demonstrate that CD9 limits epithelial cell proliferation in response to injury.

DISCUSSION

Inflammatory bowel disease arises through close interaction between genetics, immunology, environment, and microbiome. The development and progression of this multifactorial disorder is affected by several factors, including diet, lifestyle, and behavior. Moreover, perturbations of the gut microbiota due to antibiotic medication may also play an important role in IBD. DSS-induced colitis has become a widely used model for studying IBD in the mouse (25, 26). DSS is a chemical colitogen toxic to gut epithelial cells, interfering with intestinal barrier function and stimulating local inflammation. This

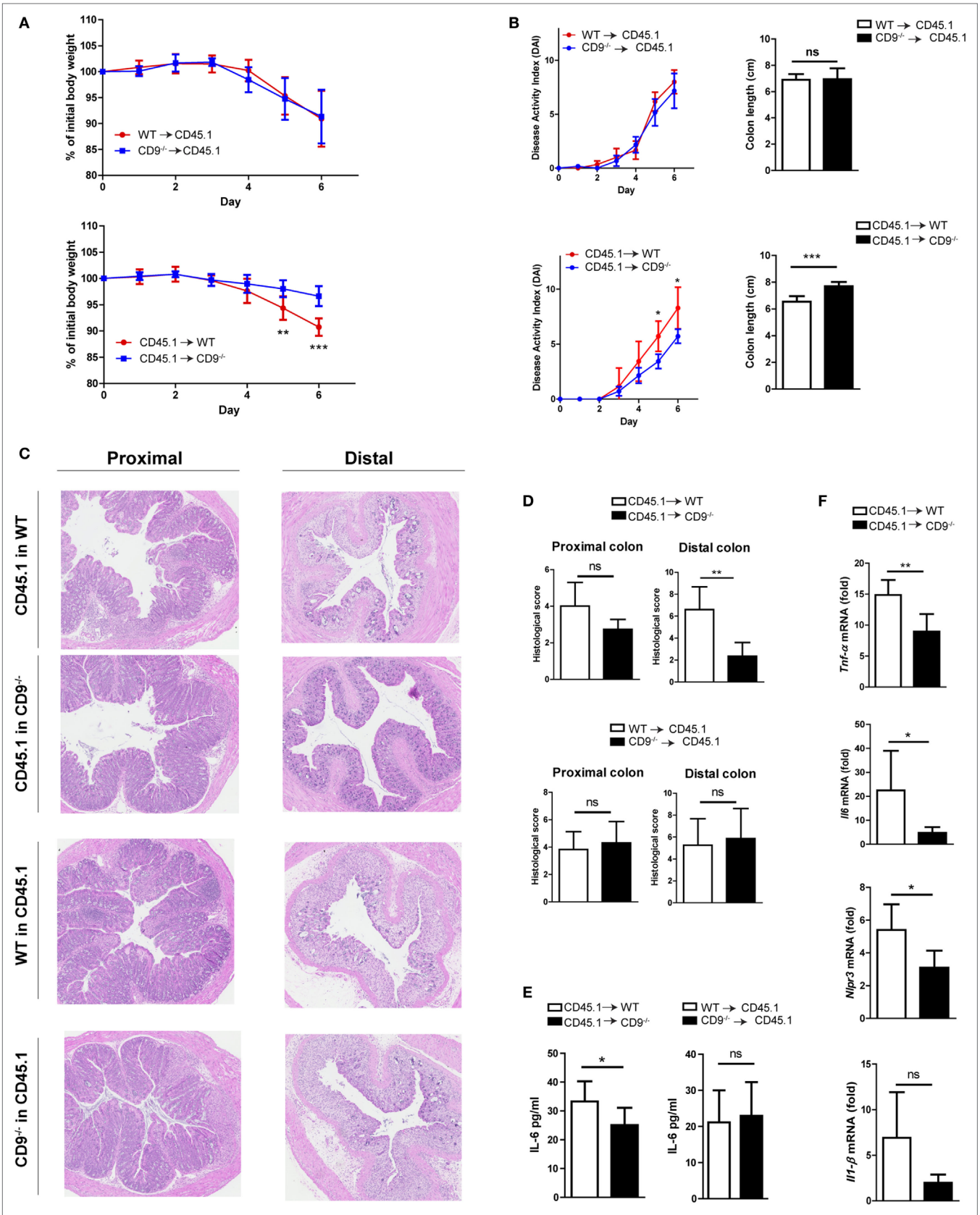

FIGURE 5 | Continued

FIGURE 5 | Lack of CD9 in the resident non-hematopoietic compartment confers the reduced susceptibility to dextran sodium sulfate (DSS)-mediated colitis. Lethally irradiated WT CD45.1 mice were rescued with WT or CD9$^{-/-}$ CD45.2 bone marrow, whereas lethally irradiated WT and CD9$^{-/-}$ CD45.2 mice were rescued with WT CD45.1 bone marrow. Three months post-transplantation mice were treated with 2% DSS. **(A)** Body weight evolution. **(B)** Disease activity index and colon shortening. **(C)** H&E stained proximal and distal colon sections. **(D)** Histological injury scores. **(E)** Serum IL-6 measured by ELISA. **(F)** qPCR analysis of proinflammatory cytokine expression in the colon of WT or CD9$^{-/-}$ CD45.2 recipients. Experiments were perfomed twice, giving similar results. $n = 6–7$ per group. All between-group comparisons were analyzed by unpaired *t*-test; $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$.

model is suitable for studying events triggered by temporary failure of mucosal homeostasis after epithelial cell shedding and loss of barrier integrity, and can also provide insight into the mechanisms that lead to MH after initial injury (27). Here, we report protection against DSS-induced colonic mucosal damage in CD9-deficient mice. These mice show lower DAI scores throughout treatment, larger colons, and have a less severe histological injury. The protection conferred by CD9 absence was confirmed by the increased survival of CD9$^{-/-}$ mice upon administration of a lethal 4% DSS dose. Epithelial preservation *in vivo* was demonstrated by lower colonic transepithelial FITC-dextran leakage in CD9$^{-/-}$ mice, and the importance of CD9 in the control of intestinal epithelial barrier function and integrity was further demonstrated by preserved expression of tight junction and other barrier-related genes in CD9$^{-/-}$ mice.

CD9 is ubiquitously expressed, and we therefore performed chimeric reconstitution experiments to determine which cell compartment is responsible for mediating DSS-induced toxicity. Our data clearly demonstrated that protection in CD9$^{-/-}$ animals was not dependent on the hematopoietic cell compartment. In CD9$^{-/-}$ colon, crypt and villous distortion is minimal and surface epithelium is more preserved; this keeps luminal pathogens outside the lamina propria, and therefore proinflammatory cytokine and chemokine release is lower and there is less inflammatory cell recruitment. Specifically, the myeloid-derived cytokines involved in the inflammatory response in DSS acute colitis iNOs, TNF-α, IL-6, IL-12, and the inflammasome drivers NLRP3 and IL-1β were enhanced in WT mice versus CD9$^{-/-}$ mice, but no differences were observed in either IFNγ, IL-17, or IL-22 cytokines. DSS colitis can be exacerbated by granulocyte recruitment (28–30). However, the reconstitution experiments ruled out a contribution to colon protection from CD9 deficiency in innate immune cells. The role of endothelial CD9 could not be completely discarded, and additional research with endothelium-specific deletion of CD9 would be required to resolve this issue. In addition, CD9 plays an important role in T cell activation (31–34). However, our data in the adoptive T cell transfer-mediated colitis model and *in vitro* T cell polyclonal experiments showed no significant differences in the differentiation and activation of CD9-deficient and WT Th1 and Th17 T cell subsets.

Flow cytometry and immunohistochemistry analysis revealed a higher percentage of Ki67 IECs in DSS-exposed CD9$^{-/-}$ colon. In the distal colon, the percentage Ki67^{+} cells is lower than in the proximal colon. This likely reflects the more severe colitis in the middle and distal third of the colon in DSS-exposed mice, causing a predominantly distal injury characterized by epithelial ulceration and impaired regeneration (35). Notably, the difference in proliferation was observed after 6 days of DSS exposure, suggesting that it is related to mechanisms of post-injury epithelial recovery. CD9 absence thus does not increase IEC proliferation *per se* and only supports MH after injury. Indeed, hyperplasia and dysplasia were not observed in any CD9$^{-/-}$ animals after cessation of DSS exposure.

Dextran sodium sulfate treatment leads to the exposure of the Toll-like receptors on the IEC basolateral surface. This triggers a proliferation that contributes to mucosal repair after injury, and DSS-induced colitis is exacerbated in mice with gene deletions affecting TLR signaling such as Tlr2$^{-/-}$, Tlr4$^{-/-}$, and Myd88$^{-/-}$ (36–38). TLR signaling is linked to EGFR activation (39), which is required for intestinal homeostasis in the setting of acute mucosal damage (40, 41). CD9 could be playing several roles in these settings. EGFR signaling is increased in CD9-deficient cell lines (17, 42), and CD9 also negatively regulates ADAM17 (16) metalloproteinase activity, which is known to shed some EGFR ligands. CD9 deficiency will thus translate into increased EGFR phosphorylation and activation. Aside from a direct control of proliferation, CD9 might regulate epithelial restitution, its deficiency ultimately resulting in increased epithelial proliferation (43). In this context, CD9 absence might facilitate rapid resealing of the intestinal epithelial barrier and could promote IEC migration through impaired localization of talin-1 to focal adhesions (10) or increase CXCR4/CXCL12-mediated migration (44), a route that directly regulates epithelial cell migration, barrier maturation, and restitution (45). Compared with CD9-positive cells, CD9-negative or depleted epithelial and tumor cells have a much higher migratory capacity, thereby supporting epithelial restitution (42, 46).

Inflammatory bowel disease is effectively treated with anti-TNF-α monoclonal antibody (Infliximab), either as monotherapy or in combination with other immunomodulators, and current efforts are directed toward the crucial goal of achieving MH in order to accomplish long-term remission (4, 47). However, pharmacological anti-inflammatory agents such as glucocorticosteroids or 5-aminosalicylic acid do not heal the bowel mucosa (4), and the efficacy of growth factors such as GH and EGF has yet to be established (48). There is thus a clear need to identify new therapeutic targets for MH. Our results indicate that CD9 depletion enhances IEC proliferation, resulting in a high regenerative response and reduced susceptibility to DSS-induced colitis. Our findings thus reveal a critical role for the tetraspanin CD9 in colon inflammation and suggest a novel therapeutic opportunity. Growing recent evidence suggests that targeting tetraspanins by an array of

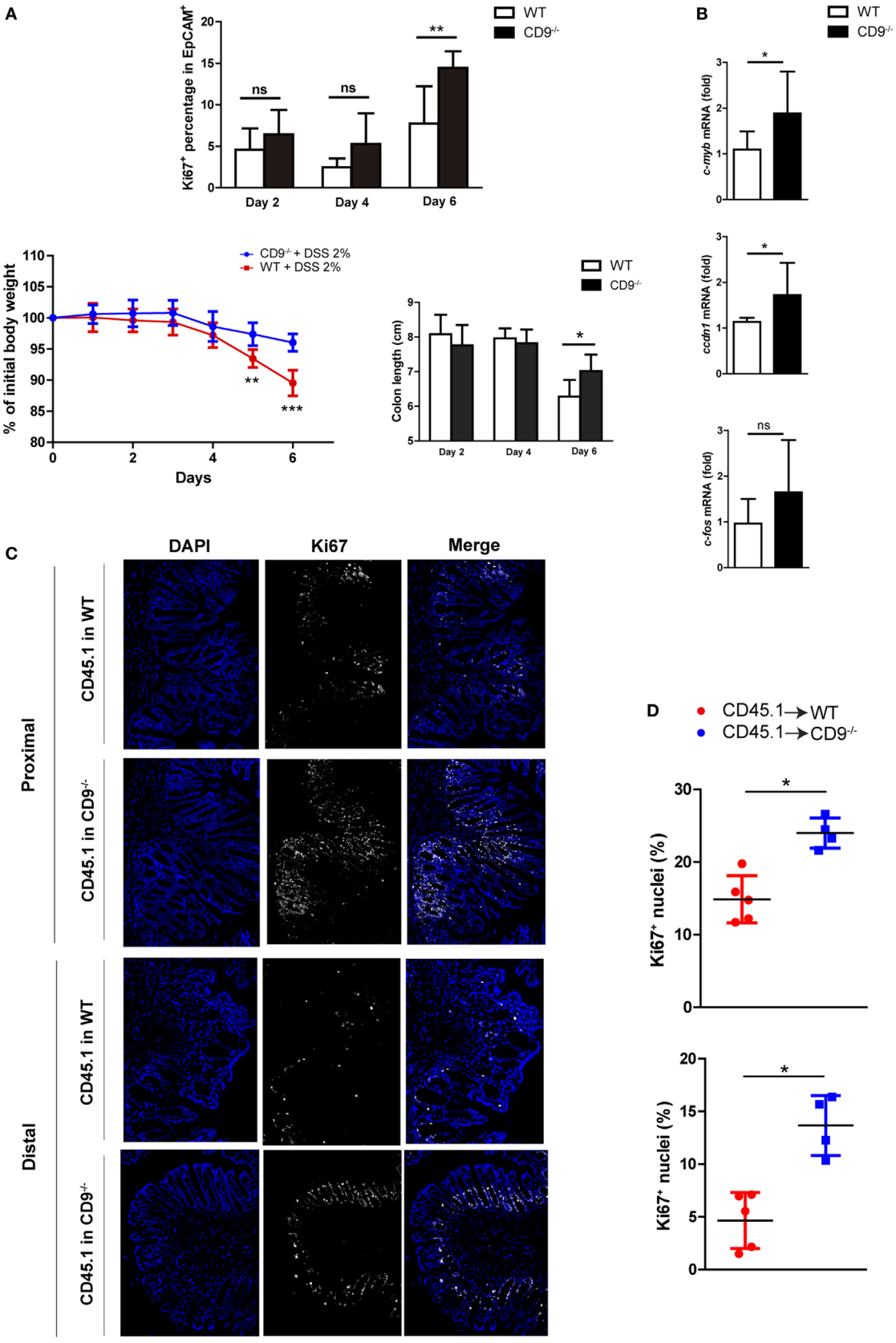

FIGURE 6 | Continued

FIGURE 6 | CD9 limits epithelial cell proliferation upon dextran sodium sulfate (DSS) challenge. **(A)** *Top*, FACS analysis of Ki67$^+$ cells in the EpCAM$^+$CD45$^-$ gated population after days 2, 4, and 6 days 2% DSS exposure (upper panel). *Bottom,* body weight loss and colon shortening, $n = 5$–6 mice per group. **(B)** qPCR analysis of colonic mRNA expression of the cell-cycle genes *c-myb*, *ccdn1*, and *c-fos*. Data are pooled from two independent experiments, $n = 4$–6 mice per group. **(C)** Ki67 immunofluorescence staining on proximal and distal colon sections from untreated or 6-day DSS-treated chimeric mice. Representative magnification images are shown. **(D)** Quantification of Ki67$^+$ cells in the epithelial layer of proximal (upper), and distal (lower) colons from 6-day DSS-fed chimeric mice. Each dot corresponds to the percentage of Ki67$^+$ nuclei from all the epithelial nuclei of whole colon sections $n = 4$–5 mice per genotype. Between-group comparisons were analyzed by unpaired *t*-test for **(A,B)** and Mann–Whitney *U*-test for **(D)**; $^*P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$.

tools including monoclonal antibodies, soluble large-loop proteins, and RNAi technology may be used to improve the course of IBDs.

ETHICS STATEMENT

All animals were housed in pathogen-free conditions at the CNIC animal facility. Experimental procedures were approved by the local research ethics committee and conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC, enforced in Spanish law under Real Decreto 53/2013.

AUTHOR CONTRIBUTIONS

MS designed research plan and performed all mice studies, analyzed and interpreted all data, and wrote the manuscript. DC helped to designed research plan and in mice experimentation, data interpretation, and writing of the manuscript. MR-H, DT, and OM-G performed experimental work. FS-M planned research, discussed results, and collaborated to write the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2017.01854/full#supplementary-material.

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MINI REVIEW
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Tetraspanin CD9: A Key Regulator of Cell Adhesion in the Immune System

*Raquel Reyes[1], Beatriz Cardeñes[1], Yesenia Machado-Pineda[1] and Carlos Cabañas[1,2]**

[1] Departamento de Biología Celular e Inmunología, Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Madrid, Spain,
[2] Departamento de Inmunología, Oftalmología y OTR (IO2), Facultad de Medicina, Universidad Complutense, Madrid, Spain

The tetraspanin CD9 is expressed by all the major subsets of leukocytes (B cells, $CD4^+$ T cells, $CD8^+$ T cells, natural killer cells, granulocytes, monocytes and macrophages, and immature and mature dendritic cells) and also at a high level by endothelial cells. As a typical member of the tetraspanin superfamily, a prominent feature of CD9 is its propensity to engage in a multitude of interactions with other tetraspanins as well as with different transmembrane and intracellular proteins within the context of defined membranal domains termed tetraspanin-enriched microdomains (TEMs). Through these associations, CD9 influences many cellular activities in the different subtypes of leukocytes and in endothelial cells, including intracellular signaling, proliferation, activation, survival, migration, invasion, adhesion, and diapedesis. Several excellent reviews have already covered the topic of how tetraspanins, including CD9, regulate these cellular processes in the different cells of the immune system. In this mini-review, however, we will focus particularly on describing and discussing the regulatory effects exerted by CD9 on different adhesion molecules that play pivotal roles in the physiology of leukocytes and endothelial cells, with a particular emphasis in the regulation of adhesion molecules of the integrin and immunoglobulin superfamilies.

Keywords: CD9, tetraspanins, integrins, ICAM1, activated leukocyte cell adhesion molecule, ADAM17, lymphocyte function-associated antigen 1, very late activation antigen 4

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Manfred B. Lutz, Universität Würzburg, Germany

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****Correspondence:***
Carlos Cabañas
ccabanas@cbm.csic.es

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INTRODUCTION

CD9, or Tspan 29 in the systematic nomenclature, is a 21–24 kDa member of the tetraspanin protein family. Tetraspanins are structurally characterized by containing four transmembrane domains, which delimit a small extracellular loop (known as SEL or EC1), a large extracellular loop (termed LEL or EC2), and short intracellular N- and C-terminal tails. Within the LEL domain five α-helices (A-E) can be distinguished, with helices A, B, and E defining a region well conserved among different members ("constant region") that is involved in tetraspanin dimerization and oligomerization, whereas helices C and D define the "variable region" of the LEL, involved in most lateral interactions of tetraspanins with other membrane proteins. The presence of a CCG motif after the B

Abbreviations: ADAM, a disintegrin and metalloproteinase; ALCAM, activated leukocyte cell adhesion molecule; BBB, blood–brain barrier; CAMs, cell adhesion molecules; CNS, central nervous system; cSMAC, central supramolecular activation cluster; DCs, dendritic cells; EAPs, endothelial adhesive platforms; ER, endoplasmic reticulum; ICAM-1, intercellular adhesion molecule 1; Ig-SF, immunoglobulin superfamily; IL-2, interleukin-2; IS, immune synapse; LDL, low density lipoproteins; LFA-1, lymphocyte function-associated antigen 1; LPS, lipopolysaccharide; mAb, monoclonal antibody; MMP, matrix metalloproteinase; NK, natural killer cells; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin; pSMAC, peripheral supramolecular activation cluster; TEMs, tetraspanin-enriched microdomains; TLR4, toll like receptor-4; TNF-α, tumor necrosis factor-alpha; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late activation antigen 4.

helix in the LEL domain, with its two cysteines engaged in the formation of intradomain disulfide bonds with other conserved cysteines, membrane-proximal palmitoylations, and hydrophilic residues within transmembrane domains are also defining features of tetraspanin members. Tetraspanins are glycoproteins which generally contain several N-glycosylation sites in the LEL domain (1), and in this regard, CD9 is peculiar as it contains only one N-glycosylation site that is located in its SEL domain (2).

CD9 shows a wide cellular and tissue distribution and was initially identified as a lymphohematopoietic marker (3) and then implicated in a range of cellular functions, including motility, proliferation, differentiation, fusion, and adhesion [reviewed in Ref. (1, 4–6)]. Not surprinsingly, given its involvement in such a range of crucial cell activities, CD9 plays a major role in critical physiological and pathological processes, including sperm–egg fusion, neurite outgrowth, myotube formation, viral infections, tumorigenicity, and metastasis [reviewed in Ref. (7–9)]. As for other members of the tetraspanin family, the biological functions of CD9 greatly depend on the multitude of dynamic interactions that this molecule is able to establish with other transmembrane and cytoplasmic proteins within the context of a specific type of membrane domains, called tetraspanin-enriched microdomains (TEMs) (10, 11). CD9 in TEMs can thus affect, either directly or indirectly, the activity of numerous transmembrane and intracellular proteins such as metalloproteinases and other enzymes, ion channels, receptors for growth factors, cytokines and chemokines, transporters, signaling transducers, and cytoskeletal linkers (**Table 1**). CD9 can potentially alter the activity of these molecules through different mechanisms including their selective confinement in TEMs or segregation into separate microdomain compartments, which would hinder their access to their cognate substrates or binding of their extracellular or intracellular ligands (10, 11).

It is striking that the basal expression of CD9 in the major types of leukocytes from freshly drawn blood [monocytes, natural killer (NK) cells, B cells, $CD8^+$ T cells, and $CD4^+$ T cells] is usually low (49, 50). However, its expression in these leukocyte subpopulations increases following their culture, being particularly high in monocytes (8, 49, 50). Likewise, CD9 is also clearly detected in activated lymphoblasts derived from PHA/interleukin-2-stimulated PBMCs (21). CD9 is also expressed by the different subsets of human and murine dendritic cells (DCs), with the exception of mouse plasmacytoid DCs (51). In contrast to the relatively low expression on resting leukocytes, the expression of CD9 is particularly high on endothelial cells (52), in keeping with the crucial role this tetraspanin plays in regulating the firm adhesion and transendothelial migration of leukocytes (24, 25).

Several excellent reviews have already covered the topic of how tetraspanins, including CD9, participate in the regulation of specific functions in different cells of the immune system (4, 8, 53, 54). In this review, however, we will focus particularly on discussing the regulatory effects exerted by CD9 on different adhesion molecules that play pivotal roles in the physiology of leukocytes and endothelial cells, with a particular emphasis in the regulation of adhesion molecules of the integrin and immunoglobulin superfamilies.

TABLE 1 | List of molecules reported to associate with CD9.

CD9-associated molecules	Other associated tetraspanins	Reference
Adhesion molecules		
Integrins		
α1β1		(12)
α2β1	CD151	(13, 14)
α3β1	CD63, CD81, CD82, CD151, NAG-2, Co-029	(13, 14)
α4β1 [very late activation antigen 4 (VLA-4)]	CD53, CD81, CD82, CD151	(15, 16)
α5β1 (VLA-5)	CD151	(16)
α6β1	CD63, CD81, CD82, CD151, NAG-2, Co-029	(15, 17)
α7β1	CD151	(18)
αIIbβ3	CD151	(19, 20)
α6β4	CD151	(13)
αLβ2 (lymphocyte function-associated antigen 1)	CD81, CD82	(21–23)
Immunoglobulin-SF members		
Intercellular adhesion molecule 1 (CD54)	CD151	(24, 25)
EpCAM (CD326)	Co-29, Tspan8, D6.1A	(26)
B-CAM/Lu (CD239)		(27)
Activated leukocyte cell adhesion molecule (cD166)		(28, 29)
Vascular cell adhesion molecule-1 (CD106)	CD151	(24)
Other adhesion receptors		
CD42		(20, 30)
CD44	Co-29, Tspan8, D6.1A	(13)
CD47		(20)
Claudin-1		(31)
Syndecan		(13)
Immune system molecules		
HLA-DR	CD153, CD81, CD82	(15)
CD2	CD53	(32)
CD3	CD81, CD82	(33)
CD4	CD81, CD82	(33)
CD5		(33)
CD19	CD81	(34)
CD46		(12)
Growth factors		
Pro-TGF-α		(35)
Pro-HB-EGF		(36)
Signaling molecules		
CD117	CD63, CD81	(37)
GPCR56	CD81	(38)
PI4K	CD81, CD151, CD63	(39)
PKC	CD53, CD81, CD82, CD151	(27, 40)
Other proteins		
ADAM2	CD81	(41)
ADAM10	CD81, CD82	(42, 43)
ADAM17		(42, 44)
CD36		(45)
CD26 (DPPIV)		(27, 46)
CD224	CD37, CD81, CD53, CD82	(27)
CTL1/CD92		(27)
CTL2		(27)
EWI-2	CD63, CD81, CD82, CD151	(47)

(*Continued*)

TABLE 1 | Continued

CD9-associated molecules	Other associated tetraspanins	Reference
EWI-F	CD81	(48)
Hem-1		(27)
TADG-15		(27)
Syntaxins 3 and 4A		(27)

CD9 REGULATES ADHESION MOLECULES AT THE IMMUNE SYNAPSE (IS) AND T LYMPHOCYTE ACTIVATION

Recognition of antigenic-peptides bound to MCH-I and MCH-II molecules on the surface of antigen presenting cells (APCs) is essential for activation of $CD8^+$ and $CD4^+$ T cells, respectively, and triggers the initiation of T cell-mediated immune responses. This process requires the establishment of a dynamic structure at the APC-T cell contact area, termed as IS. In its mature form, the IS contains a clearly distinguishable central region (cSMAC), where clusters of TcR/CD3, costimulatory and signaling molecules concentrate, and a peripheral region (pSMAC) highly enriched in integrins and other adhesion molecules (55, 56). The establishment of IS proceeds through a series of spatiotemporal segregated events, including the initial scanning of antigen peptide-loaded MHC molecules, the specific recognition of antigen-loaded MHC, and signaling by the TcR/CD3 complex and stabilization of the IS. The interaction of different adhesion receptors on either side of the IS (i.e., on the APC or on the T cell) with their specific ligands on the opposing side is essential during the initial scanning and stabilization of the IS. In particular, interactions of integrin lymphocyte function-associated antigen 1 (LFA-1) (αLβ2) on the T cell with its ligands intercellular adhesion molecule 1 (ICAM-1) and ICAM-3 on the APC surface are crucial for IS formation and stabilization. During the initial scanning of MHC-peptide complexes on the surface of APCs, the affinity of T cell LFA-1 for its ligands is low/intermediate, but inside-out signaling triggered from the TcR/CD3 complex upon recognition of the cognate MHC-peptide induces the high-affinity state of this integrin, leading to strong ligand binding and stabilization of the IS (57, 58). TcR and LFA-1 molecules on immune cells are preorganized in nanoclusters that coalesce into larger aggregates following ligand binding (59–62). CD9 has been shown to engage through its LEL domain in direct interactions with LFA-1 on the surface of different types of leukocytes, including T lymphocytes and monocyte/macrophage-like cells (21). Through its association with LFA-1, CD9 controls the state of aggregation and adhesive capacity of this integrin. Through the use of different strategies, such as monoclonal antibody (mAbs) specific for CD9, as well as genetic approaches based on its silencing or neoexpression, it was demonstrated that CD9 exerts a negative regulation on the adhesive capacity of LFA-1. The mechanism involved in this inhibitory effect of CD9 on LFA-1 activity does not rely upon changes in the integrin affinity state, as inferred from the unaltered expression of the activation-reporter 24 epitope (21). These findings concur with the observation that talin relocalization, which is required for the induction of conformational changes in LFA-1 and acquisition of increased affinity through inside-out signaling [reviewed in Ref. (63)], is not altered in T cells with silenced CD9 and CD151 (64). Thus, the negative regulation exerted by CD9 on the adhesive capacity of LFA-1 is rather related to alterations in its state of aggregation and the control of its binding valency (avidity). Interestingly, confocal and TIRF microscopy analyses showed that expression of CD9 in lymphocytic and monocytic cells induces LFA-1 molecules to become organized in a larger number of clusters but individually displaying a smaller size, which would account for the reduced integrin adhesive capacity (21) (**Figure 1A**). The association of LFA-1 with the actin cytoskeleton has long been known to control the avidity of this integrin through regulation of its dynamic reorganization into microclusters (65). Thus, although the exact mechanism by which CD9 alters the state of aggregation of LFA-1 molecules has not been resolved, it likely relies upon the linkage of this tetraspanin with the microfilaments of the actin cytoskeleton through the Ezrin–Radixin–Moesin (ERM) proteins (66).

In addition to LFA-1, VLA-4 (α4β1) is another integrin predominantly expressed on hematopoietic cells, which also concentrates at the pSMAC playing a role in the stabilization of the IS and in T cell co-stimulation, though the precise ligand for this integrin on the APC is still unknown (67). The two classic ligands of integrin α4β1, alternatively spliced fibronectin and VCAM-1, are not expressed on APCs. Alternative ligands of this integrin are members of the junctional adhesion molecules (JAM) family, but it has not been demonstrated so far whether these molecules play a role in the IS. Contrary to CD81, which strongly colocalizes with CD3 and contributes to cSMAC formation, becoming segregated from LFA-1 which is mainly found at the pSMAC, CD9 (along with CD151) shows less colocalization with CD3, indicating that it is not involved in the reorganization and clustering of CD3 at the IS (64). In contrast, CD9 does associate with integrin α4β1 on T cells and drives both the accumulation of the high-affinity form of this integrin at the IS and the subsequent downstream signaling (64). CD9 (or CD151) silencing results in diminished relocalization of integrin α4β1 to the IS, reduced accumulation of high-affinity β1 integrins at the cell–cell contact area, and decreased downstream integrin signaling.

Besides their adhesive function, integrins also work as efficient bidirectional signaling molecules (68). In T cells, LFA-1 costimulation lowers the TcR-mediated activation threshold (69, 70). Similarly, ligation of integrin α4β1 also provides costimulatory signaling (71). Downstream signaling from these adhesion receptors is mediated through interactions with a number of adaptor and signaling molecules (72, 73), whose activity, location, and aggregation may be regulated by tetraspanins through their selective inclusion in, or exclusion from, specific TEMs. CD9 and CD151 have been shown to increase integrin-dependent ERK1/2 signaling, and the knockdown of these tetraspanins reduces the phosphorylation of focal adhesion kinase (FAK) and ERK1/2 integrin downstream targets and impairs the enrichment of phosphorylated FAK at the IS (64).

Activated leukocyte cell adhesion molecule (ALCAM) or CD166 is a member of the immunoglobulin superfamily of CAMs that can engage in homophilic (ALCAM–ALCAM) as well

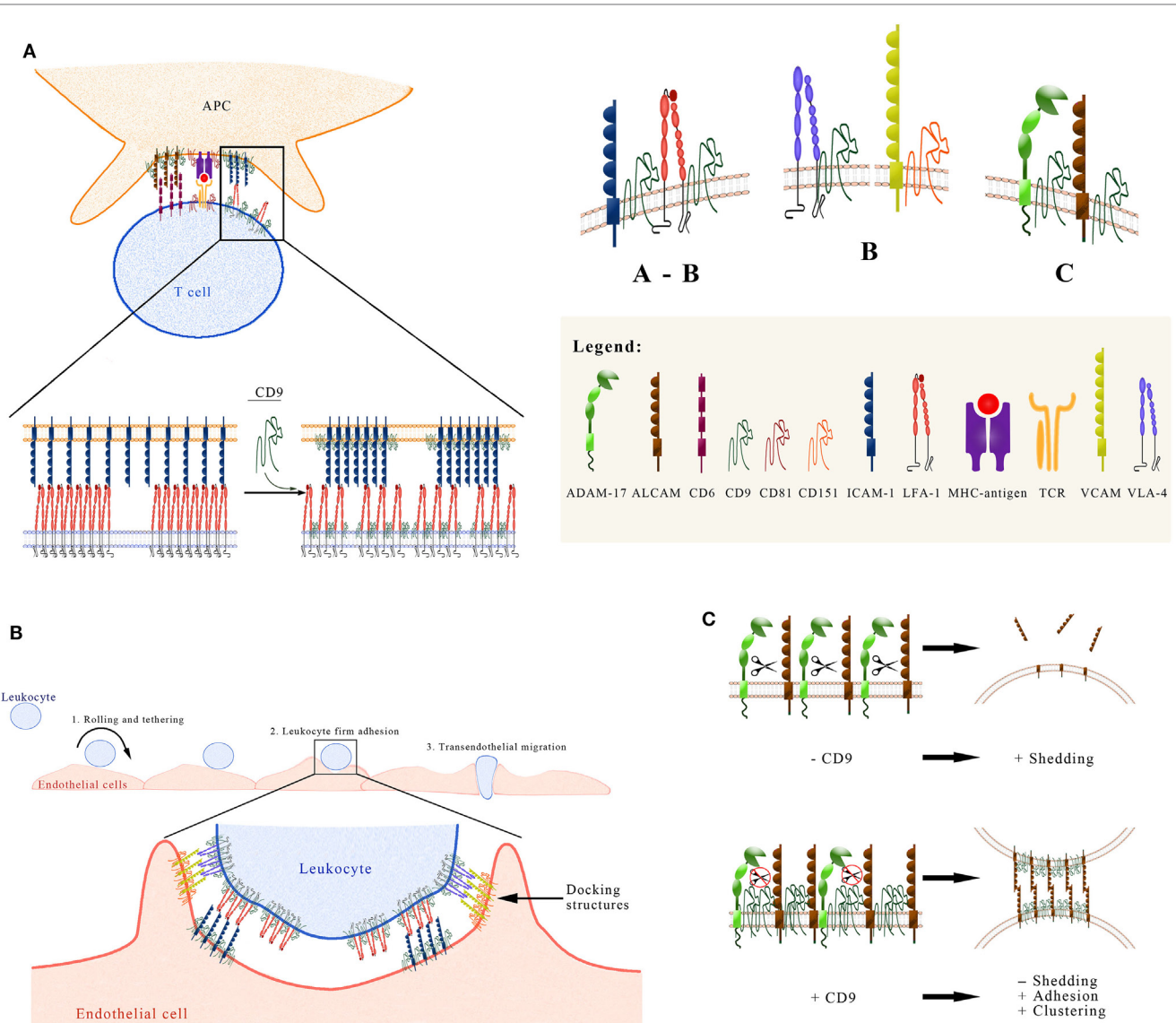

FIGURE 1 | Functional regulation exerted by CD9 on the activity of some immune system adhesion molecules. **(A)** CD9 regulates ICAM and lymphocyte function-associated antigen 1 (LFA-1) at the immune synapse (IS). Interactions between LFA-1 on the T cell, and its ligand intercellular adhesion molecule 1 (ICAM-1) on the APC surface, take place at the peripheral area of the IS (pSMAC) and are crucial for IS formation and stabilization. The tetraspanin CD9 plays an important role in the IS in two different ways: (1) Through its association with LFA-1 on the T cell, CD9 controls the state of aggregation and adhesive capacity of this integrin. Neoexpression/overexpresion of CD9 reduces the integrin adhesive capacity by generating a larger number of clusters of LFA-1 molecules that individually display a smaller size. (2) On the APC surface CD9 recruits ICAM-1 into TEMs, thus increasing its adhesive capacity. **(B)** CD9 regulates leukocyte firm adhesion on endothelial cells. The multi-step paradigm of the leukocyte extravasation cascade includes the initial tethering and rolling of the leukocyte on the endothelial surface, followed by the firm adhesion step and transmigration either between two endothelial cells or through the body of an endothelial cell. The firm adhesion step is mediated by the high-affinity interaction of leukocyte integrins LFA-1 ($\alpha L\beta 2$) and Mac-1 ($\alpha M\beta 2$) with their endothelial counter-receptor ICAM-1, and of integrin VLA-4 ($\alpha 4\beta 1$) with its endothelial ligand VCAM-1. ICAM-1 and VCAM-1 are preorganized in endothelial adhesive platforms (EAPs), through their association with CD9 and CD151 respectively. After leukocyte binding, EAPs evolve into three-dimensional docking structures that emanate from the endothelial surface and embrace the leukocyte. **(C)** CD9 affects the shedding of leukocyte adhesion molecules mediated by ADAM17. The recruitment of ADAM17 into CD9-organized TEMs (low panel), following the overexpression or neoexpression of this tetraspanin, exerts a negative regulation on the sheddase activity of ADAM17 against different substrates on leukocytic cells, including activated leukocyte cell adhesion molecule (ALCAM). This negative regulation on ADAM17 activity accounts for an increased expression of ALCAM on the cell surface. Additionally, CD9 also induces the aggregation of ALCAM and the concomitant increase in its avidity. Therefore, CD9 augments ALCAM-mediated cell–cell adhesion through this dual mechanism.

as heterophilic (ALCAM–CD6) interactions. ALCAM-mediated adhesion is crucial in different physiological and pathological situations, with particular relevance in immune responses, collective cell migration, cancer metastasis, neuronal development, and leukocyte migration across blood–brain barrier (BBB) in multiple sclerosis and autoimmune encephalomyelitis. ALCAM on APCs engages in heterophilic interactions with CD6 on T cells, with both molecules becoming redistributed to the IS in

an antigen-dependent manner, which have been shown to play an important role in the formation and stabilization of the IS (74). They also provide costimulatory signals and thus contribute to T cell activation and proliferation (75–77). ALCAM-mediated APC-T cell adhesion requires upregulation of ALCAM avidity for CD6, which is brought about by the aggregation of ALCAM molecules on the cell surface, a process that is in turn controlled by their dynamic association with the actin cytoskeleton. Such dynamic linkage with the actin cytoskeleton takes place through the interaction of a C-terminal KTEA PDZ-binding motif and a membrane-proximal positively charged stretch in ALCAM's cytoplasmic tail, with the adaptor proteins syntenin-1 and the ERM protein ezrin, respectively (28, 78).

In this context, CD9 has been shown to play an important role in the regulation of ALCAM-mediated cell adhesion and T cell activation. On leukocytes, CD9 acts as a scaffold in ALCAM-containing TEMs, engaging in direct lateral interactions through its LEL domain with the extracellular region of ALCAM, therefore, upregulating its adhesive capacity and co-stimulation in T cells through its ligand CD6 (29). Importantly, this enhancement of ALCAM avidity and functional activity induced by CD9 is mediated by a dual mechanism involving, on the one hand, augmented clustering of ALCAM molecules and, on the other hand, upregulation of ALCAM surface expression due to the inhibition of ADAM17/TACE sheddase activity (the latter will be discussed below) (**Figure 1C**). As CD9 has been shown to be part of TEMs that contain a number of proteins directly linked to ERM proteins (66), this tetraspanin could contribute an additional level of regulation to the dynamic association with the actin cytoskeleton that ultimately controls ALCAM avidity and costimulatory capacity.

ENDOTHELIAL CD9 REGULATES LEUKOCYTE ADHESION, EXTRAVASATION, AND INFLAMMATION

Migration across the endothelial cell layer that lines the inner surface of blood vessels is required for leukocyte recirculation during immune surveillance and their accumulation at sites of tissue infection and inflammation. Circulating leukocytes exit the blood vessels at certain preferred sites, such as the high endothelial venules of secondary lymphoid organs or the postcapillary venules at sites of inflammation. Leukocyte transendothelial migration proceeds through a series of steps which are mediated by a set of complex and sequential interactions between adhesion receptors and counter-receptors expressed on the leukocyte and the endothelial apical surface. This sequence of events represents the multi-step paradigm of the leukocyte extravasation cascade (79–81). These steps include the initial tethering and rolling of the leukocyte on the endothelial surface, followed by the firm adhesion, subsequent crawling on the endothelial apical surface in search for an appropriate exit site and the actual transmigration either between two endothelial cells (paracellular route) or through the body of an endothelial cell (transcellular route) and finally, leukocyte migration in the interstitial tissue following specific chemotactic cues. Specifically, the step of firm leukocyte adhesion to the endothelium is fundamentally mediated by the high-affinity interaction of leukocyte integrins, LFA-1 (αLβ2) and Mac-1 (αMβ2), with their endothelial counter-receptor ICAM-1, and of integrin VLA-4 (α4β1) with its endothelial ligand VCAM-1. These high-affinity interactions require the activation of integrins in response to intracellular signals triggered upon recognition by leukocyte chemokine receptors of chemokines immobilized on heparan sulfate proteoglycans on the endothelial apical surface.

Vascular endothelium plays an active role in transendothelial leukocyte migration by controlling the expression level and organization of adhesive molecules. Following exposure to proinflammatory cytokines (such as TNF-α and IL-1β), an increase in the expression of E- and P-selectins (main mediators of leukocyte tethering and rolling steps) and integrin ligands ICAM-1 and VCAM-1 is induced on the apical surface of endothelial cells, thus facilitating leukocyte adhesion to the endothelium and their subsequent transmigration. Interestingly, ICAM-1 and VCAM-1 on the endothelial surfaces are preorganized in adhesive clusters, so-called endothelial adhesive platforms (EAPs), by their inclusion in tetraspanin nanoplatforms through their association with CD9 and CD151 (24, 25). Analysis by FLIP-FRET microscopy evidenced that specificity exists in these associations, with CD9 preferentially associating with ICAM-1 whereas CD151 preferentially associates with VCAM-1 (24) (**Figure 1B**). Upon leukocyte binding, EAPs evolve into three-dimensional docking structures formed by elongated microvilli and long filopodia that emanate from the endothelial surface and embrace the adherent leukocytes, preventing their detachment under physiological hemodynamic flow conditions. The formation of these structures requires not only the tetraspanin-mediated clustering of ICAM-1 and VCAM-1, but also an active participation of the actin cytoskeleton and associated proteins, such as ERMs (ezrin–radixin–moesin), α-actinin, vinculin, filamin, cortactin, and vasodilator-stimulated phosphoprotein (VASP), as well as signaling molecules like Rho A/ROCK, MLCK, src, pyk-2, and PIP_2 (82–84). Importantly, in the absence of CD9 the formation of microvilli required for the development of full docking structures is inhibited, crucially underlying the relevance of this particular tetraspanin in regulating the firm adhesion and transendothelial migration of leukocytes (85).

ALCAM is also abundantly expressed on endothelial cells in central nervous system (CNS) and has been shown to participate in the formation of docking structures (or transmigratory cups) required for leukocyte diapedesis (86). Interestingly, unlike ICAM-1 and VCAM-1, ALCAM mediates the transmigration of both lymphoid and myeloid leukocytes (86), and seems to be particularly relevant in the extravasation of monocytes rather than T cells across the BBB (87). Blockade of ALCAM reduced the transmigration of $CD4^+$ lymphocytes and monocytes across the BBB and reduced the severity and delayed the time of onset of experimental autoimmune encephalomyelitis in animal models, highlighting the potential usefulness of ALCAM as a therapeutic target in multiple sclerosis (86). ALCAM has also been found to associate with tight junction molecules that maintain BBB integrity (88). The interaction of CD9 with ALCAM on endothelial cells has not been as yet properly explored, but considering that ALCAM participates in the formation of docking structures and

what has been reported on leukocytic cells (29), it is likely that CD9 could also crucially regulate both the organization and the level of expression of ALCAM molecules on the CNS endothelium, critically controlling the transmigration of lymphocytes and monocytes across the BBB.

CD9 REGULATES THE SHEDDING OF LEUKOCYTE ADHESION MOLECULES MEDIATED BY ADAM10 AND ADAM17 METALLOPROTEASES

Another important mechanism by which CD9 is capable to influence the activity of adhesion molecules with relevance in the immune system is through the modulation of ADAM10 and ADAM17 metalloproteinases. These two closely related members of the a disintegrin and metalloproteinase (ADAM) family are key enzymes responsible for the cleavage and release from the cell surface of the ectodomains of a large variety of transmembrane proteins, a process known as *ectodomain shedding*. Through this process, cells can rapidly alter their surface phenotype by releasing a variety of soluble protein ectodomains that can subsequently trigger autocrine, juxtacrine, paracrine, or endocrine cellular signaling and responses in specific target cells. The list of identified protein substrates that can be shed either by ADAM10 or by ADAM17 has kept on growing over recent years and currently comprises over a hundred different cell surface proteins, including growth factors, cytokines, many types of receptors, and numerous cell adhesion molecules (CAMs). Among the CAMs shed by ADAM10 or ADAM17 there are members of the immunoglobulin (ICAM-1, L1-CAM, Ep-CAM, VCAM-1, ALCAM, and JAM-A), integrin, cadherin, and selectin families as well as other adhesion receptors like CD44 (89–91). Many of the protein substrates of these two proteases are shared, indicating that their function may sometimes be complementary or redundant, but the mechanisms that selectively regulate their individual activities and substrate preference are still poorly understood. Regulation of ADAMs' shedding activities can be achieved at different levels, impacting upon their intracellular trafficking and maturation, metalloprotease activity, and accessibility of substrates [as reviewed in Ref. (92, 93)].

Tetraspanins are emerging as important regulators of ADAMs' shedding activities. By dynamically regulating their inclusion in, or exclusion from specific TEMs, tetraspanins are shown to influence the relative localization of ADAMs and their substrates on the plasma membrane, thus exerting crucial regulatory effects on their sheddase activity [reviewed in Ref. (42, 94)].

CD9 and other tetraspanins (including CD53, CD81, CD82, and CD151), have been reported to associate with ADAM10 under mild solubilization conditions. Additionally, antibody engagement of CD81, CD82, or CD9 did indeed stimulate ADAM10-dependent shedding of its substrates TNF-α and EGF (43). However, subsequent experiments using more stringent detergent conditions indicated that these tetraspanins do not associate directly with ADAM10 (95). Such interactions, instead, seem to be mediated through a distinct subfamily of tetraspanins (TspanC8), whose specific and direct association with ADAM10 regulates its exit from the ER, enzymatic maturation, intracellular trafficking, subcellular localization, and its metalloprotease activity against different cell surface protein substrates (94, 96, 97).

Importantly, CD9 is the only tetraspanin that has so far been reported to associate directly with ADAM17 on the surface of leukocytes and endothelial cells. This direct association was demonstrated by image and biochemical techniques, including confocal microscopy, proximity ligation, co-immunoprecipitation, covalent crosslinking, and pull-down assays. Heterologous expression/overexpression of CD9 or treatment with agonist CD9-specific antibodies inhibited ADAM17-mediated shedding of different substrates from leukocytic cells, including TNF-α and the adhesion molecules ICAM-1 and ALCAM/CD166. Thus, while anti-CD81 mAbs enhanced the release of TNF-α mediated by ADAM10 (43), anti-CD9 mAbs regulated negatively the stimulated release of that proinflammatory cytokine mediated by the closely related sheddase ADAM17 (44). Additionally, CD9 knockdown increased ADAM17-mediated shedding of its substrates (29, 44). CD9-mediated inhibition of ALCAM shedding by ADAM17 resulted in increased ALCAM levels on the cell surface and augmented ALCAM-mediated cell adhesion (29) (**Figure 1C**). These findings were later confirmed for additional ADAM-17 substrates, like LR11 (SorLa), a member of the low density lipoprotein receptor family which binds ApoE and has a key role in cell migration, adhesion, and drug resistance (98).

CONCLUDING REMARKS

CD9 is emerging as an important regulatory molecule that controls the expression and activity of different adhesion molecules of crucial importance in the immune system. At the IS, CD9 on the T cell side regulates the clustering and adhesive activity of integrins LFA-1 (αLβ1) and VLA-4 (α4β1), whereas on the APC side, CD9 regulates the expression of ALCAM/CD166 and the avidity of its interaction with CD6. Through the control exerted by CD9 on the expression and/or activity of these adhesive receptors, this tetraspanin regulates the stabilization of the IS and the subsequent activation of T lymphocytes.

On activated endothelial cells exposed to inflammatory cytokines, tetraspanins CD9 and CD151 play a crucial role in the step of firm leukocyte adhesion by arranging endothelial ICAM-1 and VCAM-1 in preorganized adhesive clusters, called EAPs. Upon leukocyte binding to the luminal endothelial surface, EAPs evolve into docking structures that embrace the leukocytes, preventing their detachment under flow conditions.

CD9 is the only tetraspanin that has been shown to associate directly with ADAM17 on leukocytes and endothelial cells. Through this direct interaction with ADAM17, CD9 inhibits the sheddase activity of this metalloproteinase against its substrates, thus regulating the balance between the membrane and soluble forms of crucial adhesion molecules, such as ICAM-1 and ALCAM, which are key in the processes of leukocyte extravasation and recruitment into inflamed tissues and stabilization of the IS.

These important regulatory effects exerted by CD9 on the activity of adhesion molecules with relevance in the immune system are summarized in the three panels of **Figure 1**.

AUTHOR CONTRIBUTIONS

CC conceived and wrote the manuscript. RR and CC designed the figures. BC, YM-P, and RR commented and edited the manuscript. All authors read and approved the final manuscript.

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MINI REVIEW
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Regulation of Leukocytes by TspanC8 Tetraspanins and the "Molecular Scissor" ADAM10

*Alexandra L. Matthews[1], Chek Ziu Koo[1,2], Justyna Szyroka[1], Neale Harrison[1], Aditi Kanhere[1] and Michael G. Tomlinson[1,2]**

[1] School of Biosciences, College of Life and Environmental Sciences, University of Birmingham, Birmingham, United Kingdom, [2]Centre of Membrane Proteins and Receptors (COMPARE), University of Birmingham, Birmingham,United Kingdom

A disintegrin and metalloproteinase 10 (ADAM10) is a ubiquitous transmembrane protein that functions as a "molecular scissor" to cleave the extracellular regions from its transmembrane target proteins. ADAM10 is well characterized as the ligand-dependent activator of Notch proteins, which control cell fate decisions. Indeed, conditional knockouts of ADAM10 in mice reveal impaired B-, T-, and myeloid cell development and/or function. ADAM10 cleaves many other leukocyte-expressed substrates. On B-cells, ADAM10 cleavage of the low-affinity IgE receptor CD23 promotes allergy and asthma, cleavage of ICOS ligand impairs antibody responses, and cleavage of the BAFF–APRIL receptor transmembrane activator and CAML interactor, and BAFF receptor, reduce B-cell survival. On microglia, increased ADAM10 cleavage of a rare variant of the scavenger receptor triggering receptor expressed on myeloid cells 2 may increase susceptibility to Alzheimer's disease. We and others recently showed that ADAM10 interacts with one of six different regulatory tetraspanin membrane proteins, which we termed the TspanC8 subgroup, comprising Tspan5, Tspan10, Tspan14, Tspan15, Tspan17, and Tspan33. The TspanC8s are required for ADAM10 exit from the endoplasmic reticulum, and emerging evidence suggests that they dictate ADAM10 subcellular localization and substrate specificity. Therefore, we propose that ADAM10 should not be regarded as a single scissor, but as six different scissors with distinct substrate specificities, depending on the associated TspanC8. In this review, we collate recent transcriptomic data to present the TspanC8 repertoires of leukocytes, and we discuss the potential role of the six TspanC8/ADAM10 scissors in leukocyte development and function.

Keywords: a disintegrin and metalloproteinase 10, tetraspanins, TspanC8s, leukocytes

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***Correspondence:**
Michael G. Tomlinson
m.g.tomlinson@bham.ac.uk

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INTRODUCTION

The proteolytic cleavage, or "shedding," of the extracellular region (ectodomain) of transmembrane proteins is an important mechanism for the regulation of leukocyte development and function. Shedding can initiate intracellular signal transduction *via* the cell-associated cleavage fragment (e.g., Notch signaling to drive cell fate decisions), downregulate signaling or adhesion that requires cell surface receptor expression, or activate paracrine signaling through the release of growth factors, cytokines, and chemokines (1). The ADAMs (a disintegrin and metalloproteinases) are one

of the main proteinase families that function as sheddases and can be regarded as "molecular scissors." A total of 22 ADAM genes have been identified in humans, of which 12 (ADAM8, 9, 10, 12, 15, 17, 19, 20, 21, 28, 30, and 33) are active proteases with the consensus sequence (HExGHxxGxxHD) required for Zn^{2+}-dependent protease activity (1).

THE "MOLECULAR SCISSOR" ADAM10

One of the best-characterized ADAMs is ADAM10, due to its essential role in ligand-dependent cleavage of Notch proteins to initiate Notch signaling (2). Indeed, ADAM10-knockout mice die at embryonic day 9.5, phenocopying double knockout mice for two of the four Notch proteins, Notch 1 and 4 (3, 4). ADAM10 has an N-terminal signal sequence, an inhibitory prodomain, a metalloproteinase domain, followed by disintegrin, cysteine-rich, transmembrane, and cytoplasmic domains (**Figure 1**). The prodomain is removed by proprotein convertases during biosynthesis to generate a mature sheddase (5). The first crystal structure of the mature ADAM10 ectodomain suggests that the metalloprotease exists in a closed conformation in which the cysteine-rich domain partially occludes the catalytic site, but with the catalytic site in position to cleave substrates close to the plasma membrane (6). ADAM10 has at least 40 substrates, including amyloid precursor protein (7), cellular prion protein (8), cadherins (9–11), and the platelet-activating collagen/fibrin receptor GPVI (12, 13). ADAM10 has a number of substrates that are expressed by leukocytes, or which impact on leukocyte function, including the low-affinity IgE receptor CD23 (14, 15), the endothelial cell–cell adhesion molecule vascular-endothelial (VE)-cadherin (11), the B-cell costimulatory molecule ICOS ligand (16), B-cell homeostasis proteins transmembrane activator and CAML interactor (TACI) (17) and BAFF receptor (BAFFR) (18), and triggering receptor expressed on myeloid cells 2

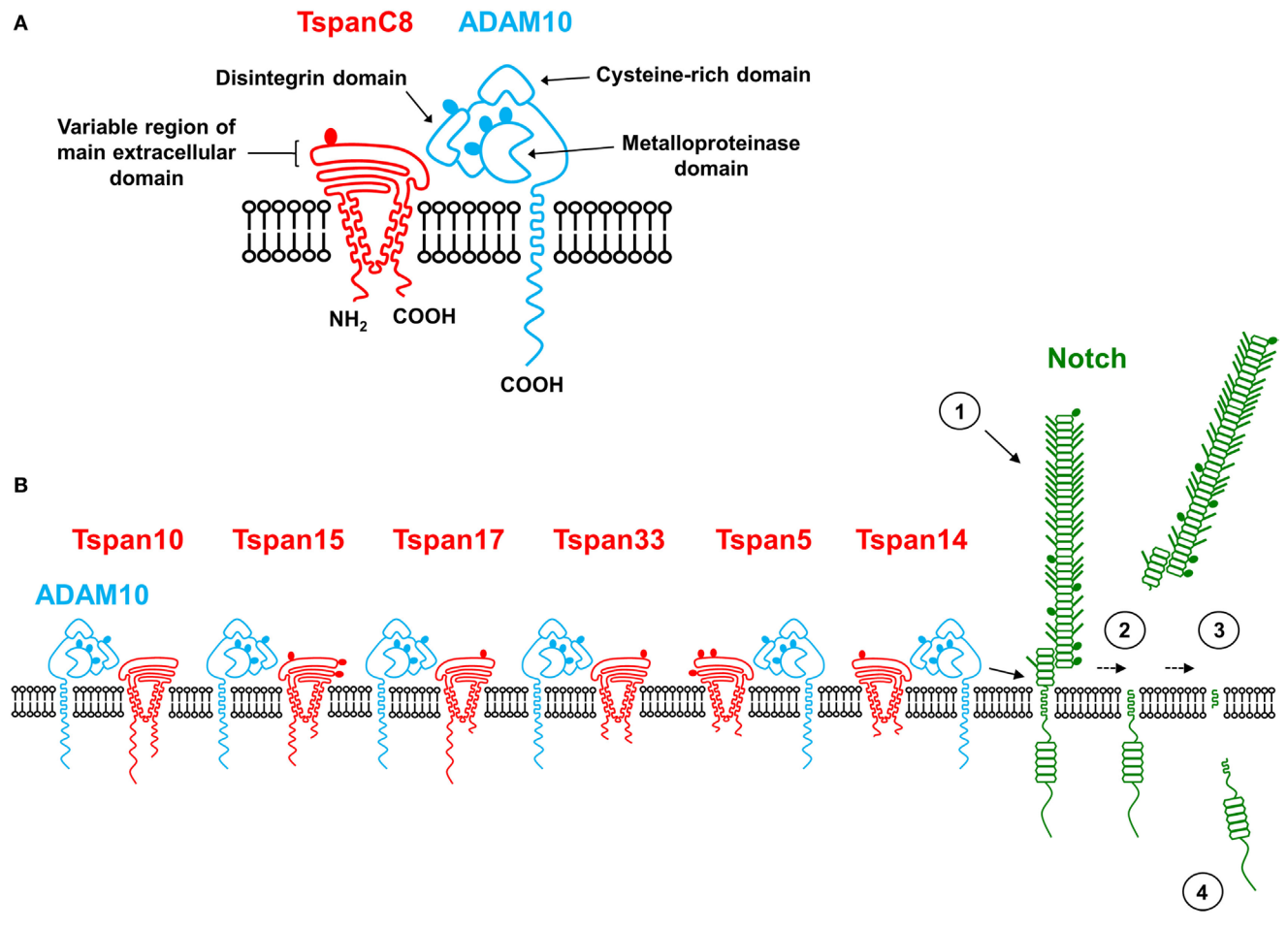

FIGURE 1 | Six ADAM10 scissors: Tspan14 and Tspan5 may be important for Notch activation in immune cells. **(A)** A schematic representation of a TspanC8 and ADAM10. **(B)** A model figure to show the six different TspanC8/ADAM10 complexes, which have different subcellular localizations, distinct substrate specificities, and may have different ADAM10 conformations. Notch cleavage is initiated following engagement with a Notch ligand on another cell (1), which induces a conformational change that we hypothesize allows cleavage by a Tspan14/ADAM10 or Tspan5/ADAM10 scissor (2), followed by intramembrane cleavage by γ-secretase (3), to release the intracellular domain to act as a transcription factor and drive cell fate decisions (4). Tspan10 has also been implicated in Notch activation, but its relatively low expression by immune cells suggests no substantial role in these cells. N-linked and O-linked glycosylation sites are indicated by filled ovals and short lines, respectively.

(TREM2) (19, 20). ADAM10 has been implicated in myriad immune diseases including T-cell acute lymphoblastic leukemia (T-ALL), asthma, atherosclerosis, and Alzheimer's disease (2).

TETRASPANINS AS "MEMBRANE ORGANIZERS"

The tetraspanins are a superfamily of transmembrane proteins expressed in multicellular eukaryotes. They are characterized by four transmembrane domains and small and large extracellular loops (**Figure 1**), the latter containing structurally important cysteine residues. Mammals express 33 tetraspanins and each cell type has a distinct repertoire of tetraspanins (21); leukocytes express at least 20 tetraspanins (22). Tetraspanins are "membrane organizers" that form dynamic nanoclusters (21). Visualization of tetraspanins CD37, CD53, CD81, and CD82 on human B-cells and dendritic cells, by super-resolution microscopy, suggests that approximately 10 tetraspanins of a single type cluster together into individual nanodomains (23). Tetraspanins also associate directly with specific non-tetraspanin proteins, termed "partner proteins," to regulate their intracellular trafficking, clustering, lateral mobility, and compartmentalization (21). Relatively well-studied tetraspanin–partner interactions are tetraspanin CD151 with the laminin-binding integrins (24), tetraspanin CD81 with the B-cell co-receptor CD19 (25), and Tspan12 with the Wnt/Norrin receptor Frizzled-4 (26). In each case, tetraspanin mutations yield phenotypes that are consistent with impaired partner protein function. For example, in CD81-deficient humans and mice, CD19 fails to traffic to the B-cell surface and antibody generation is impaired (25). The recently published crystal structure of full-length CD81, which is the first such structure for a tetraspanin, shows CD81 to be cone shaped with an intramembrane cholesterol-binding cavity within the transmembranes (27). Molecular dynamics simulations suggest that CD81 may exist in two different conformations, a closed conformation when cholesterol is present, and an open conformation when cholesterol is absent, in which the large extracellular loop swings upwards (27). Therefore, tetraspanins could function as "molecular switches" that control the activity of their partner proteins through cholesterol-regulated conformational change.

TSPANC8 TETRASPANINS REGULATE ADAM10: THE "SIX SCISSOR" HYPOTHESIS

In 2012, we and others showed that ADAM10 interacts with six tetraspanins that are closely related by protein sequence: Tspan5, Tspan10, Tspan14, Tspan15, Tspan17, and Tspan33 (28–30). We termed these the TspanC8 subgroup due to the eight cysteine residues in their large extracellular loops (28, 29); other tetraspanins have four, six, or seven cysteines. The TspanC8s are essential for promoting ADAM10 exit from the endoplasmic reticulum, its subsequent maturation in the Golgi through removal of the prodomain, and trafficking to the cell surface or other membrane compartments (28–30). The functional association between TspanC8s and ADAM10 has been demonstrated in TspanC8-knockout mice (29, 31) and the fruit fly *Drosophila* (28), and is reinforced by recent data demonstrating reciprocal regulation of Tspan5 exit from the endoplasmic reticulum by ADAM10 (32). Moreover, emerging evidence indicates that each TspanC8 can target ADAM10 to different subcellular localizations and to different substrates, and ADAM10 may adopt distinct conformations dictated by the associated TspanC8 (28, 32–35). For example, we and two other groups reported Tspan15 as the only TspanC8 to promote ADAM10 cleavage of neuronal (N)-cadherin (30, 33, 34). These *in vitro* data are supported by data from the recently characterized Tspan15-deficient mouse, which has strikingly reduced N-cadherin cleavage in the brain, despite only a subtle decrease in mature ADAM10 expression (31). ADAM10 shedding of Notch is promoted by Tspan5, Tspan10, and Tspan14 (**Figure 1**), but not by Tspan15 and 33 (28, 32, 33, 36). In addition, we have shown Tspan5 and Tspan17 to regulate VE-cadherin expression on endothelial cells (35). Taking these data together, we now propose that ADAM10 should be regarded as six different TspanC8/ADAM10 scissor complexes, rather than a single scissor (37, 38). This idea has implications for therapeutic targeting of ADAM10, which may be impractical due to toxic side effects. However, targeting one of the TspanC8/ADAM10 complexes, *via* the tetraspanin, may minimize toxicity while enabling substrate- and disease-specific targeting.

We and others have recently reviewed our current understanding of how TspanC8s regulate ADAM10 (37–39). In this review, we will analyze and present published RNA-Seq transcriptomic data for TspanC8 expression in different leukocyte subsets. We will discuss these expression profiles in the context of our current knowledge of ADAM10's role in the development and function of T-cells, B-cells, and myeloid cells.

REGULATION OF T-CELL DEVELOPMENT AND TRANSMIGRATION BY ADAM10 AND TSPANC8s

Two publications have shown that ADAM10 is important for normal T-cell development, most likely through regulation of Notch signaling (40, 41). In the first, the embryonic lethality of ADAM10-knockout mice was circumvented by the generation of transgenic mice that express dominant negative ADAM10 under the control of the T-cell-specific Lck promoter (40). The dominant negative ADAM10 construct yields a similar phenotype to T-cell-specific deletion of Notch1, the Notch family member with the major role in thymocyte development. Thymocyte numbers are reduced by 60–90% due to a partial block in the CD4/CD8 double negative to double positive transition, with accompanying reduction in expression of T-cell receptor (TCR) β (40). Defective Notch signaling is the probable mechanism, since expression of Notch-responsive genes is partially reduced, and partial rescue is achieved by transgenic overexpression of the Notch ligand Delta-1, or a dominant active form of Notch1, in thymocytes. The dominant negative construct lacks the metalloproteinase domain and is expressed at several-fold higher levels than endogenous ADAM10 (40); one mechanism of action may be the sequestration of endogenous TspanC8s,

since we have shown such a construct to interact with TspanC8s (34). In the second publication, conditional T-cell-specific ADAM10-knockout mice were generated by crossing ADAM10 floxed mice with mice expressing Cre recombinase driven by the Lck promoter (41). This model phenocopies T-cell-specific Notch1 deletion in showing a twofold to threefold reduction in thymocyte numbers, due to a partial block in development from the double negative to double positive stage. There is reduced expression of Notch target genes but, unlike the dominant negative ADAM10 model, no defect in TCRβ expression is observed (41).

The most highly expressed TspanC8 in human and mouse T-cells is Tspan14, followed by Tspan5 and Tspan17; Tspan15 is also expressed by human T-cells but not mouse (**Figures 2A,B**). However, it is important to note that such publically available transcriptomic data have not been independently validated, nor have the expression profiles been confirmed using validated antibodies. Nevertheless, since both Tspan14 and Tspan5 promote Notch signaling (28, 32, 33), we hypothesize that Tspan14/ADAM10 will have a major role in thymocyte development *via* activation of Notch1, while Tspan5/ADAM10 may have a minor role. The future analyses of the respective knockout mice will help to test this hypothesis; the Tspan14-knockout mouse has yet to be made, while the Tspan5-knockout mouse is viable but functionally uncharacterized (32). It is possible that Tspan14 and/or Tspan5 play a role in the aggressive blood cancer T-ALL. Approximately 50% of T-ALL is driven by activating mutations in Notch1, some of which require ADAM10 for full activation in a ligand-independent manner. Knockdown of ADAM10 reduces Notch signaling and T-ALL proliferation (42). Targeting Tspan14 or Tspan5 may achieve a similar result, yet without the toxicity of global ADAM10 inhibition. Interestingly, antibody targeting of Tspan5 can impair Notch signaling (32).

In addition to Notch proteins, a number of other proteins have been reported to be cleaved by ADAM10 on T-cells: CD40 ligand (44), Fas ligand (45, 46), LAG-3 (47), CD44 (48), and T-cell immunoglobulin and mucin domain 3 (Tim-3) (49). How important these cleavage events are to T-cell function has yet to be determined, and for CD40 ligand, LAG-3 and Tim-3 is complicated by their additional cleavage by ADAM17 (44, 47, 49), an ADAM10-related metalloproteinase.

We have recently reported that endothelial cell-expressed ADAM10 promotes the transmigration (also known as extravasation or diapedesis) of T-cells in an *in vitro* model of inflammation (35). The mechanism involves ADAM10 regulation of VE-cadherin expression levels, since ADAM10 knockdown results in 50% elevated VE-cadherin expression and delayed transmigration, which is rescued by partial VE-cadherin knockdown to wild-type levels. This promotion of transmigration by endothelial ADAM10 appears restricted to T-cells, since no effects on neutrophils or B-cell transmigration are observed (35), nor on monocytes in a separate study (50). Among the TspanC8s, we have previously reported that Tspan14 is the most highly expressed on endothelial cells (29, 51). However, knockdown experiments show that Tspan5 and Tspan17 are the tetraspanins that promote ADAM10 regulation of VE-cadherin (35). Consistent with this common function, Tspan5 and Tspan17 are the most highly conserved pair of tetraspanins, with 72% protein sequence identity in human (35). However, it remains to be determined whether this role for Tspan5/17 holds true *in vivo*. Tspan17-knockout mice have yet to be made; ultimately a Tspan5/17 double knockout may be required to overcome functional redundancy.

REGULATION OF B-CELL DEVELOPMENT AND FUNCTION BY ADAM10 AND POTENTIAL ROLE FOR TSPANC8s

An important role for ADAM10 in B-cell development and function has been demonstrated using B-cell-specific ADAM10-knockout mice, made by crossing ADAM10 floxed mice with CD19-Cre mice (52). Early development of B-cells in the bone marrow of these mice is unaltered, with normal numbers of pro-, pre-, and immature B-cell populations. B1 cell numbers in the peritoneal cavity are also normal. However, ADAM10-knockout immature B-cells entering the spleen fail to develop into marginal zone B-cells; these cells act as a first line of defense by rapid antibody generation against blood-borne pathogens that become trapped in the spleen. By contrast, follicular zone B-cell numbers in the spleen are slightly elevated. The mechanism underlying defective marginal zone B-cell development in the absence of ADAM10 appears to be defective Notch2 activation (52), and consistent with this, B-cells express relatively high levels of Notch2 and low levels of Notch 1, 3, and 4, and Notch2 is critical for development of marginal zone but not follicular B-cells (53–55).

B-cell-specific ADAM10-knockout mice have a striking reduction in antibody responses following immunization, associated with impaired germinal center formation in secondary lymphoid tissues (56). The mechanism responsible appears to be the upregulated expression of TNFα, a pro-inflammatory cytokine which is important for the maintenance of lymphoid tissue architecture, and ADAM17, which sheds TNFα (57). How ADAM10-deficiency leads to ADAM17 and TNFα upregulation in B-cells remains unknown. To investigate the function of ADAM10-knockout plasma cells in the context of normal germinal centers, ADAM10 floxed mice were crossed with IgG1-Cre mice to delete ADAM10 post-isotype switching. Antibody responses are strikingly reduced, despite normal plasma cell numbers (58). This may be due to elevated expression of ICOS ligand, a recently identified ADAM10 substrate, on ADAM10-knockout B-cells (16). The engagement of ICOS ligand with its receptor ICOS on T-cells is required for T-cell-dependent antibody responses. In ADAM10-knockout B-cells, elevated ICOSL causes a substantial reduction in surface ICOS by promoting its internalization (16), thus providing a mechanism for impaired antibody responses.

With the possible exception of Notch2, the best-studied ADAM10 substrate on B-cells is CD23, the low-affinity IgE receptor, which regulates allergic and inflammatory responses (14, 15). Indeed, on ADAM10-knockout B-cells, CD23 expression is increased approximately threefold, while soluble CD23 levels in plasma are substantially reduced (52). In an IgE-dependent asthma model, B-cell-specific ADAM10-knockout mice have

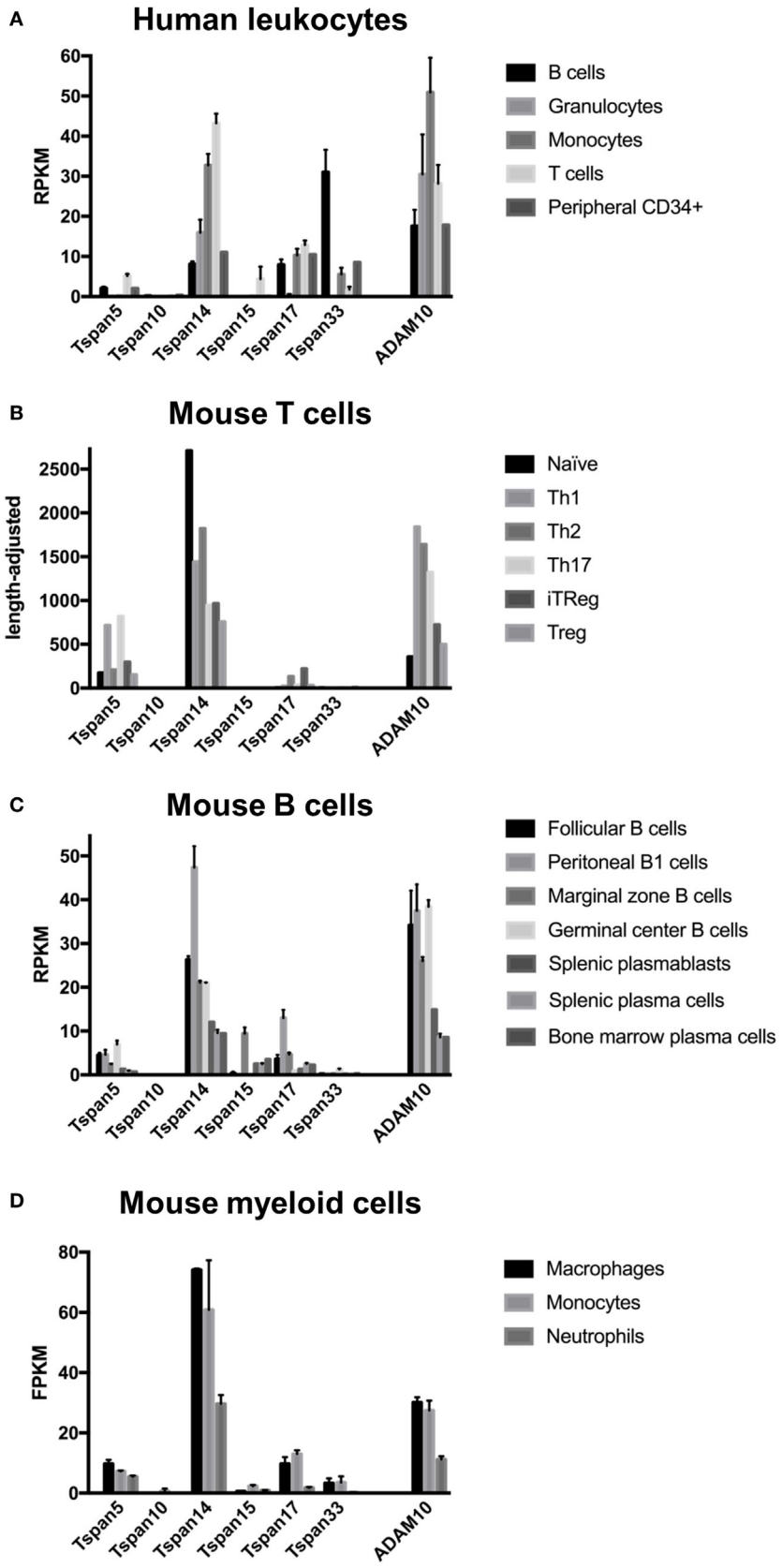

FIGURE 2 | Continued

FIGURE 2 | Leukocytes express ADAM10, but different cell subsets have distinct TspanC8 repertoires. Publically available RNA-Seq data for **(A)** human leukocytes [Gene Expression Omnibus (GEO) accession GSE51984], **(B)** mouse T-cell subsets (43), **(C)** mouse B-cells (GEO accession GSE60927), and **(D)** mouse macrophages, monocytes, and neutrophils (GEO accession GSE59831). Data are presented as reads per kilobase of transcript per million mapped reads (RPKM) **(A,C)**, as length-adjusted values that provide a measure equivalent to RPKM (43) **(B)**, or as fragments per kilobase of transcript per million mapped reads (FPKM) **(D)**. Error bars represent the SD. Number of samples are as follows: five for panel **(A)**, except for CD34+ peripheral cells (hematopoietic stem cells from the blood) which has one; two for panel **(C)**, except for splenic plasmablasts and bone marrow plasma cells which have one, and splenic plasma cells which have three; and two for panel **(D)**, with the exception of neutrophils which have two.

strikingly reduced signs of allergic inflammation in the lung (59). Allergic patients and allergy-prone Th2 mice have increased expression of ADAM10 on B-cells and increased soluble CD23 and IgE levels in plasma (60). Although the regulation of IgE expression by CD23 is complex and not fully understood, these data have lead the authors to propose ADAM10 as a therapeutic target for asthma (59).

ADAM10 is emerging as a regulator of the BAFF–APRIL system which controls B-cell homeostasis. The two ligands are BAFF and APRIL, while the three receptors are B-cell maturation antigen, TACI, and BAFFR, the latter of which binds BAFF but not APRIL. ADAM10 can shed TACI to release a soluble ectodomain that acts as a decoy receptor, binding to BAFF and APRIL and so inhibiting survival of B-cells (17). In addition, cell survival can be reduced by ADAM10 or ADAM17 shedding of BAFFR (18).

In human B-cells, Tspan33 expression is highest, followed by Tspan14, Tspan17, and then Tspan5 (**Figure 2A**). In mouse, Tspan14 expression is highest, followed by Tspan5, Tspan15, and Tspan17, while Tspan33 expression is minimal (**Figure 2C**). Therefore, Tspan14 is likely to be the main regulator of Notch2 in B-cells, with a less important role for Tspan5. A role for TspanC8s in regulating CD23 shedding has not been reported, but Tspan5, Tspan14, Tspan17, and/or Tspan33 are candidates; this is important work for the future, because such a tetraspanin is a potential therapeutic target for asthma. Tspan33 expression in human B-cells has been confirmed at the protein level (61), but the ADAM10 substrates that it regulates have not been investigated.

REGULATION OF MYELOID CELL FUNCTION BY ADAM10 AND POTENTIAL ROLE FOR TSPANC8s

Dendritic cell-specific ADAM10-knockout mice have been generated by crossing ADAM10 floxed mice with CD11c-Cre mice (62). These mice have strikingly impaired Th2 responses, but Th1 and Th17 responses are unaffected. As a consequence, the mice are protected from IgE-mediated anaphylaxis and allergic lung inflammation. This appears to be due to defective Notch signaling, since rescue is observed by transgenic expression of the Notch1 intracellular domain, and dendritic cell-specific Notch1-knockout mice have a similar phenotype (62). Our RNA-Seq analyses have found Tspan14 to be most highly expressed in human dendritic cells, with lower expression of Tspan17 and Tspan33 (data not shown). Therefore, Tspan14 is likely to be important in dendritic cells for Notch signaling.

Specific knockout of ADAM10 in myeloid cells has been investigated by crossing ADAM10 floxed mice with LysM-cre mice (63). This does not achieve complete knockout in myeloid cells, but surface levels of ADAM10 on bone marrow-derived macrophages are reduced by 85%. No major abnormalities in the mice are observed, and leukocyte populations are present in normal numbers. However, ADAM10-knockout results in a reduced inflammatory phenotype in macrophages and a reduced capacity to migrate and to degrade extracellular matrix (63). Macrophages play a central role in the initiation and progression of the inflammatory disease atherosclerosis, and can take up lipids to become pro-inflammatory foam cells within atherosclerotic plaques. To investigate the role of macrophage ADAM10 in this disease, bone marrow from myeloid-specific ADAM10-knockout mice was transplanted into atherosclerosis-prone low-density lipoprotein receptor knockout mice. Consistent with a less inflammatory macrophage phenotype, atherosclerotic plaques appear more stable, with higher collagen content, although plaque size is similar to wild type (63). Nevertheless, this has important implications for human disease; stable plaques are less susceptible to the rupture that causes thrombosis, vessel occlusion, and heart attack or stroke. Interestingly, Notch signaling promotes an inflammatory macrophage phenotype, and blockade of Notch signaling in an atherosclerosis model reduces atherosclerosis development while increasing plaque stability (64). Macrophages express relatively high levels of the Notch-promoting Tspan14 (**Figures 2A,D**), highlighting the macrophage Tspan14/ADAM10 complex as a potential therapeutic target for maintaining plaque stability in atherosclerosis.

An additional study generated mice with leukocyte- and myeloid-specific ADAM10 deficiency by crossing floxed ADAM10 mice with Vav-Cre and LysM-Cre mice, respectively. In an inflammatory lung model, neutrophil and monocyte recruitment are reduced by approximately 50% in the absence of ADAM10 (65). The underlying mechanism is not clear. Tspan14 appears to be the only TspanC8 expressed by human granulocytes and is also highly expressed by mouse neutrophils, which have relatively weak Tspan5 expression (**Figures 2A,D**). Therefore, Tspan14/ADAM10-induced Notch activation may potentially promote neutrophil inflammatory responses.

Finally, the scavenger receptor TREM2 has been recently identified as an ADAM10 substrate with a potential role in Alzheimer's disease (19, 20). TREM2 is expressed on macrophages, microglia, osteoclasts, and dendritic cells. A rare H157Y variant of TREM2 is associated with increased risk of Alzheimer's disease; amino acid 157 is at the cleavage site for ADAM10, and H157Y is shed more readily. The loss of TREM2 renders macrophages less phagocytic, and the authors propose that this renders the individual more prone to Alzheimer's disease (19, 20). It will be

interesting to determine if any macrophage-expressed TspanC8, namely Tspan5, Tspan14, Tspan17, or Tspan33, can promote TREM2 cleavage, and could thus be considered a therapeutic target for TREM2-associated Alzheimer's disease.

CONCLUDING REMARKS

The six scissor hypothesis suggests that ADAM10 should be studied in the context of its regulatory tetraspanins. In leukocytes, the relatively high expression of Tspan14, together with its capacity to promote Notch activation, suggest that the Tspan14/ADAM10 complex may be critical for leukocyte development and function. The future analyses of cells and mice deficient in Tspan14, and other TspanC8s, will determine which scissor cleaves which substrates. This may direct therapeutic targeting of individual TspanC8/ADAM10 complexes, using antibodies or small molecules, to modulate a specific substrate while avoiding the toxicity of global ADAM10 targeting. Such an approach could provide new treatments for ADAM10-associated diseases, including T-ALL, asthma, atherosclerosis, and Alzheimer's disease.

AUTHOR CONTRIBUTIONS

AM, CK, JS, NH, and MT: conception, design, and writing of the manuscript. AK: data analysis and editing of the manuscript.

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REVIEW
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Tetraspanins as Organizers of Antigen-Presenting Cell Function

Maria Laura Saiz[1,2], *Vera Rocha-Perugini*[1,2]* *and Francisco Sánchez-Madrid*[1,2,3]*

[1] Servicio de Inmunología, Hospital de la Princesa, Instituto de Investigación Sanitaria La Princesa, Madrid, Spain,
[2] Vascular Pathophysiology Research Area, Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain,
[3] CIBER Cardiovascular, Madrid, Spain

Professional antigen-presenting cells (APCs) include dendritic cells, monocytes, and B cells. APCs internalize and process antigens, producing immunogenic peptides that enable antigen presentation to T lymphocytes, which provide the signals that trigger T-cell activation, proliferation, and differentiation, and lead to adaptive immune responses. After detection of microbial antigens through pattern recognition receptors (PRRs), APCs migrate to secondary lymphoid organs where antigen presentation to T lymphocytes takes place. Tetraspanins are membrane proteins that organize specialized membrane platforms, called tetraspanin-enriched microdomains, which integrate membrane receptors, like PRR and major histocompatibility complex class II (MHC-II), adhesion proteins, and signaling molecules. Importantly, through the modulation of the function of their associated membrane partners, tetraspanins regulate different steps of the immune response. Several tetraspanins can positively or negatively regulate the activation threshold of immune receptors. They also play a role during migration of APCs by controlling the surface levels and spatial arrangement of adhesion molecules and their subsequent intracellular signaling. Finally, tetraspanins participate in antigen processing and are important for priming of naïve T cells through the control of T-cell co-stimulation and MHC-II-dependent antigen presentation. In this review, we discuss the role of tetraspanins in APC biology and their involvement in effective immune responses.

Keywords: tetraspanins, tetraspanin-enriched microdomains, antigen-presenting cells, immune receptors, cell migration, antigen presentation

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***Correspondence:**
Vera Rocha-Perugini
verapfrp@gmail.com;
Francisco Sánchez-Madrid
fsmadrid@salud.madrid.org

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INTRODUCTION

Professional antigen-presenting cells (APCs), which include dendritic cells (DCs), monocytes/macrophages, and B cells, are essential players of the immune system. Once an infection occurs, the innate immune system is stimulated, beginning the inflammation process to prevent the infection from spreading. Then, adaptive immune responses are required for the effective and specific clearance of the pathogen. This vital task lies on APCs, which operate at the interface between the innate and adaptive immunities. First, APCs detect foreign pathogens thanks to specialized receptors, known as pattern recognition receptors (PRRs). PRRs recognize conserved repeated motifs in microbial species, called pathogen associated molecular patterns (PAMPs), and enable APCs to discriminate between self and non-self (1). After engulfment of exogenous pathogens, APCs use their unique machinery to break down molecular antigens into small peptides and present a representative repertoire of these through a specialized immune receptor, namely, the major histocompatibility complex class II (MHC-II) molecule. This process triggers APC activation and maturation, with

upregulation of surface expression of MHCII and co-stimulatory molecules. APC migration from peripheral tissues to secondary lymphoid organs is a key step for the generation of proper adaptive immunity, since antigen presentation to naïve T lymphocytes by APCs takes place primarily in secondary lymphoid organs (2). DCs have been extensively characterized and different subsets have been described (3, 4). Moreover, these cells precisely alternate their sentinel capacities with their antigenic presentation properties to favor antigen detection and migration, and antigen processing and presentation.

Tetraspanins belong to a family of small proteins (20–30 kDa) that contain four transmembrane regions spanning the plasma membrane. They also share other structural features: a small and a large extracellular loop with conserved residues, and short N- and C- terminal tails (5). In humans and mice, 33 tetraspanin members have been identified. These proteins are widely distributed in cells and tissues. Some of them are ubiquitous (CD81, CD82, CD9, or CD63), whereas others have a tissue-restricted expression (CD37 or CD53 in immune cells) (6). Tetraspanins do not have the characteristics of prototype membrane receptors. They have small cytoplasmic tails that lack known motifs involved in signal transduction (5), and there are only few reports claiming tetraspanin ligands (7). Instead, tetraspanins function as molecular organizers of multimolecular membrane complexes, which facilitate signal transduction processes (8). Through the association with proteins and lipids, they organize specific membrane microdomains with a particular composition and detergent-solubilization properties, conforming the so-called tetraspanin-enriched microdomains (TEMs) (9, 10). TEMs are distinct from other well-known membrane domains, like lipid rafts, caveolae, and GPI-linked protein nanodomains (10).

Early studies using biochemical approaches have shown that TEMs follow a hierarchical network of associations based on the strength of the interactions (5, 9). The first level comprises the direct and specific interaction of a tetraspanin with its protein partner and is resistant to strong detergent conditions. The second level is characterized by interactions between tetraspanins. These interactions are more labile, resistant to mild detergents, and regulated by palmitoylation. Cutting edge fluorescence microscopy techniques, as single-molecule tracking, phasorFLIM-FRET and super-resolution microscopy, have more recently demonstrated that TEM organization and composition is highly dynamic (10–14). Accordingly, several studies have suggested that TEM composition can differ between cells. Through the organization of TEMs, tetraspanins regulate the function of their associated partners, finely tuning a breadth of biological processes. They may have overlapping functions in some cases or can have unique roles or even opposing functions. Their importance for several pathological and physiological processes has been discussed in detail elsewhere (15–22).

Tetraspanins have been widely studied in the mammalian immune system, and thanks to the generation of tetraspanin knockout mice a deeper comprehension is being achieved. Interestingly, the existence of tetraspanins in the innate immune system of invertebrates and non-mammalian vertebrates has also been described. Marine gastropod mollusks show ubiquitous expression of CD63 and Tspn33, which are upregulated upon different immune stimulation challenges, like toll-like receptor (TLR) ligands, bacteria or viral infection (23). Similarly, CD9 expression is induced in lamprey fish after LPS stimulation (24), or in turtles after bacterial infection (25). CD37 expression is highly increased in Atlantic salmons after a secondary viral infection (26). Conversely, treatment with several immune stimulators downregulate CD9, CD53, and CD63 expression in leukocytes from teleost fishes (27, 28). The study of the innate defense mechanisms in non-mammalian vertebrates can give additional hints for the comprehension of vertebrate innate immunity. In mammals, tetraspanins are master regulators of APC function, mediating the crosstalk between the immunogenic environment and APCs, and the interplay between innate and adaptive immune cells.

Herein, we will review the function of tetraspanins in regulating each step of APC function: at the cellular level, by modulating clustering and trafficking of immune receptors; during the process of APC migration, and finally during MHC-II-dependent antigen presentation. We will also discuss the growing evidence on tetraspanins as markers of specific DC subsets.

Tetraspanins, Negative Regulators of PRRs

Recognition and uptake of microbial antigens by APCs is mediated by PRRs, which bind conserved pathogen structures known as PAMPs (1). Membrane-bound PRRs include TLRs, C-type lectin receptors (CLRs), scavenger receptors (SRs) and NOD-like receptors. The efficiency of antigen recognition greatly depends on the supramolecular organization of PRRs at the APC surface, and tetraspanins play an important role in this process (**Figure 1**).

Toll-like receptors multimerization at the APC surface promotes the recruitment of signaling molecules (29), a process influenced by the inclusion of TLRs and associated co-receptors into TEMs. LPS stimulation triggers TLR-4 and CD81 co-clustering in peripheral blood monocytes (30). How CD81 regulates TLR-4 signaling has not been assessed; however, it has been shown that CD9 restricts LPS-induced macrophage activation and TNF-α production by preventing the TLR-4 co-receptor CD14 localization into lipid rafts (**Figure 1**). Through this mechanism, CD9 deficiency in mice enhances macrophage infiltration and lung inflammation after *in vivo* intranasal LPS administration (31). In DCs, bacterial antigens can be recognized by TLR-dependent pathways, sensing cell surface or endosomal antigens, and by cytosolic pathways, like the cytosolic sensor stimulator of IFN genes (STING) (32). Interestingly, CD81 negatively regulates STING/IFNAR signaling through its interaction with Rac1 and the inhibition of STAT-1 activation, thus leading to reduced TNF-α and NO production by inflammatory monocytes and DCs (**Figure 1**). As a consequence, CD81 deficient mice are protected against systemic *Listeria monocytogenes* infection (33).

Among the CLRs, Dectin-1 specifically recognizes β-glucans in fungal cell walls and is important for efficient immune response against fungi (34). Dectin-1 associates with tetraspanins CD37 and CD63 at the membrane of APCs when using CHAPS 1% (35, 36), a mild detergent extraction condition that only keeps third level molecular interactions within TEMs (7, 10). Dectin-1 direct association with CD37 was however observed in transfected HEK293 cells when using Triton X-100 1%, which preserves

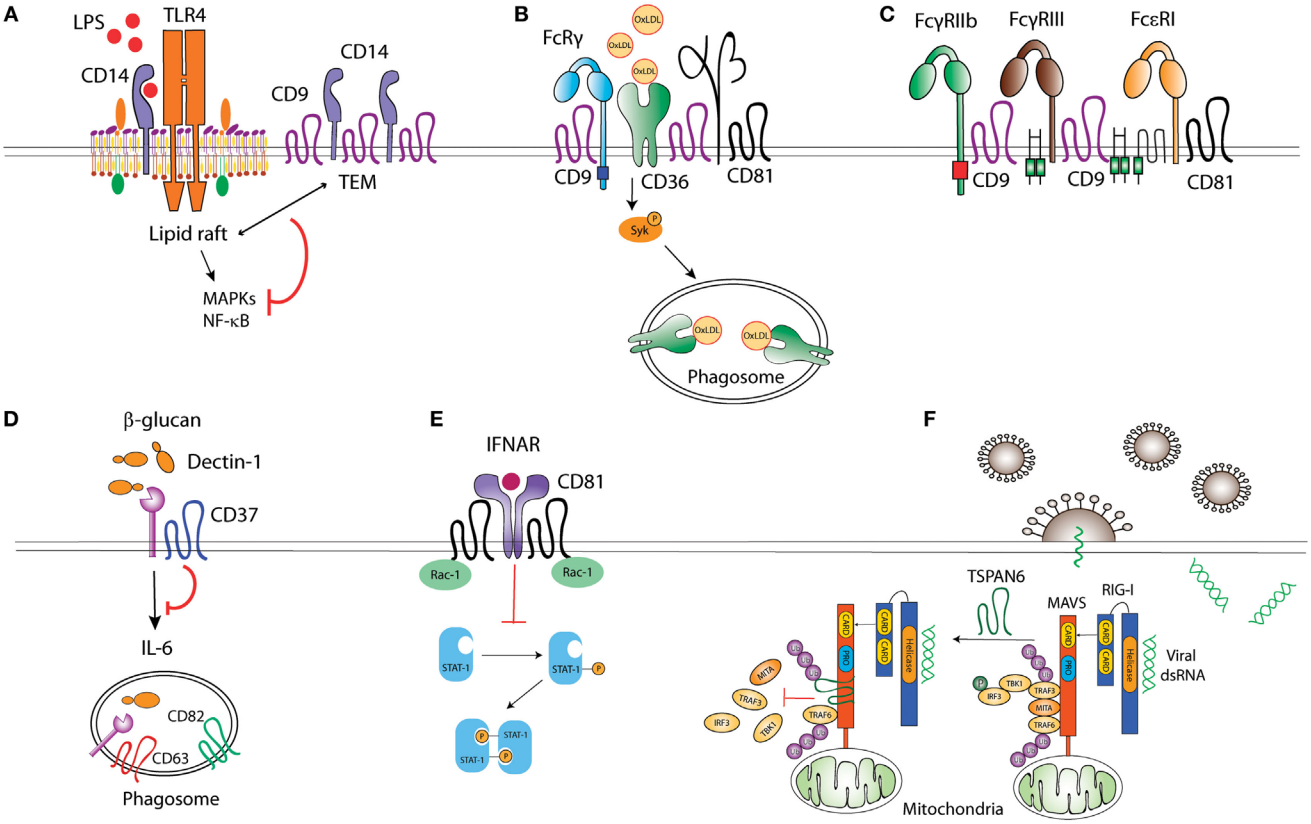

FIGURE 1 | Tetraspanins act as molecular organizers of pattern recognition receptors (PRRs). **(A)** CD9 sequesters TLR4 co-receptor CD14 into tetraspanin-enriched microdomains (TEMs), impairing its localization into lipid rafts, and thus preventing TLR4 downstream signaling. **(B)** CD36 coupling to the adaptor FcRγ is mediated by the complex formed between CD36, β1, and/or β2 integrins, and CD9 and/or CD81 tetraspanins. CD36 association with tetraspanins regulates its engagement with Src and Syk kinases, and its internalization when bound to ligands like oxidized low-density lipoproteins. **(C)** CD9 collaborates with FcγRIIb and FcγRIII upon macrophage activation, and, together with CD81, associates with FcεRI. **(D)** CD37 stabilizes membrane C-type lectin receptor Dectin-1 and inhibits IL-6 production. **(E)** CD81 interaction with Rac-1 prevents STAT-1 activation downstream of interferon-α/β receptor (IFNAR) stimulation. **(F)** The interaction between ubiquitinated Tspan6 and MAVS (mitochondrial antiviral signaling) interferes with RIG-I (retinoic acid-inducible gene I)-induced recruitment of downstream molecules TRAF3, MITA, and IRF3 to MAVS after viral RNA detection.

tetraspanin-partner primary complexes, but not in B cells (36), indicating that this interaction could be affected by other proteins expressed on APCs or that it is dynamically dependent on the cell activation status. CD37 stabilizes Dectin-1 surface expression and impairs its internalization, and Dectin-1-mediated TNF-α and IL-6 production in response to yeast cell walls (36) (**Figure 1**). Accordingly, CD37$^{-/-}$ mice are protected against systemic *Candida albicans* infection, producing high levels of IL-6 and specific IgA antibodies (37). On the other hand, CD37 mRNA expression positively correlates with Dectin-1 and IL-6 mRNA in brains of mice infected with *Toxoplasma gondii* (38); however, further studies are necessary to evaluate this effect at the protein level and if there is any causal relationship. CD63 also seems to cooperate with Dectin-1 during yeast phagocytosis by human monocyte-derived DCs (MoDCs) (35), being specifically recruited to phagosomes containing *Cryptococcus neoformans* (39) in a process dependent on acidification and thought to be required for tethering the antigen-loading machinery together.

CD36 is a SR that recognizes proteinaceous or lipidic antigens from microbes, or self-ligands. In mouse macrophages, CD81 and CD9 are required for CD36 internalization after binding to oxidized low-density lipoprotein (oxLDL) ligands (40, 41). CD9 would be important for signaling in response to oxLDL, since oxLDL uptake and subsequent JNK phosphorylation are impaired in CD9$^{-/-}$ macrophages (40). Moreover, CD9 and CD81-dependent scaffolding of CD36, and β1 and β2 integrins in membrane multimolecular complexes is essential for CD36 association with FcγR (Fc receptor for IgG) and with Src and Syk kinases; and for its subsequent antigen uptake (41) (**Figure 1**). CD9 is also associated with the scavenger-like receptor CD5, which recognizes β-glucans expressed on fungi (42), although there is no experimental evidence about the functional implications of this interaction.

Pathogens can be opsonized with IgGs produced in response to microbial invasion, and recognized by FcγRs associated with PRRs. This combined stimulation triggers cytokine production and pathogen-specific innate immune responses. FcγRs seem to be included in TEMs in phagocytic cells. CD9 antibody cross-linking, but not Fc fragment alone, stimulates intracellular signaling dependent on FcγRIIB and FcγRIII, thus promoting mouse macrophage

activation (43). Antibody cross-linking of tetraspanin CD82 enhances FcR-dependent activation of intracellular signaling in human monocytic cell lines (44). Importantly, IgG-opsonized HIV-1 particles are targeted to TEMs in endosomes of immature DCs (45). Other Fc receptors are also associated with TEMs, as the FcεRI (Fc receptor for IgE), which is a molecular partner of CD9 and CD81 in human monocytes and skin-derived DCs (46) (**Figure 1**). The importance of TEMs as organizers of FcεRI signalosome in mast cells has been recently reviewed elsewhere (47).

Tetraspanins can also regulate signaling of cytoplasmic PRRs, like the RIG-I-like receptors (RLRs). RLRs recognize viral RNA and trigger signaling pathways that induce type I IFN responses (48). In the presence of viral RNA, ubiquitination of human tetraspanin 6 (Tspn6) promotes its interaction with RIG-I, MDA5, and mitochondrial antiviral signaling (MAVS) signalosome, impairing the activation of IFN-stimulated response element (ISRE), NF-κB, and IFN-β promoters (49) (**Figure 1**).

In summary, increasing evidence shows that tetraspanins usually act as negative regulators of PRR clustering and/or signaling. Thus, tetraspanins constitute key players to avoid uncontrolled immune responses, which are harmful to the host.

Tetraspanins Tightly Control APC Migration

Leukocyte migration is of fundamental importance for the efficient development of immune responses against pathogens. Innate immune cells capture antigens in peripheral tissues and then migrate to secondary lymphoid organs where antigen presentation to T lymphocytes takes place. Immune cells can also migrate out of the bloodstream toward the inflammation site, where adaptive immune responses occur (**Figure 2**). Thus, leukocytes modify their adhesive properties depending on the immune scenario (50). Innate immune cells usually need inflammation signals to initiate migration, whereas naïve lymphocytes efficiently migrate to secondary lymphoid organs, and after activation signals acquire specific migratory patterns. Tetraspanins have emerged as key regulators of cell migration, since they modulate the function of proteins involved in cell-cell adhesion, cell-ECM (extracellular matrix) adhesion, cytoskeletal protrusion/contraction, and proteolytic ECM remodeling. Indeed, tetraspanins associate with integrins, cadherins, members of the Ig superfamily, signaling molecules like Rac and Rho GTPases, and matrix metalloproteinases (MMP); regulating their membrane compartmentalization, intracellular trafficking, and proteolytic activity. Most of the information on tetraspanin regulation of cell migration comes from studies with adherent and tumor cells and has been reviewed in detail (20, 51). In this section, we will delineate the importance of tetraspanins for migration and extravasation of APCs.

Early studies employed cross-linking with monoclonal antibodies (mAbs) to investigate the role of tetraspanins in immune cell migration. Human MoDC *in vitro* migration toward MIP-5 and MIP-1α chemokines was increased by the treatment with mAbs against CD9, CD63, CD81, or CD82 (35). These chemokines are

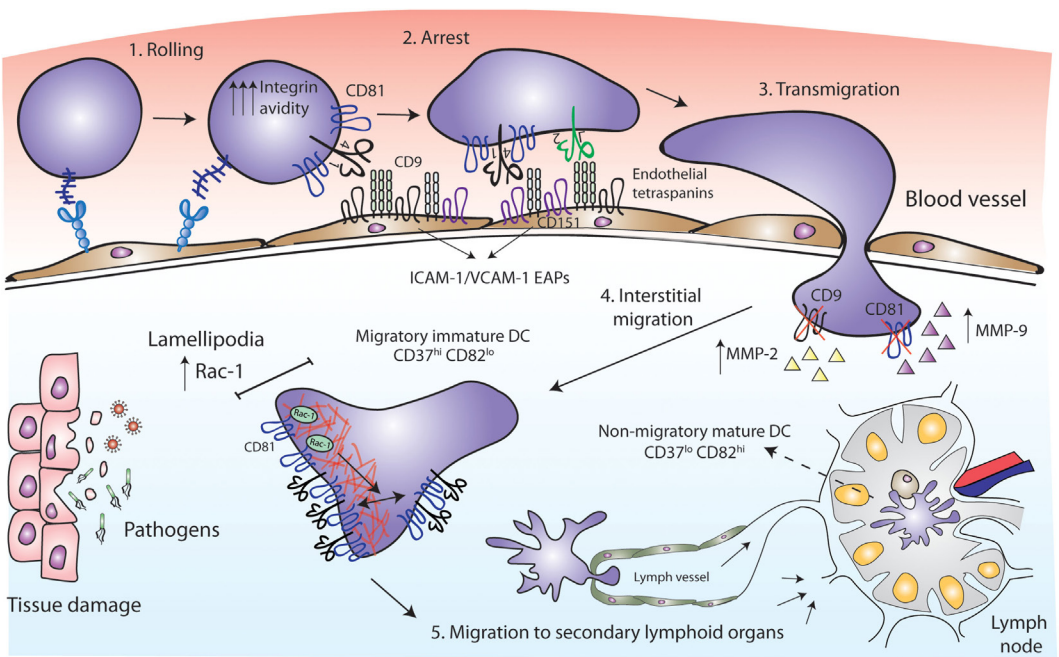

FIGURE 2 | Tetraspanins act as key players in antigen-presenting cell (APC) migration. CD81 facilitates rolling and arrest under shear flow, increasing the avidity of VLA-4 integrin. Tetraspanins CD9 and CD151 congregate the endothelial adhesion receptors (ICAM-1 and VCAM-1) in clusters called endothelial adhesive platforms, thus controlling their adhesive properties and leukocyte extravasation. CD9 and CD81 deficiency results in an increase of MMP-2 and MMP-9 metalloproteinases production and activity, required for interstitial migration. Once in the tissue, CD81 tetraspanin controls cell migration *via* Rac-1-dependent mobilization of preformed integrin clusters at the leading edge and contributes to the formation of lamellipodia. While migratory immature dendritic cells (DCs) are $CD37^{hi}$ and $CD82^{lo}$, mature DCs at the lymph nodes are $CD37^{lo}$ $CD82^{hi}$, and display reduced migratory capacity and efficient antigen presentation machinery.

strong chemoattractants required for the recruitment of inmature DCs to the surrounding tissue at the sites of injury (52, 53). After antigen capture, DCs mature, lose their responsiveness to inflammatory chemokines and express CCR7 (54, 55). CCR7 is the receptor for CCL19 and CCL21, which are chemokines highly present in lymphoid T-cell zones of secondary lymphoid organs (56), where DCs home to present their processed antigen to T lymphocytes. Opposite to that observed with MoDC migration toward MIP-5 and MIP-1α, the same mAb against CD81 (clone JS-81) or a CD81 ligand [the Hepatitis C Virus E2 envelope glycoprotein (57)] inhibited MoDC migration in response to CCL21 *in vitro* (58). These contradictory results could be due to different chemokine stimuli or to technical issues. Subsequent studies were all in line with a positive role for tetraspanins in cell migration. Monocyte transmigration across brain endothelial cell monolayers was significantly inhibited by an anti-CD9 mAb and several anti-CD81 mAbs, in both rodent and human *in vitro* models, by acting on the leukocyte side and on endothelial tetraspanins (59, 60). Accordingly, CD81 mAb (clone Eat2) administration reduced spinal cord inflammation *in vivo*, alleviating autoimmune encephalomyelitis (EAE) (59). Ly6C$^+$ monocytes, which can derive in MoDCs (61), are key determinants for Th17 differentiation in the EAE mouse model (62, 63). Moreover, since ICAM-1 and VCAM-1 adhesion molecules are the ligands of leukocyte integrins Mac-1 (αMβ2) and LFA-1 (αLβ2) (for ICAM-1) and VLA-4 (α4β1) (for VCAM-1), we must emphasize the importance of endothelial tetraspanins as organizers of ICAM-1 and VCAM-1 containing docking structures during leukocyte extravasation (11, 64). Importantly, loss-of-function studies have demonstrated that CD81 is essential for cell rolling, arrest, and migration. In both monocytic cell lines and mouse primary splenocytes, CD81 facilitates rolling and arrest under shear flow, increasing the avidity of integrin VLA-4 (65) (**Figure 2**). The link between tetraspanins and MMP during immune cell migration has also been investigated. Bone marrow-derived macrophages (BMDMs) from CD9 and CD81 double deficient mice show reduced motility, through a mechanism dependent on the regulation of MMP-2 and MMP-9 expression and activity (**Figure 2**). Interestingly, CD81 and CD9 double deficient mice spontaneously develop pulmonary emphysema, with elevated numbers of alveolar macrophages and increased MMP activity (66). A similar increase in MMP-2 and MMP-9 production and activity was observed in BMDMs from CD9-deficient mice, which showed decreased macrophage motility with an increase in macrophage infiltration after intranasal administration of LPS (31).

It is important to mention that DC motility behavior depends on the environmental context. DC migration on two-dimensional (2D) surfaces, like endothelial cell surfaces of the circulatory system, require adhesive forces and integrin functionality; whereas migration in three-dimensional (3D) environments, as interstitial ECM, is ameboid and less adhesive, and largely driven by cytoskeletal deformability (67–69). Importantly, tetraspanins fine-tune DC migratory capabilities by tightly controlling Rac1 and RhoA spatiotemporal activation. CD81 controls the migration of MoDCs, by regulating the formation of lamellipodia, and the mobilization of preformed integrin clusters at the leading edge of migratory cells (70). This tetraspanin is essential for the formation of actin protrusions through a mechanism dependent on its interaction with the small GTPase Rac-1 (70, 71) (**Figure 2**). Integrin adhesiveness and lamellipodia formation are required for DCs migration on 2D surfaces, thus this kind of migration is impaired in the absence of CD81. However, CD81 is not required for DCs migration within 3D collagen scaffolds, corresponding with unaffected Rho-A activity (70) and pointing out the differential molecular requirements of DCs migration. CD37 also promotes Rac-1 activation, while CD82 inhibits RhoA (72). Consequently, CD37 deficient DCs have impaired migration from the skin to the draining lymph nodes *in vivo*, and reduced *ex vivo* DC migration in response to CCL19 (73). CD82 deficient DCs display the opposite phenotype (72). Absence of CD37 in BMDCs also reduces adhesion to fibronectin under low shear flow, and cell spreading (73), while CD82 deficiency increases DC spreading (72). Thus, CD37^{hi}CD82^{lo} DCs would correspond to immature cells, showing increased migration and reduced capacity to activate naïve T cells, while CD37^{lo}CD82^{hi} DCs would have an activated phenotype, being less motile and endowed with the proper presentation machinery to efficiently activate naïve T cells (72) (**Figure 2**). It is becoming increasingly clear that through the regulation of cytoskeletal rearrangement, integrins, and signaling molecules, tetraspanins constitute key players in APCs migration.

MHC-II Trafficking and Antigen Presentation Take Place Within TEMs

Upon their arrival to the lymph nodes, DCs transfer the information collected at peripheral tissues to T lymphocytes triggering adaptive immune responses. This process of antigen presentation is mediated by MHC-II molecules, which are able to stably bind to antigenic peptides, and then present these fragments of exogenous proteins to effector T lymphocytes. MHC-II is expressed on professional APCs and associates with several tetraspanins, including CD9, CD37, CD53, CD81, and CD82, at the surface of APCs (74–76). It has been suggested that different tetraspanins may play a role in MHC-II clustering (**Figure 3**). CD37 negatively regulates MHC-II clustering, thus limiting antigen presentation by mouse splenic CD11c$^+$ DCs. CD37 knock-out splenic DCs show increased T-cell stimulatory capacity, by a mechanism strictly dependent on peptide-bound MHC-II signals (77). CD81 and CD9 co-immunoprecipitate with I-A MHC-II molecules in mouse BM-derived DCs and B blasts, and I-A/I-E heterologous multimerization is reduced in CD9-deficient BM-derived DCs (78). However, functional analyses were not performed in the study of Unternaehrer and collaborators. Another study has also suggested that MHC-II, together with HLA-DM and CD86, was included in TEMs containing tetraspanins CD9, CD63, CD81, and CD82 (79). This study was performed using the CDw78 antibody, which recognizes a specific determinant on an MHC-II subpopulation. However, this biochemical analysis of MHC-II multimerization was performed using mild detergent conditions (CHAPS 1%). It was later demonstrated that CD9 and CD81 co-immunoprecipitation with MHC-II I-A/I-E multimers only occurs under these mild detergent conditions (80), not being observed when using more stringent conditions (Triton X-100). Thus, deficiency in CD9 or CD81 does not affect MHC-II clustering at the surface of mouse BM-derived DCs, while surface

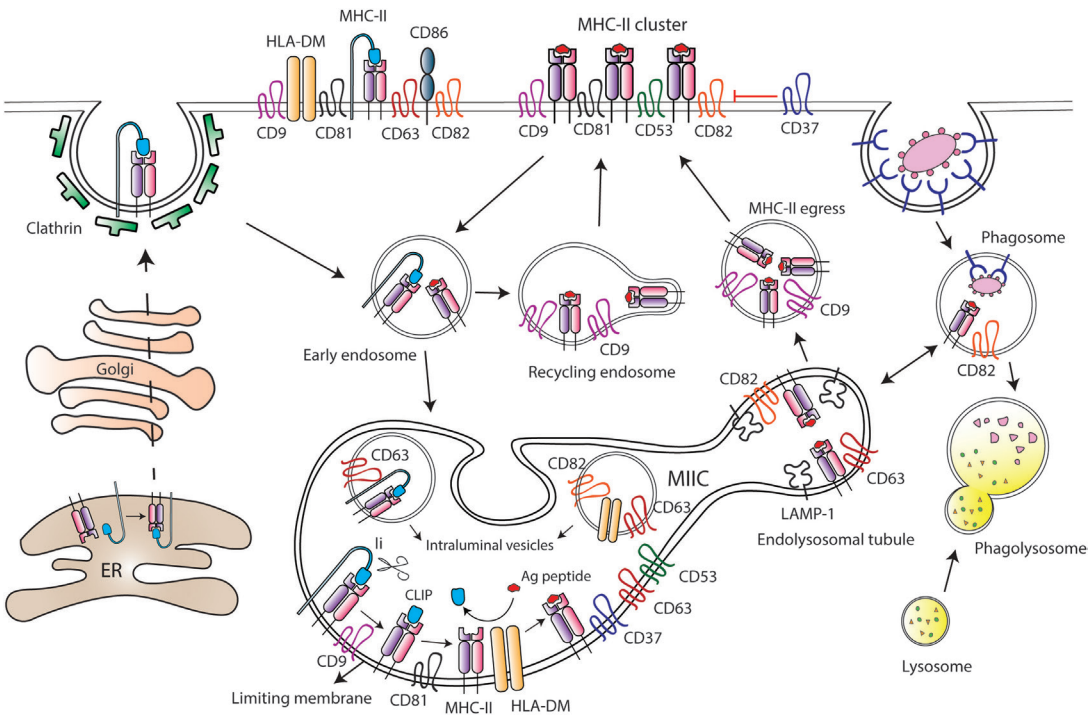

FIGURE 3 | Tetraspanins regulate major histocompatibility complex class II (MHC-II) trafficking and surface expression at antigen-presenting cells (APCs). After synthesis, MHC-II molecules are transported to the plasma membrane of APCs in association with the chaperone Ii, which drives MHC-II internalization through clathrin-coated pits. Then, Ii is sequentially degraded by different proteases at the MHC-II-enriched endosomal compartment (MIIC), until MHC-II molecules remain bound only to the CLIP (class II-associated invariant chain peptide) fragment. Peptides derived from internalized antigens are subsequently loaded to MHC-II molecules with the help of the class-II-like chaperone HLA-DM. Several tetraspanins are greatly enriched at the MIIC compartment, and peptide-bound MHC-II molecules coupled to Ii are included into tetraspanin-enriched microdomains (TEMs). Tetraspanin CD9 is required for the efficient egress of MHC-II from MIIC to the cell surface, and it is essential for MHC-II endocytosis and recycling. CD82 together with CD63 accumulate in LAMP-1-enriched endolysosomal tubules that emanate from the MIIC. In MIIC intraluminal vesicles, CD63 is associated with MHC-II and HLA-DM; while CD82 only interacts with HLA-DM. In addition, CD82 is preferentially associated with peptide-bound MHC-II, and it is found in phagosomes prior to their fusion with lysosomes. At the APC surface, tetraspanins CD9, CD53, CD81, and CD82 are associated with MHC-II, and tetraspanin CD37 negatively regulates MHC-II clustering. Moreover, TEMs containing CD9, CD63, CD81, and CD82 include MHC-II, HLA-DM, and CD86 molecules.

cholesterol content is essential for multimerization (80, 81). In addition, it was later demonstrated that the CDw78 determinant also recognizes peptide-bound MHC-II molecules coupled to the chaperone class-II associated invariant chain (Ii) (82). Intracellular trafficking of MHC-II molecules in APCs is a tightly regulated process, essential for proper antigen internalization, processing and subsequent presentation to T lymphocytes. Newly synthesized MHC II molecules associate with the chaperon Ii in the endoplasmic reticulum, which prevents premature peptide loading of MHC-II until MHCII-Ii complex enters the endocytic pathway (83) (**Figure 3**). The observation that peptide-bound MHC-II molecules coupled to the chaperone Ii (recognized by the CDw78 determinant) are included in TEMs (79, 82) suggests that tetraspanins could regulate MHC-II trafficking at the MHC Class II compartment (MIIC). Accordingly, there is considerable evidence supporting this hypothesis.

The MIIC is a multilamellar compartment that has similarities with late endosomes, being enriched in classical late endocytic markers, like LAMP-1, and in resident proteases, like cathepsins (84–86). Several tetraspanins, including CD37, CD53, CD63, CD81, and CD82, are highly enriched at the MIIC of human MoDCs and B cell lines (76, 87–89) (**Figure 3**). MHC-II diffusion rates are comparable to the diffusion values of CD63 and CD82, indicating inclusion into TEMs (89). Indeed, CD63 associates with MHC-II at both intraluminal vesicles and limiting membranes of the MIIC, and with the chaperone HLA-DM at the intraluminal vesicles. On the contrary, CD82 associates with HLA-DM at MIIC intraluminal vesicles and limiting membranes, but it only associates with MHC-II molecules at the limiting membrane (88, 89). CD82 would be mostly associated with peptide-bound MHC-II molecules, since it does not interact with MHC-II-coupled to Ii (88). Accordingly, CD82 and MHC-II are recruited together to phagosomes containing fungi or bacteria, before the fusion with lysosomes (90). Moreover, CD82 deficiency in DCs slightly reduces the maturation of MHC-II/peptide complexes (72). However, despite abundant evidence that tetraspanins dynamically interact with MHC-II and HLA-DM at the MIIC, they do not seem to be essential for peptide loading to MHC-II molecules. Downregulation of CD9, CD63, CD81, and CD82 in human cell lines does not affect surface expression of peptide-bound MHC-II (89), and CD9 deficiency in BMDCs does not affect antigen proteolysis (91). The dynamic interactions between these molecules

at the MIIC compartment would rather indicate that TEMs are important organizers of MHC-II trafficking in APCs.

After being loaded with antigenic peptides, MHC-II molecules egress from the MIIC to the APC surface, a process that remains largely undefined. Recently, it has been reported that tetraspanin CD9 is important for MHC-II egress to the surface of mouse immature MoDCs (91). CD9-deficient MoDCs display increased accumulation of MHC-II molecules in acidic compartments, in which MHC-II colocalizes with LAMP-1. As a consequence, surface expression of MHC-II is decreased in the absence of CD9 (91). Upon DC maturation, tubular extensions emanate from the MIIC in a process dependent on microtubules and microtubule-adaptor proteins (92–94), thus transporting peptide-bound MHC-II molecules to the plasma membrane (95, 96). In mouse BMDCs stimulated with LPS, these dynamic tubular extensions are enriched in LAMP-1 and tetraspanins CD63 and CD82 and show accumulation of fluorescent OVA protein (93) (**Figure 3**). In mature MoDCs, CD9 is not involved in MHC-II egress from the MIIC to the plasma membrane, which would take place only in a CD9-independent manner (91). Therefore, transport of peptide-bound MHC-II to the cell surface might be dependent on different TEMs, whose composition would be tightly controlled before and after cell maturation.

After arriving at the APC plasma membrane, peptide-bound MHCII molecules are actively endocytosed and then recycled back to the surface *via* early endocytic compartments. MHC-II endocytosis occurs through clathrin- and dynamin-independent pathway(s) (83). Early studies suggested that in immature DCs, MHC-II internalization is facilitated through ubiquitination by the ubiquitin E3 ligase MARCH-I (97, 98). MHC-II ubiquitination would be less efficient in mature DCs due to reduced MARCH-I expression, which would result in an increase in MHC-II surface expression (98, 99). However, subsequent studies have challenged this view (100–102). MHC-II ubiquitination enhances the kinetics of degradation of peptide-bound MHC-II molecules in immature DCs (101) and prevents recycling of internalized molecules back to the membrane (102), without affecting endocytosis. MHC-II recycling back to the surface is highly increased upon DC maturation, greatly contributing to boost MHC-II surface expression (102). Other members of the MARCH family have been shown to be involved in tetraspanin turnover. CD81 is targeted to lysosomes in the presence of MARCH-IV and -VIII, but not MARCH-I. Accordingly, MARCH-IV downregulation by siRNA increases CD81 surface expression (103). The effect of MARCH proteins on CD81 turnover could also affect the expression levels of CD81-interacting partners included in TEMs. Importantly, a recent study showed that CD9 is essential for MHC-II endocytosis in both immature and mature MoDCs, by a mechanism independent on MHC-II ubiquitination. Moreover, CD9 deficiency prevents MHC-II recycling in mature MoDCs (91). Tetraspanins are therefore important players in MHC-II trafficking and surface expression at APCs.

Tetraspanins are relevant for antigen presentation to T lymphocytes. Early studies have suggested that disruption of TEMs by cholesterol depletion, which is an essential component of these microdomains (104), affects the capacity of APCs to stimulate T cell activation (79, 81). However, cholesterol depletion can also disrupt lipid rafts, which are also required for proper antigen presentation (105). More recently, the specific functions of individual tetraspanins during antigen presentation have been established. CD37 negatively regulates MHC-dependent antigen presentation to $CD4^+$ and $CD8^+$ T cells, while CD151 inhibits T-cell co-stimulation by mouse $CD11c^+$ splenic DCs. As a consequence, mouse deficiency in those tetraspanins triggers $CD4^+$ and $CD8^+$ T-cell hyperstimulation (77). Sheng and collaborators have suggested that CD37 and CD151 could negatively regulate MHC clustering; however, despite the functional evidence demonstrated in their study, the molecular mechanisms behind CD37 and CD151 function remain to be determined. A similar phenotype was also observed with $Tssc6^{-/-}$ and $CD37^{-/-}Tssc6^{-/-}$ mice, through a mechanism independent on DC costimulatory signals (106). CD63 knock-down in human B cell lines also enhances MHC-II-dependent $CD4^+$ T cell stimulation, but in this case, the mechanism seems to be related with increased production of extracellular vesicles (107). In this sense, T cell activation can be induced by extracellular vesicles derived from mature DCs (18, 108), which are enriched in MHC-I and MHC-II, and several tetraspanins, like CD9, CD63, CD81, and CD82 (87, 109). MHC-II sorting into extracellular vesicles has been suggested to depend on its recruitment to TEMs (110–112). Together, these data suggest negative roles for some tetraspanins during antigen presentation. However, other tetraspanins can have the opposite effect. Indeed, mouse $CD9^{-/-}$ MoDCs induce less $CD4^+$ T-cell activation and proliferation than wild-type MoDCs, due to reduced surface expression of MHC-II (91). Strikingly, CD9-deficient Flt3L conventional DC (cDC) showed similar T-cell stimulatory capacity as wild-type cDCs, triggering comparable $CD4^+$ T proliferation *in vivo* (91). The role of CD9 in antigen presentation seems therefore to be DC subset-specific, and it would be interesting to investigate the molecular mechanisms behind this difference. CD9 interacts with MHC-II, and engagement of this tetraspanin with antibodies promotes the formation of antigen-dependent conjugates between human $CD14^+$ monocytes and T cells (113). CD9 could also play a role in antigen presentation through extracellular vesicles, since both are found at the MIIC and exosomes from mature splenic mouse DC lines (114). Together, these studies indicate that the strength of antigen presentation by professional APCs can be tightly regulated by TEM composition, with some tetraspanins playing positive roles while others limit T-cell activation signals.

Tetraspanins Define Distinct DC Subsets

Dendritic cells can be classified in several subsets that differentially control the strength and duration of T-cell responses. The main populations that have been described are plasmacytoid DCs (pDCs) and cDCs, which can be divided into several subpopulations. Monocytes can also be precursors of different subsets of DCs found in different tissues in the steady state and can generate MoDCs during inflammatory reactions (61). Both human and mice individual DC subsets display different TEM composition (115). In addition, expression of specific tetraspanins can be modulated by DC differentiation and maturation. For instance, CD9 is differentially expressed on conventional and pDCs (115, 116).

Regarding cDCs, it has been suggested that they have a higher capacity to sense, process and present phagocytosed antigens to T cells than pDCs. cDCs are classified in two main subsets: $CD141^+$ ($BDCA3^+$) in humans and $CD8\alpha^+$ ($CD11b^-CD11c^+$) in mice; or

CD1c$^+$ (BDCA1$^+$) in humans and CD4$^+$ (CD11b$^+$CD11c$^+$) in mice (3, 4). Murine and human DC subsets have some similarities in their functional properties. In mice, CD8α$^+$ cDC are found in lymphoid tissues and show similar phenotype and functional specialization to CD103$^+$ cDCs, which are found in non-lymphoid organs. Both subsets express comparable levels of TLRs, CLRs, and chemokine receptors and have a higher capability to cross-present antigens to CD8$^+$ T lymphocytes compared to CD11b$^+$ DCs (3). Interestingly, CD141$^+$ human and CD8α$^+$ mouse cDCs show high expression of tetraspanins CD9, CD53, and CD81 (115), which associate with MHC-I (75, 117). CD141$^+$ cDCs also display high levels of CD37, CD82, CD151, and Tspan31 (115). The other main subset of cDC is CD11b$^+$ cDCs, which seem to be more efficient in MHC-II-dependent antigen presentation to CD4$^+$ T lymphocytes, thus triggering polarization to Th2 and Th17 responses (3). The tetraspanin expression profile was somewhat variable when comparing CD1c$^+$ human and CD11b$^+$CD11c$^+$ mouse cDCs (115). Indeed, CD1c$^+$ human cDCs express very high levels of CD37, CD53, and CD81 and display intermediate to high levels of CD9, CD82, and CD151. In mice, CD4$^+$CD11b$^+$ cDCs show intermediate to low levels of CD9, CD53, CD81, and CD151 (115). As previously discussed, several of these tetraspanins are described to regulate different steps of MHC-II trafficking and antigen presentation by APCs. However, further studies are necessary to ascertain whether specific tetraspanin expression profiles can be used as markers of cDC subsets and/or define APC functions.

Plasmacytoid DCs, both in humans and mice, have the capacity to produce large amounts of type I interferons (IFN-α/β) in response to invading pathogens (118, 119). pDCs (BDCA2$^+$ in humans, and B220$^+$ in mice) are a small subset, and in mice express low levels of MHC-II, co-stimulatory molecules, integrin CD11c, and PRRs (119). Importantly, tetraspanins can be used as markers for the identification of different mouse and human pDC subpopulations. CD9 expression allows the recognition of immature and mature mouse pDCs subsets. CD9$^+$Siglec-H^{low} pDCs have an immature phenotype, producing high levels of type I IFN and other pro-inflammatory cytokines. These cells are mainly present in mouse bone marrow and spleen, and when stimulated can induce strong CD4$^+$ and CD8$^+$ T cell responses *in vitro* and *in vivo* (120). In contrast, tissue resident pDCs are negative for CD9, do not produce IFN-α, and have a tolerogenic phenotype, increasing the numbers of Foxp3$^+$CD4$^+$ Treg cells in tumor-draining lymph nodes (120). Therefore, these two pDC subsets (CD9$^+$ and CD9$^-$) define cells at different maturation stages at steady state. Upon infection, cell activation would induce migration of CD9$^+$ pDCs to the periphery, allowing the secretion of inflammatory cytokines at the infection site. Interestingly, upon maturation, CD9$^+$ pDC upregulate markers of pDC differentiation but gradually lose CD9 expression (120). Distinct pDC mouse subsets can also be distinguished when looking at tetraspanin CD81. A small subpopulation of B220$^+$CD5$^+$CD81$^+$ cells could be observed in blood, spleen, and bone marrow. This small subset does not produce IFN-α, while splenic CD5$^-$CD81$^-$ pDCs secrete high amounts of the cytokine (121). Similar CD81$^-$ and CD81$^+$ pDC subpopulations were observed in humans. Human pDCs are divided in two subsets depending on CD2 expression (122), and it has been recently demonstrated that CD2^{high} pDCs include CD2^{hi}CD5$^-$CD81$^-$ and CD2^{hi}CD5$^+$CD81$^+$ cells (121). Similarly to mice, human CD2^{hi}CD5$^+$CD81$^+$ pDCs represent a relatively rare subpopulation that produce little or no IFN-α (121). This subset can, however, secrete other pro-inflammatory cytokines, like IL-12p40 and IL-6, and is capable of inducing B-cell proliferation and differentiation to plasma cells. In addition, CD2^{hi}CD5$^+$CD81$^+$ pDCs are efficient inducers of CD4$^+$ T cell proliferation and Treg differentiation (121). Interestingly, antibodies against CD81 and CD9, but not CD63, specifically inhibited IFN-α production by pDCs when co-cultured with HCV-infected hepatoma cells. This effect was specifically related to CD81 expression in pDCs and required Rac GTPase activity (123). Hence, the absence of tetraspanins CD9 and CD81 seems to identify small pDC subpopulations that do not produce type I IFN. However, whether these tetraspanin expression profiles define overlapping pDC subsets and/or if differential expression of tetraspanins is associated with specific APC phenotypes remain to be determined.

CONCLUSION

In APCs, surface immune receptors and adhesion molecules, such as MHC molecules, co-receptors, PRRs, and integrins, associate with tetraspanins. Through the inclusion of these receptors in TEMs, tetraspanins can regulate their clustering, internalization, and intracellular trafficking, then affecting their downstream signaling. TEMs are thus important regulators of proper antigen uptake, processing and presentation. In addition, by modulating cytoskeleton-dependent processes, like outside-in integrin signaling, actin polymerization and cell spreading, tetraspanins are also key players in APC migration. Increasing evidence shows that different subsets of DCs having distinct requirements for antigen presentation and/or motility capabilities express specific repertoires of tetraspanins. This fine-tuned regulation warrants appropriate adaptive immune responses. Therefore, tetraspanins are potential targets for therapeutical interventions aiming to balance exaggerated immune responses in pathological inflammations and in immune-mediated chronic diseases.

AUTHOR CONTRIBUTIONS

MS and VR-P designed and wrote the review. VRP and FSM coordinated and edited the manuscript.

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REVIEW
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Expression and Function of Tetraspanins and Their Interacting Partners in B Cells

Fagui Zou[1,2†], Xu Wang[1,2†], Xinxin Han[1,2], Gerson Rothschild[3], Song Guo Zheng[4,5], Uttiya Basu[3] and Jianbo Sun[1,2]**

[1] Guanghua School of Stomatology, Hospital of Stomatology, Sun Yat-Sen University, Guangzhou, China, [2] Guangdong Provincial Key Laboratory of Stomatology, Guangzhou, China, [3] Department of Microbiology and Immunology, Vagelos College of Physicians and Surgeons, Columbia University, New York, NY, United States, [4] Department of Medicine, Milton S. Hershey Medical Center at Penn State University, Pennsylvania, PA, United States, [5] Center for Clinic Immunology, Third Affiliated Hospital at Sun Yat-Sen University, Guangzhou, China*

Tetraspanins are transmembrane proteins that modulate multiple diverse biological processes, including signal transduction, cell–cell communication, immunoregulation, tumorigenesis, cell adhesion, migration, and growth and differentiation. Here, we provide a systematic review of the involvement of tetraspanins and their partners in the regulation and function of B cells, including mechanisms associated with antigen presentation, antibody production, cytokine secretion, co-stimulator expression, and immunosuppression. Finally, we direct our focus to the signaling mechanisms, evolutionary conservation aspects, expression, and potential therapeutic strategies that could be based on tetraspanins and their interacting partners.

Keywords: tetraspanin, B cell, partner, immune regulation, therapy strategy

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****Correspondence:***
Uttiya Basu
ub2121@cumc.columbia.edu;
Jianbo Sun
sunjb3@mail.sysu.edu.cn

[†]These authors have contributed equally to this work.

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ORIGIN, DEVELOPMENT, FEATURES, AND FUNCTIONS OF B CELLS

Origin, Subtypes, and Development of B Cells

Conventional B cells—a type of white blood cell—were first defined in 1965 by Cooper (1). They originate from hematopoietic stem cells in mammalian bone marrow or in the bursa of Fabricius of birds, where they pass through several developmental stages and become IgM$^+$ immature B cells capable of recognizing antigen (1, 2). The immature IgM$^+$ B cells subsequently migrate to secondary lymphoid tissues and develop into three groups of mature naïve B cells: follicular B cells, marginal zone B cells (MZB), and B-1b cells. When bound with antigen, mature naïve B cells are activated, selected, and differentiated into plasmablasts and then antibody producing plasma B cells. These are conventional B cells and also named as B-2 cells. There are additional B cell populations (named B-1 cells) generated in the fetal liver or spleen which undergo self-renewal in the periphery and secrete IgM and IgG3 natural antibodies to facilitate immune responses. B-1 cells have a distinct developmental lineage from B-2 cells. The exact origin and development of B-1 cells is uncertain (3). Accumulated evidence indicates the existence of yet other B cells, named regulatory B cells (Breg), and associates their function with suppression of immune responses. Whether Breg is a distinct lineage of B cells is still unknown (4). More details of B cell development stages and B cell subsets are summarized in **Figure 1**.

Functions of B Cells

B cells play pivotal roles in the immune system. As outlined in **Figure 2**, B cells can promote an immune response through presentation of antigens and production of diverse antibodies, proinflammatory cytokines, and co-stimulators (5). B cells can also suppress immune responses through a

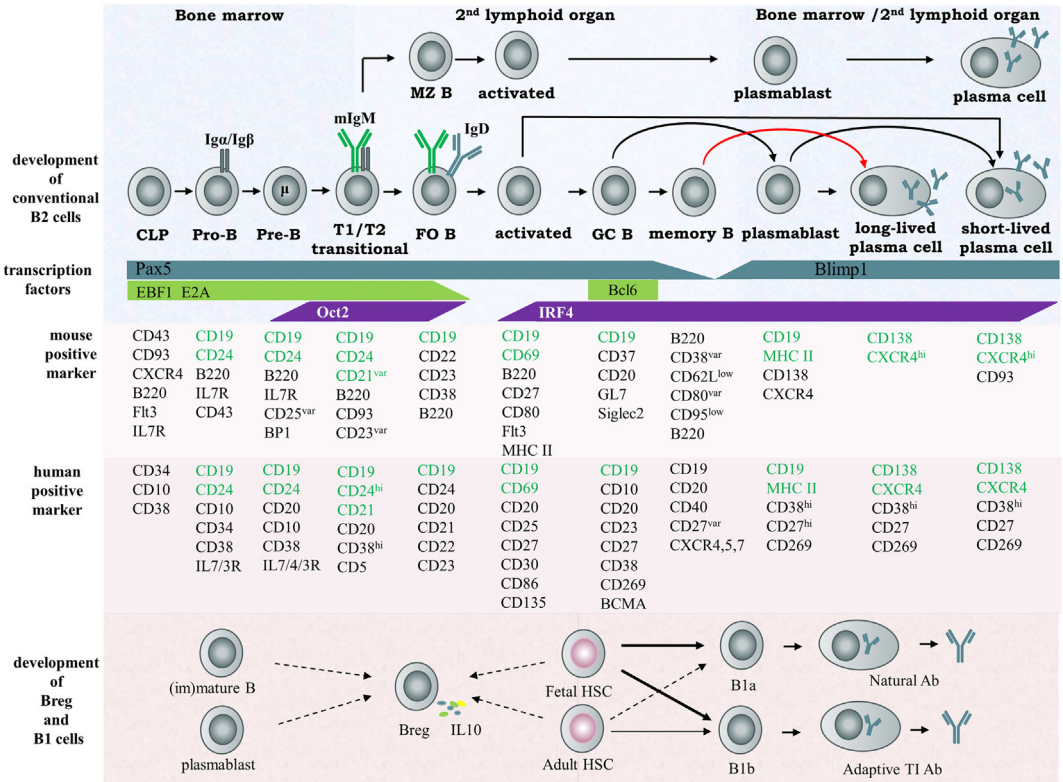

FIGURE 1 | Scheme of B cell development and subsets in humans and mice. According to their individualized origin, surface marker, anatomic localization and functional property, B lymphocytes can be divided into several subsets, including B-1a cell, 1b cell, Breg cell, and B2 cell, the latter considered the conventional B cell. In the early stage, B cells differentiate from hematopoietic precursors into pro-B, pre-B within the bone marrow, then migrate to the spleen and progress through the transitional T1 and T2 stages. These immature cells then differentiate into FO or MZ naïve B cells depending on their special B cell receptor. MZ B cells rapidly develop into plasma cells secreting IgM during the early stage of pathogen infection and function as the first defense line against blood-borne pathogens. FO B cells enter germinal centers and undergo class switch recombination (CSR), somatic hypermuation (SHM), and affinity maturation and terminally differentiate into memory B cells or plasma cells. The important transcription factors and surface markers in human or murine involved in conventional B cell development are shown. The origin of regulatory B cells and B1 cells is still not identified. Here, the solid arrows represent known developmental routes while the dashed arrows represent possible development directions. Abbreviation: CLP, common lymphoid progenitor.

variety of mechanisms, such as production of IL-10, IL-35, and TGFb1, induction of regulatory T cells, and clearance of auto antigens (4). Many cell surface molecules are involved in B cell development and function. Tetraspanins are one such important family of molecules.

GENERAL FEATURES AND FUNCTIONS OF TETRASPANINS

Structure and Evolutionary Conservation of Tetraspanins

Tetraspanins belong to a protein family in which members contain intracellular N- and C-termini, two extracellular domains (EC1 and EC2), and specifically four transmembrane domains (**Figure 3A**; 6, 7). Each phylum has evolved its own particular tetraspanins with distinction in the variety and abundance in different species. Despite this, the chemical composition of tetraspanins is highly conserved among species with four or more cysteine residues in a highly conserved "CCG" motif in the EC2 domain (8). There are 33 tetraspanins found in humans (**Tables 1** and **2**) and most of them preserve the characteristics of the ancient sequence in domain EC2.

General Interactions Among Tetraspanins and Their Partners

Tetraspanins act as scaffold proteins to anchor multiple proteins—including other tetraspanins, partners of tetraspanins, and other proteins—to one area of the cell membrane, and form a tetraspanin-enriched microdomain (TEM) or tetraspanins web (10, 11). A recent study with super resolution microscopy provided a close view of TEM and demonstrated that TEM is composed of individual nanoclusters (<120 nm). There are no more than 10 CD53 molecules in a single tetraspanin cluster of CD53. The study also evaluated the distances between the individual clusters, including CD53, CD37, CD81, CD82, and the tetraspanin partners such as CD19 and major histocompatibility complex class II (MHC II) (12). Based on the sensitivity and stringency to different detergents, the interactions of tetraspanins and partners in TEM were classified into three categories (13, 14).

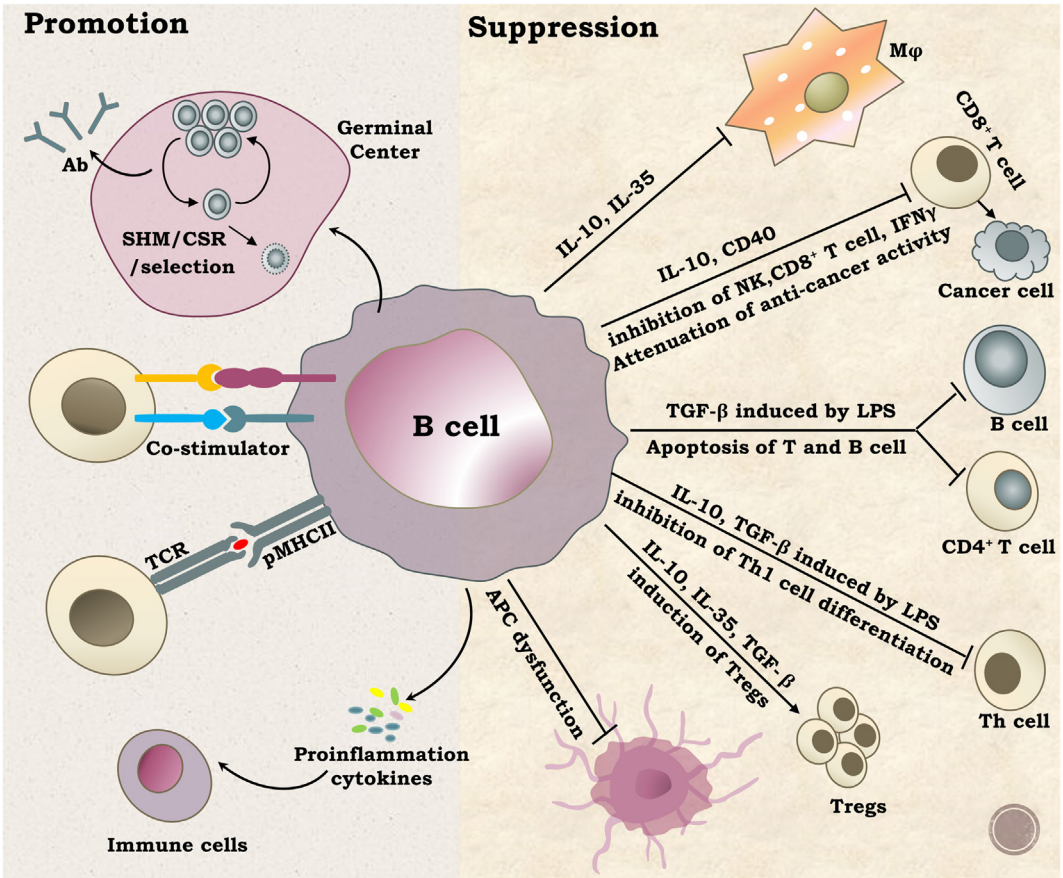

FIGURE 2 | Functions of B cells. B lymphocytes perform diverse and complex roles *in vivo* mainly through promotion or suppression of immune responses. The well-known function of B cells is antibody production by plasma cells after SHM selection and CSR. B cells can also activate other immune cells by providing co-stimulation signals, serving as antigen-presenting cells or secreting multiple proinflammation cytokines, such as IL2, IL4, IL6, TNF-α, and INF-γ. On the other hand, B cells can suppress immune responses by regulating certain types of immune cells through multiple ways. Abbreviations: SHM, somatic hypermutation; CSR, class switch recombination; Ab, antibody.

This model allows for dynamic and adaptable interactions between tetraspanins and other surface proteins based on a descriptive categorization without correlation to functionality in the living cell. A recent review proposed a new applaudable classification of tetraspanin interactions based on their function in the formation of TEM: interactions (a) necessary to maintain tetraspanin structure, (b) that support tetraspanin web formation, (c) that add functional partners to the web, and (d) that facilitate intracellular events (6).

Three hypothetical models could be postulated to decipher the ways that tetraspanin microdomains enhance or regulate cellular signals and exert effects on fundamental biological processes. One model is that tetraspanins be considered a transmembrane linker connecting and augmenting signal transduction between membrane partners and intracellular-signaling proteins (15). Another model could propose that tetraspanins are involved in gathering partner membrane proteins which subsequently result in increased avidity and/or enhanced interaction with their ligands (16). The third hypothesis is that tetraspanins function as regulators by sequestering partners from signal transduction (17) thus preventing inappropriate signals and responses in resting cells. Without favoring any of these models at the present time, we now direct our attention to signal transduction and/or regulation by tetraspanins in immune cells (**Figure 3B**).

Interaction of Tetraspanin CD81 and Its Partners in B Cell Receptor (BCR) Activation Pathway

In B cells, tetraspanins CD81 interacts with the CD19/CD21 signal-transducing complex to lower the threshold for BCR signaling (**Figure 3**B1). The multiprotein complex BCR consists of two parts: membrane immunoglobulin (Ig) with integral membrane domain, and signal transduction moiety Ig-α/Ig-β (also known as CD79A/CD79B) heterodimer tethered by disulfide bridges (18). When antigen binds to Ig, Src family kinase-like Lyn phosphorylates immuno-receptor tyrosine-based activation motif residues on the cytoplasmic tails of Ig-α/Ig-β, sequentially recruiting and activating Syk and Btk kinases, then initiating downstream signaling cascades of the Ras–MAPK pathway and PLCγ2 (19). The CD19/CD21 complex is thought to augment BCR signaling by decreasing the signaling threshold for

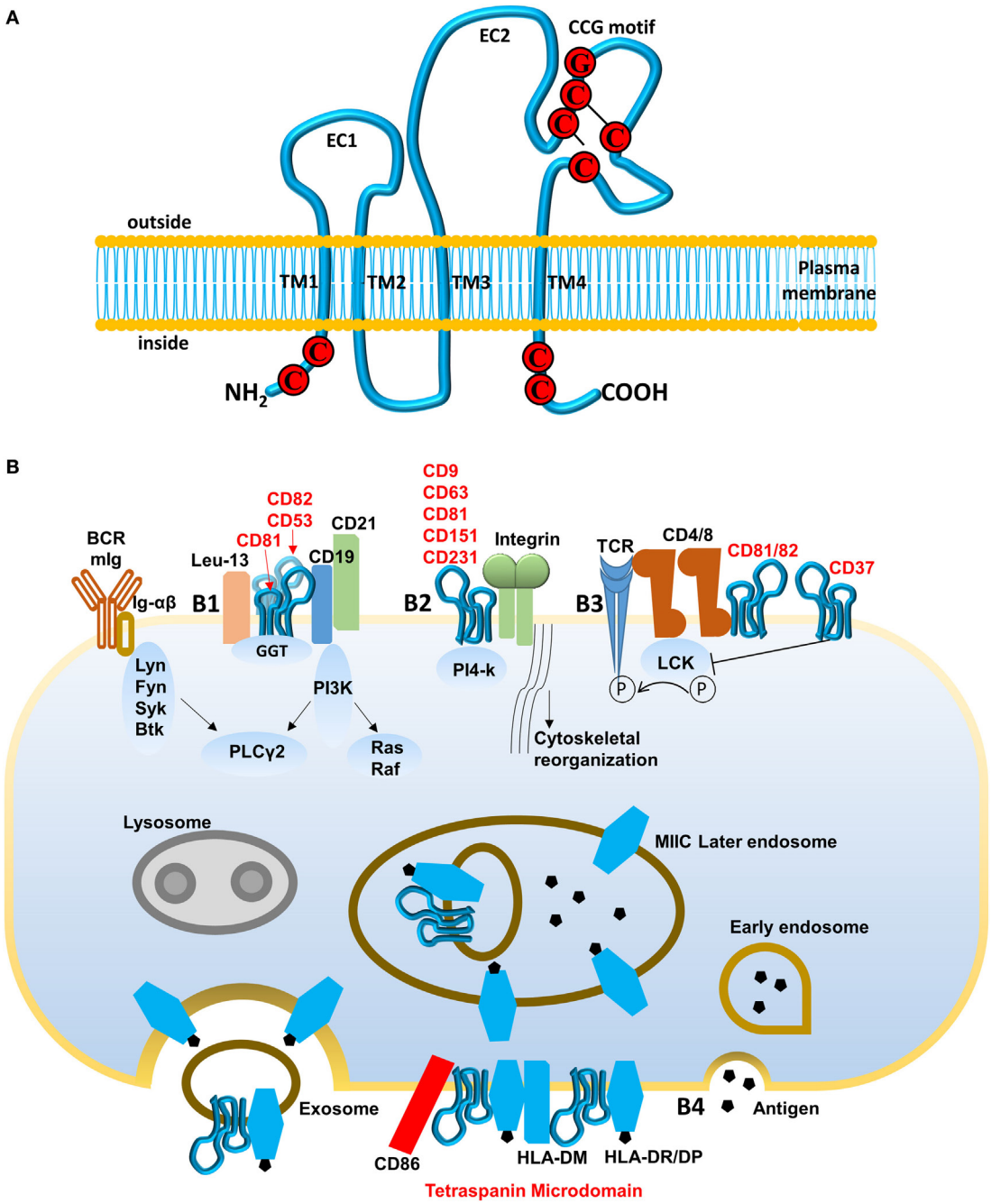

FIGURE 3 | Structure of tetraspanin and pathways regulated by tetraspanins. **(A)** Schematic diagram of tetraspanins. Tetraspanins present four transmembrane domains (TMs) intracellular N- and C-termini and two extracellular domains (EC1 and EC2). CCG motif is formed with cysteine–cysteine–glycine (marked by red) and two disulfide bonds (marked by black line). **(B)** Pathways regulated by tetraspanins. (B1) B cell receptor (BCR) activation mediated by CD19–CD81–CD21 complex. Ig-α/β receive signals and are phosphorylated by Src kinase (Lyn, Fyn, or Btk), then recruit Syk kinase for initiating downstream signal pathway PLCγ2, Ras/Raf. Tetraspanin CD81, associated with CD52 and CD82, binds C19/CD21/Leu-13 signal-transducing complex and actives PLCγ2 through PI3K, which lowers the threshold for BCR signaling. (B2) Integrin-mediated cell adhesion. PI4-k, associated with various tetraspanins (CD9, CD63, CD81, CD151, and CD231), interacts with and promotes integrins to modulate cell spread and migration. (B3) T cell-B cell contact (TCR) pathway mediated by tetraspanins CD81, CD82, and CD37. CD4 and CD8 associate with Lck kinase to activate TCR signaling but their interaction with CD81, CD82, and CD37 interferes with phosphorylation of Lck kinase and may inhibit TCR signaling. (B4) Endocytic pathway for antigen presentation. Recognized antigens are internalized, processed, and loaded onto MHC class II molecules during the late endosome stage. Major histocompatibility complex class II mediates transport to the cell surface and the release of exosomes. Tetraspanin microdomains in antigen-presenting cell membranes are enriched for specific peptide–MHC class II complexes, peptide editor human leukocyte antigen-DM, and CD86 among other proteins. This selecting domain probably facilitates antigen presentation and T-cell activation, increasing MHC avidity.

TABLE 1 | The regulation and function of tetraspanins and their interacting partners.

Protein	Regulate[a]	Regulated by[a]	Binds[a]	Role in cell[a]
TSPAN1	N/A	TP73, mir-8	NFKBIB	Endocytosis by proliferation
TSPAN2	CTNNB1, Jnk, BAX	TCF7L2, calmodulin, ERN1, RTN4, TGFB3, dexamethasone, D-glucose	SNX13, MTCH1, REEP6, PTGFRN, ARF6, GLP1R, LPCAT3, ZDHHC6, LGALS3, TSPAN3, DAGLB, LCLAT1, HSDL1, FAM241A, SNX14	Myelination, degeneration, development, differentiation, formation, upregulation, activation in, apoptosis
TSPAN3	N/A	FAS, neutrophils, PAX3, camptothecin, L-dopa, mir-197	ITGB1, LPAR1, RNF13, GABARAP, RDH14, MAP1LC3B2, SNX17, UGCG, FAM189B, GOLGA7, ZDHHC9, TNFRSF10B, RNF149, STX6, CDC6	Migration, proliferation
TSPAN4	Protein–protein complex	PLAG1, HK cells, MGEA5, hydrogen peroxide, CLOCK, estrogen	ITGA3, ITGB1, ITGA6, LOC100996763/ NOTCH2NL, CREB3, UI94, CD81, CD9, CLDN11, peptide, miR-1-3p (and other miRNAs w/seed GGAAUGU)	N/A
TSPAN5	N/A	CST5, WT1, MGEA5, TNFSF11, STAT4, beta-estradiol	ATP2B3, LCLAT1, RDH14, FAM210B, KLHL2, THAP11, TMEM87A, PIEZO1, NAT14, BSCL2, AGPAT3, TVP23C, ALG11, SOAT1, SNX25	Adhesion, proliferation, osteoclastogenesis
TSPAN6	N/A	Seocalcitol, SOX4, RBM5, HRAS, TP53, SNX27, retromer	EVA1C, TMEM185A, CLEC5A, TNFRSF17, ASIC4, CDS1, TMEM30B, VNN2, SERPINA12, LYPD4, GPR141, LRRTM1, MAVS, TMEM173, IFIH1	N/A
TSPAN7	N/A	MYC, EZH2, HOXD3, IL15, LMO1, TAL1, NEUROG1, HDAC4, influenza A virus [A/Bangkok/RX73(H3N2)], CD3, PAX3, omeprazole, LIF, NKX2-1, large T antigen	HAVCR2, PI4KA, CREB3, BBS1, NEF, ADCY5, CACNA1A, KPTN, RBL1, LGALS3	Shape change, cell spreading
TSPAN8	GCG	FGF10, TCF, STAT5A, CBX5, CTNNB1, 2-bromoethylamine, SMARCA4, AR, doxorubicin, indomethacin, captopril, hexachlorobenzene, cyclophosphamide, lomustine, puromycin aminonucleoside	ITGB1, ITGA3, EPCAM, integrin, ITGA6, CLDN7, ACTA1, ATP1A1, integrin alpha 6 beta 1, integrin alpha6 beta1, CD44, PDX1, miR-125b-5p (and other miRNAs w/seed CCCUGAG)	Cell movement
TSPAN9	N/A	EAhy926 cells, tamoxifen, ESR1, dexamethasone, cyclosporin A, NEF, CD3	ELAVL1	N/A
TSPAN10	N/A	PRDM1	HSD17B13, ADAM10, ADGRG5, PNLDC1	N/A
TSPAN11	N/A	N/A	POMC, CYB5R3, IGLL1/IGLL5, TM9SF4, ITGA7, ITGA6, REEP5, NRP2, ESYT1, ARL6IP5, ITGB1	N/A
TSPAN12	ADAM10, APP	GATA2, MGEA5, CLDN7, UPF2	FTT0715, TFCP2, LRP5, NDP, FZD4, TSPAN12, ADAM10	Proteolysis in, maturation in
TSPAN13	Cyclic AMP	CBX5, STAT5A, SMARCA4, PMSG, ESR2, HDAC4, TNFSF11, TGFB3, Cg, UPF2	GAG, GLP1R, APP, ELAVL1	Osteoclastogenesis, accumulation in
TSPAN14	GP6	Tretinoin, TGM2	ADAM10, PIK3R2, DPY30, PIK3CA, PIK3R3, ATP13A2, ELAVL1, HNF4A, REST	Molecular cleavage in
TSPAN15	CDH2	TCF7L2, F2RL1	P2RY12, AGTR1, RETREG3, LPAR6, ADAM10, SLC7A1, SYPL2, SLC22A16, IPPK, FZD10, C3AR1, HTR3A, GYPB, ADGRE5, CLCC1	Molecular cleavage in
TSPAN16	N/A	N/A	N/A	N/A
TSPAN17	N/A	KLF3, SATB1, calmodulin	PRAF2, FAM210B, ATP2A3, DHRS7, CCDC115, FAM189B, AGPAT3, TYW1, GHDC, PNPLA6, SLC44A1, RNF149, RETREG3, EPHX1, GP1BB	N/A
TSPAN18	N/A	2-amino-5-phosphonovaleric acid	FITM2, RNF130, iucD	N/A
TSPAN19	N/A	N/A	N/A	N/A
UPK1B	N/A	UPK3A, UPK2, CNR1, TP63, PD 153035, rosiglitazone, troglitazone	UPK3A, SNX31, BCL2L13, BNIP2, CCDC155	Differentiation

(*Continued*)

TABLE 1 | Continued

Protein	Regulate[a]	Regulated by[a]	Binds[a]	Role in cell[a]
UPK1A	N/A	TP63, ATG16L1, OSM, SLC13A1, Caco2 cells, troglitazone, PD 153035	ECEL1, DIRC2, TMEM223, TMEM62, LMF2, TUSC3, SOAT1, FZD3, DPY19L1, TMEM39A, FZD1, LRRC8A, NAT14, PIGO, CIB1	Differentiation
PRPH2	RHO, ROM1, 26s proteasome	CRX, RHO	PRPH2, ROM1	Quantity, formation, function, synaptic transmission, overload in, length, generation, cell death, morphology
ROM1	N/A	AHI1, PLAG1, PRPH2, NRL, NRG2, influenza A virus [A/Bangkok/RX73(H3N2)], NRG1, dihydrotestosterone, EGF	PRPH2, SPTLC2, PHGDH, ITSN1, EPN1	Electrophysiology, degeneration, abnormal morphology, length, synaptic transmission, apoptosis, size
CD151	ITGA3, ITGB1, ERK1/2, PRKCA, PTK2, P38 MAPK, PI4KA, Akt, CD63, PRKCB, collagen type I, CD81, CD151, laminin (complex), FN1	MYC, ZDHHC2, PAX3, RET, integrin alpha 6 beta 4, mir-193, MITF, SMARCD3, T, BCL6, MKL2, MKL1, MGEA5, MYL2, valproic acid	ITGB1, ITGA3, ITGA6, ITGB4, CD9, ITGB3, ITGA5, GRAMD1C, integrin alpha 3 beta 1, TMED10, PI4KA, CD81, CD63, PRKCB, TMPRSS11B	Migration, adhesion, proliferation, abnormal morphology, thickness, lack, effacement, morphology, activation in, cell spreading
CD53	BCL2L1, BAX, DHX32, KRT20, GLS, TPPP3, TFDP1, MRPL32, FAM43A, CD53, PRKCA, PRKCB, caspase, Akt	Tretinoin, 17-alpha-ethinylestradiol, DYSF, CD3, RARA, IL15, RUNX1T1, RUNX1, MGEA5, SOX4, CREBBP, EP300, paclitaxel, PRDM1, vitamin E	PRKCA, PRKCB, ITGB1, CD81, GGT1, CD82, ITGA4, CD37, CD2, CD9, miR-224-5p (miRNAs w/seed AAGUCAC)	Apoptosis, invasiveness
CD37	IgG1, Immunoglobulin, IgG, CD4, LCK, CD8, IgM, IGHG1, adenosine, Lfa-1, RAC1, ICAM1, IL2	IL13, IGF1R, fluvoxamine, lipopolysaccharide, B lymphocytes, plasma cells, RAF1, PD98059	ACPA, PURL, YBTQ, PG8786 084, CD19, CD53, SYK, KARS, PTPN6, LYN, PIK3CD, PIK3CG, CD81, MHC class II (complex), CR2	Proliferation, adhesion, activation, chemotaxis, transendothelial migration, recruitment, cell death, cell division, internalization by, activation in
CD82	CD82, EGFR, BCAR1, MET, PRKCA, RAC1, CRK, focal adhesion kinase, GRB2, ITGA3, CANX, ITGA5, ITGB1, PRKCB, SHC1	IL1B, CD82, NFKBIA, ERBB2, APP, APBB1, IL6, TP63, NFkB (complex), ZFPM1, GSK3B, AURKB, P38 MAPK, mir-15, NEUROG1	CD81, CD19, CD9, ITGA3, ITGB1, ITGA6, EGFR, PRKCA, MET, NFKB1, CD1D, PRKCB, ITGA5, CREB3, integrin beta 1	Migration, invasion, transcription in, adhesion, accumulation in, motility, anoikis, invasion by, differentiation, signaling in
CD81	CD19, IFNG, MMP14, TNF, CD81, IgM, IgA, ERK1/2, PRKCA, IgG1, IgG, Igg3, dopamine, SP1, GTF3A	CD81, phorbol myristate acetate, hepatitis C virus JFH-1, LY9, WIPF1, curcumin, HIST1H1T, Hist1h1a, hydrogen peroxide, butyric acid, ZBTB16, HRAS, laminin 5, interferon alpha, ADORA2A	E2, CR2, ITGB1, CD19, ITGA3, CD9, CD82, IGSF8, PTGFRN, CLDN1, ITGA5, HNRNPD, RAC2, E1, CD151	Proliferation, abnormal morphology, adhesion, migration, motility, differentiation, number, phosphorylation in, entrance, binding
CD9	CD9, IL2, ITGA3, ITGA5, PRKCA, ITGB1, SRC (family), MMP9, CBL, ERK1/2, CD69, YAP1, DPP4, CASP3, ERVW-1	Decitabine, trichostatin A, forskolin, CD9, ZDHHC2, BCAP31, PRDX1, FOLR1, methylprednisolone, lactacystin, CCR5, MYC, MYCT1, E2F1, CXCR4	ITGB1, ITGA3, IGSF8, ITGA5, CD81, PTGFRN, CD151, ITGA6, ITGB3, CD82, ITGA2, ITGB4, Psg18 (includes others), PRKCA, CD36	Fusion, adhesion, proliferation, aggregation, migration, binding, apoptosis, motility, accumulation in, fertilization
CD63	KDR, PLC gamma, SRC, PTK2, ERK1/2, Akt, VTN, laminin (family), FN1, collagen, ITGB1, TNF	F2, cytochalasin B, IL3, IFNG, collagen(s), C5, IL5, CSF2, guanosine triphosphate, LEP, roscovitine, CDK5R1, AP3B1, NEUROG3, ZFPM1	ITGB1, LGALS8, RETREG3, LGALS3, LGALS9, TIMP1, ITGB3, ITGA3, PI4KA, CD151, MHC class II (complex), AP3M1, TSPAN2, TSPAN3, RNF13	Adhesion, differentiation, endocytosis by, internalization in, tubulation by, sprouting in, phosphorylation in, tyrosine phosphorylation in, migration, growth
TSPAN31	TSPAN31	Benzo(a)pyrene, TSPAN31, IRF4, CREBBP, EP300, heavy metal, TFAP4, EAhy926 cells	ELAVL1	Proliferation
TSPAN32	IL2	HOXA3, GATA2	N/A	Proliferation, organization, activation, aggregation
TSPAN33	PTGS2, NOTCH1, IFNB1, NOS1, NFkB (complex), ADAM10, Notch	NOTCH1, NOTCH2, *Mycobacterium tuberculosis* H37Rv, MAP3K8, tretinoin, IFNG, TLR4, TLR2, TLR3, dexamethasone	PLEKHA7, MSN, PDZD11, ADAM10, EZR	number, abnormal morphology, quantity, maturation in, signaling in, expression in, erythropoiesis

[a]Organized from the information of each gene collected from thousands of publications by Ingenuity Pathway Analysis (IPA) program. Please note that relationships for the proteins in the list of the "Regulate" and "Regulated by" may be not direct or supported directly by experiments although most of them are derived from experimental data mining by IPA from published papers.

TABLE 2 | Expression of tetraspanins on B cells.

Name	Gene synonyms	Subcellular location	Expression on human B cell (TPM)		
			CD38⁻ naïve B cell	Isotype switched memory B cell[a]	Memory B cell
TSPAN1	NET-1, TSPAN-1	Nucleoplasm, vesicles	0	0	0
TSPAN2	FLJ12082, TSN2, TSPAN-2	Nucleoplasm	0	0.8	0
TSPAN3	TM4-A, TM4SF8, TSPAN-3	Nucleoplasm, Golgi apparatus	26	15	28
TSPAN4	NAG-2, TETRASPAN, TM4SF7, TSPAN-4		0	0	0
TSPAN5	NET-4, TM4SF9, Tspan-5		2	1	1
TSPAN6	T245, TM4SF6, TSPAN-6	Cytosol	0	0	0
TSPAN7	A15, CD231, DXS1692E, MRX58, MXS1, TALLA-1, TM4SF2		0	0	0
TSPAN8	CO-029, TM4SF3	Nucleoplasm	0	0	0
TSPAN9	NET-5	Nucleoplasm, Golgi apparatus, cytosol	0	0	0
TSPAN10	OCSP		0.6	0	0
TSPAN11		Vesicles	0	0	0
TSPAN12	NET-2, TM4SF12	Vesicles, microtubules	0	0	0
TSPAN13	NET-6, TM4SF13	Nucleus	72	13	17
TSPAN14	DC-TM4F2, MGC11352, TM4SF14	Vesicles	4	2	3
TSPAN15	NET-7, TM4SF15	Nucleoplasm, cytosol	0	0	0
TSPAN16	TM-8, TM4-B, TM4SF16		0	0	0
TSPAN17	FBX23, FBXO23, TM4SF17	Nucleoplasm	2	2	2
TSPAN18	TSPAN		0	0	0
TSPAN19					
UPK1B	TSPAN20, UPK1		0.6	0	0
UPK1A	TSPAN21		0	0	0
PRPH2	CACD2, rd2, RDS, RP7, TSPAN22		0	0	0
ROM1	ROM, TSPAN23	Plasma membrane, cytosol	0.5	0	0.6
CD151	PETA-3, RAPH, SFA-1, TSPAN24		1	2	2
CD53	MOX44, TSPAN25		240	185	221
CD37	TSPAN26		183	73	117
CD82	IA4, KAI1, R2, ST6, TSPAN27	Vesicles	16	28	48
CD81	TAPA-1, TAPA1, TSPAN28	Plasma membrane	15	9	12
CD9	BA2, MIC3, MRP-1, P24, TSPAN29	Plasma membrane	8	1	0.5
CD63	ME491, MLA1, TSPAN30	Vesicles	6	7	13
TSPAN31	SAS		5	3	6
TSPAN32	PHEMX, TSSC6		6	1	2
TSPAN33 (9)	MGC50844, Penumbra	Microtubules	31	11	26

The information of "Gene synonyms" and "subcellular location" is obtained from "The Human Protein Atlas" (https://www.proteinatlas.org/). TPM, transcripts per kilobase million. Except Tspan19, all tetraspanins are expressed respectively on at least one of B lymphoma cell lines. The results were obtained by searching the gene symbol plus "B cell" for Biological conditions on Expression Atlas (https://www.ebi.ac.uk/gxa/home). The expression of human tetraspanins is determined by the RNA-seq data generated by the Blueprint Consortium.

[a]*The original description in the consortium is "Class switch memory B cell."*

B cell activation (20). Indeed, the direct association of tetraspanin CD81 with CD19 as a part of the CD19/CD21/Leu-13 complex is critical for both assembly and localization of this complex (20) and CD81-deficient B cells have been found to have reduced expression of CD19 and impaired B cell signaling (21). Furthermore, probably through interaction of tetraspanins CD9, CD53, CD82 with CD81, the CD19/CD21 complex has an additional layer of control over B cell signal regulation through formation of a CD19/CD21 complex with additional functional proteins, such as glutathione and oxidative homeostasis-related enzyme γ-glutamyl transpeptidase GGT (22).

Function of Tetraspanin/Integrin Complexes in Signaling Pathway for Cell Migration and Adhesion

Tetraspanins associating with and forming tetraspanin/kinase-integrin complexes are implicated in both leukocyte and cell–cell adhesion (**Figure 3**B2) by causing signal activation and cytoskeletal reorganization. In B cells, by enhancing tyrosine phosphorylation levels, tetraspanin CD9 promotes β1 integrin-dependent mobility (23). In addition, tetraspanins CD9 as well as CD63, CD81 have been documented to associate with both PI4-kinase and integrin α3β1 in lymphoid cell lines (24). Finally, tetraspanins also have been found to enhance the avidity of integrins for neutrophil motility and T cell–B cell contact (25).

Function of Tetraspanin CD37 and Its Partners in T Cell–B Cell Contact (TCR) Activation Pathway

Tetraspanins are implicated in TCR-induced activation and proliferation (**Figure 3**B3). Interaction of peptide with the MHC activates the TCR and initiates the downstream signaling cascade of Src kinases Fyn and Lck. Lck subsequently activates the functional proteins involved in T cell activation and proliferation.

Interaction of Lck with CD4/CD8 plays crucial roles in this pathway (20); should CD4 associate with tetraspanins CD81/82 then Lck is sequestered from the TCR signaling pathway (26). Additional evidence shows that tetraspanin CD37 is coupled to TCR signal transduction mostly by influencing the dynamics of CD4-Lck distribution to TCR signal associated microdomains (27). Thus, tetraspanins regulate the T cell biologic process by influencing the TCR-CD4/CD8 cascade proximal to Lck mobilization.

Functions of Tetraspanins and Their Partners in Antigen-Presenting Processes

MHC avidity and facilitation of T cell activation is also mediated by tetraspanins (**Figure 3**B4). Tetraspanins function in antigen-presenting cells (APCs) to assist in the presentation of the MHC–peptide complex to T cells. Tetraspanins CD81, CD37, CD82, CD53, and CD63, tether with MHC and associate with stimulators on exosome vesicles which are MHC II-enriched compartments. After the cell membrane is fused with MHC, the exosomes are released and can act as stimuli for T-cell proliferation (28). But there is an additional way in which tetraspanins work with MHC. Tetraspanin microdomains are enriched for MHC II, CD86, and the class II editor human leukocyte antigen in the membrane of APCs. This complex is referred to as the "CDw78$^+$ microdomain" involved in T cell activation (20).

Through the above-enumerated regulatory pathways, the TEMs form a web for signal transduction from extracellular stimuli to intracellular-signaling components and ultimately regulate multiple biological processes, including cell activation, proliferation, adhesion, migration, and communication, as well as involvement in pathological conditions, such as autoimmune diseases, metastasis, and viral infection (Table S2 in Supplementary Material).

EXPRESSION PROFILES OF TETRASPANINS AND THEIR PARTNERS ON B CELLS

Uniquely expressed molecules in certain B cell subsets may serve as markers of the subset or have special function for that particular subpopulation. Systematic analysis of expression of tetraspanins and partners of tetraspanins on B cells may facilitate an understanding of their biological involvement in B cell biology including B cell development and function.

Expression of Tetraspanins on the Surface of B Cells

Most tetraspanins are expressed on B cells but differ in abundance in various B cell subsets at different developmental stages (**Figure 4**). mRNA transcripts of Tspan2-8, 31, 33, CD9, and CD63 are expressed at high levels in mouse progenitor B cells in the bone marrow but at very low levels, except for CD9 and Tspan31, in other B cell subsets (which mainly exist in periphery lymphoid organs). In contradistinction, CD37, CD53, CD82, and Tspan32 all show similar expression patterns of low level expression in mouse pro-B cells but high level in other B cell subpopulations. On tested human B cells (CD38$^-$ naïve B cells, isotype switch memory B cells, and memory B cells), TSPAN3, TSPAN13, CD53, CD37, CD82, CD81, CD63, and TSPAN33 show relatively high levels of mRNA (TPM > 10). TSPAN2, 5, 10, 14, 17, 31, and 32, and UPK1B, ROM1, CD151, and CD9 have detectable mRNA transcripts. But the remaining tetraspanins have no detectable mRNA. In addition, the expression of all tetraspanins except TSPAN19 is detectable in at least one strain of B cell lymphoma cell lines. More expression profiles of tetraspanins can be found in **Figure 4** and **Table 2**.

Expression of Tetraspanin Partners on the Surface of B Cells

Affinity capture assays, protein-fragment complementation assays, and two-hybrid tests in the databases of BioGRID (Table S3 in Supplementary Material) and ingenuity pathway analysis (**Table 1**; Table S2 in Supplementary Material) have allowed for the identification of hundreds of tetraspanin interacting partners. The main cell surface partner proteins of tetraspanins are other tetraspanins, integrins, G-protein coupled receptors, and transmembrane receptors like CD19. After removal of the partners expressed in the cytoplasm and the nucleus, there are 93 membrane proteins which potentially interact with extracellular tetraspanins or tetraspanins on the same membrane (Table S3 in Supplementary Material). Some of the tetraspanins which interact with other tetraspanins include CD151, CD37, CD53, CD63, CD81, CD82, CD9, ROM1, TSPAN2, TSPAN3, and TSPAN12 (Table S3 in Supplementary Material). Most of the partners show high levels of expression in more than one mouse B cell subset (**Figure 5**). In tested human B cells (CD38$^-$ naïve B cells, isotype switched memory B cells, and memory B cells), EZR, ADGRE5, ARF6, MSN, ITGB1, ITGA4, CD44, REEP5, EPN1, CR2, MET, ATP1A1, CD1D, ADAM10, APP, IGSF8, TNFRSF10B, and LGALS9 all show relatively high levels of mRNA (TPM > 10). More data can be found in **Figure 5**.

FUNCTIONS OF TETRASPANINS IN B CELLS

Tetraspanins modulate cell adhesion, migration, and invasion which are strongly involved in cancer development and progression (29). The association between tetraspanin expression and cancer prognostic is found in many kinds of cancers (**Table 1**). In B lymphoma, aberrant expression of CD9, CD81, and CD82 was linked to B-acute lymphoblastic leukemia (30–32). Increased CD37 expression was found in B cell malignancies and thus CD37 antibodies were developed to deplete malignant B cells for the treatment of chronic lymphocytic leukemia (33). The correlation of tumorigenesis and tetraspanins is discussed in another submission for this topic, so here we focus on the functions of tetraspanins and their partners on normal B cell biology without any further discussion of B cell malignancy. Functions of tetraspanins are summarized in **Figure 6** and **Table 3**.

Act as Markers Identifying B Cell Subsets

As discussed above (**Figure 4**; **Table 2**), since some tetraspanins are enriched in specific B cell subsets, they may be used

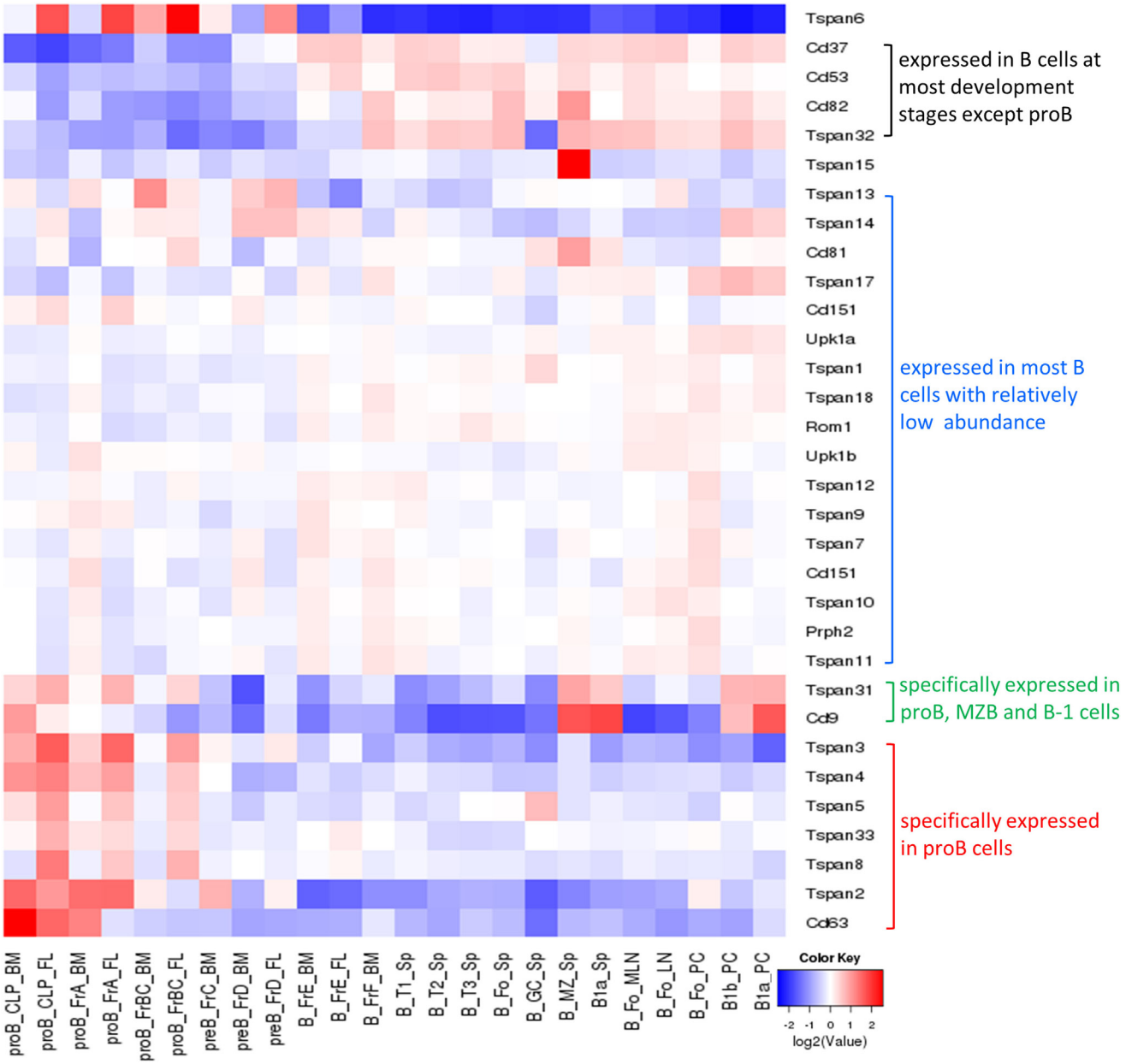

FIGURE 4 | Expression of tetraspanins in murine B cell subsets. The heat map was obtained from http://www.immgen.org/by inputting the list of tetraspanins in "My GeneSet" and choosing B cells as the populations of interest. The gene expression level is determined by Affymetrix microarrays (GEO: GSE15907).

as markers to identify B cell subsets or diagnostic markers for certain diseases. CD9 is reported to be a special shared marker by B-1 cells, MZB cells, and plasma cells in mice. A study demonstrated that CD9 is expressed by plasma cells in response to antigens independent or dependent on T cells, suggesting CD9 is acquired by T cell dependent plasma cells (34). Another study demonstrated that CD9 is a cell surface marker for precursors of human plasma cells in germinal centers. It is based on the evidence that (1) compared to $CD9^-$ cells, $CD9^+$ B cells show higher Blimp-1 but lower Bcl-6 and Pax-5 protein levels, and a faster process of differentiation into plasmablasts in the presence of PC-generating cytokines; (2) expression of CD9 was induced and gradually increased in $CD9^-$ GC-B cells under PC generating condition (35). A recent study showed that murine CD9 is a unique cell surface marker identifying IL-10 competent Bregs and their progenitors (36).

Roles in Cell Activation, Proliferation, Survival, and Development

The CD21/CD19/CD81 complex modulates signal transduction events pivotal for development of B lymphocyte and the normal

A

ID	Expression in human B cell (TPM))			Expression in murine B cell (TPM))
	CD38-naïve B cell	Class switch memory B cell	memory B cell	CD19+ B cell
EZR	334	210	313	15
ADGRE5	10	13	15	680
CD19	97	37	73	178
ARF6	64	52	77	119
MSN	98	63	95	18
ITGB1	10	153	42	
ITGA4	58	38	30	
CD44	32	25	31	31
REEP5	14	13	16	42
EPN1	3	2	3	67
CR2	23	10	18	13
MET	26	16	19	
ATP1A1	9	16	24	10
CD1D	10	10	19	3
ADAM10	14	7	8	8
APP	15	6	15	1
PDZD11	3	3	4	23
IGSF8	10	8	12	
TNFRSF10B	7	6	12	0.9
LRRC8A	6	3	5	11
GGT1	0.5	1	0.9	22
LGALS8	9	4	6	3
LGALS9	11	2	5	
TNFRSF17	4	5	5	
ADGRG5	5	3	4	
KPTN	2	0.5	0.9	8
SLC44A1	4	2	2	3
ITGA6				10
LPCAT3	3	2	3	2
SLC7A1	2	2	3	2
TUSC3				9
CD2				8
DAGLB	4	2	2	
LGALS3	0.5	2		5
POMC	2	2	2	
ITGB3				5
NAT14	2	1	2	
HTR3A	2	2		
LRP5	2	0.8	1	
ITGA3		2	0.8	
FZD3	0.8	0.7	1	
HSD17B13	1		0.7	
TIMP1		0.7	0.6	
BBS1	0.5		0.5	
HAVCR2	0.5	0.5		
ITGA5	0.9			
CLDN11	0.7			
P2RY12		0.6		
FZD1			0.5	

B

FIGURE 5 | Expression of tetraspanin partners in human and murine B cell subsets. **(A)** The expression of cell surface partners of human and murine tetraspanins on specific B cells. The partners without TPM values are not listed in the table. Human data are determined by the RNA-seq data generated by the Blueprint Consortium, and murine data are from RNA-Seq CAGE (Cap Analysis of Gene Expression) analysis of mouse cells in RIKEN FANTOM5 project. **(B)** Expression of tetraspanin partners listed in **(A)** in murine B cell subsets. The heat map was obtained from http://www.immgen.org/ by inputting the list of cell surface partners of tetraspanin partners in "My GeneSet" and choosing B cells as the populations of interest. Based on the database, ADGRE5, ADGRG5, and CD1D in **(A)** are shown as Cd97, Gpr114, and Cd1d1 in **(B)**, respectively.

function of humoral immunity. As shown in **Figure 1**, CD19, a hallmark of B cells, is sustained in its presence on B cells from the earliest pro-B cell stage to plasmablasts during development. CD19 functions as a co receptor of B cells in association with CD21 and CD81. In the complex, CD19 is the signaling molecule bound by Src-family kinases and PI-3 kinase, CD21 binds opsonized foreign antigens, and CD81 is associated with other tetraspanins including CD82, CD63, CD53, and CD9 to enable formation of TEMs (43). CoIP experiments demonstrated association of CD9, CD81, and CD82 with CD19 and digitonin treatment disrupted the CD9/CD19 and CD9/CD81 associations but not the CD81/CD19 association, implying that the association of CD9 with CD19 is through CD81 instead of another molecule. Different proteins including CD19 could be tyrosine phosphorylated which is induced by engagement of CD9, suggesting that CD9 involves B cell activation and differentiation (37). CD81 is one of the key proteins participating in controlling homeostasis of lymphocytes through modulating their proliferation. CD81 KO mice show reduced B1 cells and CD19 expression on B cells although the development of T cells and conventional B cells is normal. Moreover, in CD81 KO mice, the proliferative response of T cells is enhanced following TCR engagement, while proliferation of B cell responding to BCR cross-linking is severely impaired (21). Engagement of CD81 with hepatitis C virus (HCV) envelope protein E2 could protect B cells from apoptosis (56), and induce B cell activation (57, 58) and V_H hypermutation (59).

CD9 appears to function in B cell activation and differentiation based on its expression in specific B cell subsets and its functional interaction with CD19. CD9 is reported as a cell surface marker of B-1 B cells, MZ B cells, and plasma cells, but the development of these B cell subsets as well as the humoral immune responses to antigens appear to be normal when CD9 is knocked out (60). A recent study also confirmed that most of the tested markers expressed on total B cells are not significantly altered when CD9 is mutated. Interestingly, however, the

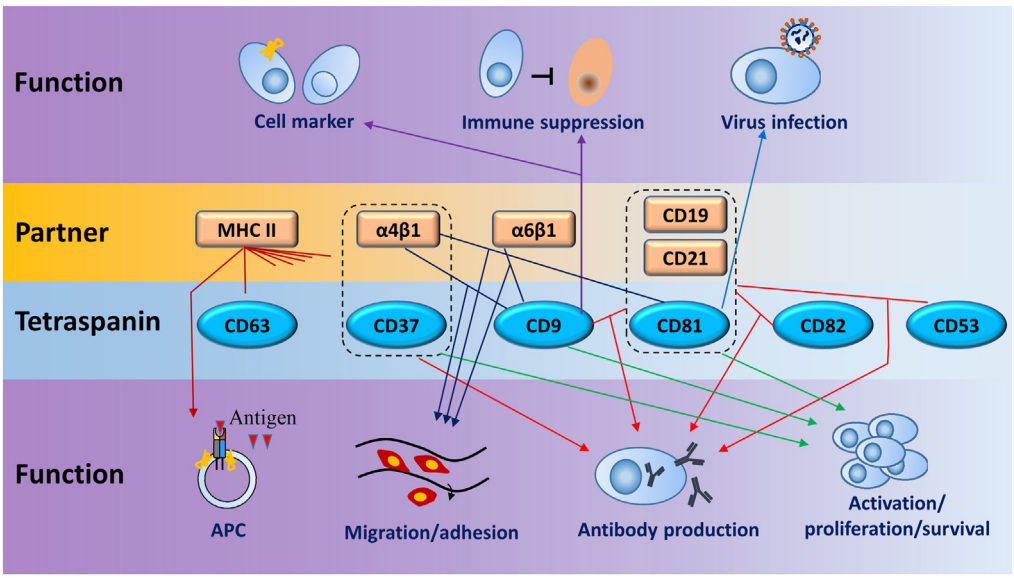

FIGURE 6 | Schematic diagram for main functions of tetraspanins and their partners in B cells. Dotted lines enclose coreceptors formed by tetraspanins and partners. Lines between tetraspanins and partners or coreceptors indicate the interactions which have functions in B cells (line with arrow) in seven biologic processes, such as cell activation, antigen presentation, and antibody production. One tetraspanin or partner can have one or more functions on B cells. Same colored lines with arrows point to similar function or regulation.

TABLE 3 | Function of tetraspanin on B cells.

Tetraspanin[a]	Keywords	Details of function on B cells
CD9	Markers, immune suppression, virus infection, activation, differentiation	Marker for murine marginal zone B cells, B-1, and plasma cells (34); a marker for plasma cell precursors in human germinal centers (35); novel cell surface marker of murine B10 cells and their progenitors (36); contributes to B cell activation and differentiation (37), and the survival of human GC B cells (38); involved in normalizing TH2- and TH17-driven airway inflammation in an IL-10-dependent manner (39); promotes inhibition of Th1-mediated contact hypersensitivity (36); enhances numbers of extracellular vesicles and improves the speed and efficiency of lentiviral gene delivery into T and B cells (40); Engagement of CD9 induces CD19 tyrosine phosphorylation (37)
CD81	Forms CD81–CD19–CD21 complex, proliferation	Controls lymphocyte homeostasis by facilitating the interaction with follicular dendritic cells through the VLA4/VCAM-1 axis (21); may interact with a putative ligand on a subpopulation of T cells to signal IL-4 production (41); determines CD19 membrane expression (42); interacts with CD19/CD21 complex and tetraspanins such as CD9, CD53, CD63, and CD82 to enable formation of tetraspanin-enriched microdomains (TEMs) (43); entry factor of hepatitis C virus (44)
CD53	Activation, adhesion, development, apoptosis, antibody production	Interferes with lymphocyte activation and cell adhesion; a direct genetic target for EBF1 which is a critical transcription factor for early B lymphocyte development, and can be induced by ectopic expression of EBF-1 (45); CD53 mediates PKCβ recruitment from cytosol to TEMs for B cell receptor activation (46). CD53 engagement with antibody against CD53 and Ig promotes activation of resting B cells into the G1 phase and induces Ig production in the presence of T cell supernatant (47)
CD63	Exosome production, virus infection, migration	Suppressor of exosome production and could regulate the exosome-mediated major histocompatibility complex class II-dependent T-cell stimulation (48); sensitized to and controls latent membrane protein 1-mediated NFkB activation for EBV persistence (49); cell migration by affecting the abundance of CXCR4 on the cell surface through IL-21-induced endocytosis and CD63-mediated endosomal recruitment (50)
CD37	Apoptosis, survival, antibody production	Regulates the membrane distribution of α(4)β(1) integrin crucial for activating the Akt survival pathways, increases apoptosis of plasma cells in germinal centers (51); initiates a cascade of events leading to apoptosis, counteracts death signals by regulating PI3K-dependent survival (52); promotes IgG1 production while inhibiting IgA immune responses *in vivo* and protects against the development of IgA nephropathy (53); control suppressor of cytokine signaling 3 (54)
CD82	Protection from cytotoxicity	Interferes with the capacity of the MHC-I complex to protect targets from NK-mediated cytotoxicity (55)

[a]*Only list the tetraspanins discussed in Section "Functions of tetraspanins on B cells."*

frequency of occurrence of IL10 competent Breg (B10 cells) is increased and CD23 expression is reduced on B10 cells when CD9 is knocked out (36). Moreover, CD9 is reported to facilitate interacting with human follicular dendritic cells through the VLA4/VCAM-1 axis and contribute to the survival of germinal center B cells (38).

In addition to its involvement in Ig production, CD53 also contributes to B cell differentiation. A study demonstrated that CD53 is a direct genetic target of EBF-1, a critical transcription factor in early B lymphocyte development. CD53 has functional binding sites for EBF-1 in its promoter elements and can be induced by ectopic expression of EBF-1 (45). CD53 transcripts are enhanced significantly by mitogenic stimulation, implying that CD53 may be involved in the transport of signals important for cell proliferation. Under conditions of serum deprivation, ligation of CD53 in B lymphoma cells triggers an AKT-mediated survival response and prompts a significant reduction in caspase activation and the number of cells that enter apoptosis (61). By using live-cell imaging and gene knockout mice, a recent study demonstrated that CD53 is specifically required for the recruitment of PKCβ (the protein kinase C family member) from cytosol to CD53-enriched TEMs on the plasma membrane to activate PKCβ for antigen-dependent BCR activation, suggesting that TEMs act as signaling hotspots (46).

The tetraspanin CD37 has profound roles in B cell proliferation and survival. CD37 regulates the plasma membrane distribution of α(4)β(1) integrins by controlling their mobility and clustering, a necessary step in activating Akt survival pathways. It is reported that the number of IgG-secreting plasma cells is reduced in lymphoid organs when CD37 is knocked out in mice, possibly due to the impaired association of VCAM-1 to the α(4)β(1) integrin for the Akt survival pathway with the corollary of increased apoptosis of plasma cells in germinal centers (51). In a recent study, CD37 knockout in mice can drive B cell lymphoma progression through constitutive activation of the IL6 pathway by losing the control of suppressor of cytokine signaling 3 (54). Although CD37 is crucial for B cells to survive and provide long lasting immune protection, another study reported that CD37 may trigger a cascade of events resulting in apoptosis when it is tyrosine phosphorylated and binds with signaling factors. The study also found that CD37 mediates SHP1-dependent death *via* its N-terminal domain, whereas it antagonizes death signals through the C-terminal domain by mediating PI3K-dependent survival (52).

CD82 associates with MHC-I at the cell surface of B cells and could interfere with the capacity of the MHC-I complex to protect targets from NK-mediated cytotoxicity (55). CD63 is reported as a suppressor of exosome production and could regulate exosome-mediated MHC II-dependent T-cell stimulation (48).

Roles in Antibody Production

In addition to its role in B cell proliferation and selection of IgG^+ plasma cells, CD37 promotes IgG1 production while inhibiting IgA immune responses *in vivo*. CD37 deficiency causes a reduction of serum IgG1 levels and alters B cell responses to T cell-dependent antigen under suboptimal costimulatory conditions (62). Besides the reduction in serum IgG1 levels, CD37 deficiency in B cells causes high levels of IL-6 and is directly responsible for the increased IgA^+ plasma cell numbers and IgA production levels in $CD37^{-/-}$ mice. $CD37^{-/-}$ mice are better protected from infection by *Candida albicans* than WT mice due to the increased *C. albicans*-specific IgA antibody levels. Neutralization of IL-6 *in vivo* could reverse the enhanced IgA response in $CD37^{-/-}$ mice (63). Therefore, it is not surprising to find that CD37 protects against the development of IgA nephropathy by controlling the formation and deposit of IgA–antigen complexes in the glomerulus (53).

The absence of CD81 on murine B cells causes a defect of antibody responses to T cell-dependent protein antigens and reduces the production of IL-4 that is specific to antigens in both spleens and lymph nodes. A putative ligand on a subpopulation of B and T cells may interact with CD81 to signal IL-4 production (41). The function of CD81 was confirmed in a patient carrying a homozygous mutation of the CD81 gene which caused the syndrome of antibody deficiency by disrupting the CD19 complex in B cells and impairing BCR activation although the CD19 alleles in the patient are normal. Otherwise, the patient had neither overt T cell subset nor functional defects, similar to CD19-deficient patients. Further study revealed that CD19 membrane expression critically depends on CD81 and no cell surface CD19 could be observed on B cells from the patient who had the mutated CD81 (42).

Besides the above proteins, CD53 also plays an important role in activation and differentiation of B cells. CD53 engagement with both the MEM-53 antibody against CD53 and a polyclonal anti-mouse immunoglobulin promotes B cell activation from resting status into the G1 phase, and induces Ig production when treated with T cell supernatant (47).

Immune Suppression

A study has shown that CD9 is a unique cell surface marker for murine B10 cells and their progenitor cells. Moreover, $CD9^+$ B cells are capable of inhibiting contact hypersensitivity mediated by Th1 cells *in vivo*. Further *ex vivo* assays demonstrated that CD9 is involved in cross-talk between B cells and T cells, which is required for $IL10^+$ B cells to suppress proliferation of T cells (36). Another study also indicated that $IL\text{-}10^+$ Bregs are enriched in a $CD9^+$ B cell subset and their homeostasis is altered by induced allergic asthma. Adoptive transfer of $CD9^+$ B cells in asthmatic mice normalizes lung function in an IL-10-dependent manner *via* inhibiting inflammation driven by Th2 and Th17 cells (39).

Roles in Virus Infection

Both CD9 and CD63 were identified and found to be transcribed by IgM^+ cells in different tissues of rainbow trout (*Oncorhynchus mykiss*). And the abundance of CD9 transcripts decreased significantly in IgM^+ splenocytes when the cells were exposed *in vitro* to viral hemorrhagic septicemia virus (64). Overexpression of CD9 caused a significantly higher yield of extracellular vesicles and improved the speed and efficiency of

lentiviral gene delivery into T and B cells with the lentivirus produced in the CD9 high cells, although the virus titers were not increased. The study indicates an important role for CD9 in membrane fusion, virus infection, and information transfer mediated by extracellular microvesicles (40).

Viral oncogene latent membrane protein 1 (LMP1) accumulates within intraluminal vesicles to avoid degradation and thus constitutively activates NF-κB which is important for EBV persistence in B cells. CD63 associates with LMP1 and facilitates the inclusion of LMP1 into vesicles lacking MHC II. Preclusion of LMP1 assembly within CD63-enriched domains by C-terminal modifications of LMP1 leads to NF-κB overstimulation. Interference through shRNAs against CD63 causes redistribution of LMP1 and leads to a dramatic increase in LMP1-induced NF-κB activity, indicating that CD63 is sensitized to and controls LMP1-mediated NF-κB activation (49).

CD81 plays important roles in HCV infection by acting as a HCV entry factor (65), promoting HCV RNA replication (66), and reducing HCV-induced immune responses (44). B cells expressing CD81 can be infected by HCV and serve as reservoirs for chronic HCV infection (67).

Cell Migration, Adhesion

CD63 plays important roles in cell migration as it can affect the abundance of CXCR4 on the cell surface through IL-21-stimulated endocytosis and endosomal recruitment. Restimulation of activated B cells with T cell-produced IL-21 accelerates CXCR4 internalization by inducing endocytosis-related GRK6 expression. The level of CD63 is strikingly elevated in activated Bcl6-deficient B cells and downregulation of CD63 mRNA with siRNAs upregulates CXCR4 expression on the B cells. Activated B cells treated with Bcl6 inhibitor have a similar phenotype to Bcl6-deficient B cells: increased CD63 mRNA expression and downregulated CXCR4 expression (50). It is reported that CD53 plays an important role in homotypic cell aggregation of lymphocytes and may interfere with lymphocyte activation and cell adhesion. HI29, an anti-CD53 monoclonal antibody, was able to induce homotypic cell aggregation in a B cell strain from a leukocyte adhesion deficiency patient. Moreover, pre-incubation with MEM53, another antibody against CD53, can block such aggregation but anti-CD44 or anti-CD49d mAbs have no blocking effects. Tetraspanins also interact with integrins which function within the area of cellular motility. Ectopic expression of CD9 has been reported to enhance B cell migration *via* interacting with integrins α6β1 and α4β1 (23). α4β1 on B cells can also be associated with CD81 (68).

FUNCTIONS OF TETRASPANIN PARTNERS EXPRESSED ON B CELLS

The partners of tetraspanins have multiple functions in B cells—including regulation of B cell activation, survival, development, antibody production, virus infection, and signal transduction—through mechanisms which may not be correlated with the interaction between tetraspanins and the partners. More details can be found in **Table 4**.

THERAPY STRATEGIES FOR IMMUNE DISEASES CORRELATED WITH TETRASPANINS AND THEIR PARTNERS

Most studies on employing tetraspanins and their partners as therapy target of diseases involve cancer treatments, a subject outside the scope of this review. For other diseases, there are some strategies using tetraspanins and their partners on B cells as therapy targets, as we explain below.

Depletion of B Cell Subsets, Blockade of Receptors or Crosslinking With Antibodies Against Certain Tetraspanins or Partners

CD21 can be used as a target for depletion of EBV positive B cells as it is a receptor for EBV on B cells. CD19 is a hallmark of B cells and could be used as a target for B cell depletion in the treatment of autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus (SLE). There is a phase I clinical trial (Identifier: NCT00639834, ClinicalTrials.gov) using anti-CD19 antibody MDX-1342 together with methotrexate for the treatment of patients with rheumatoid arthritis.

CD81 is an entry factor for HCV infection. Monoclonal antibodies with high affinity to CD81 are generated for prevention of HCV infection (108).

TNFRSF17/BCMA is preferentially expressed in $CD180^-$ B cells which produce autoantibodies and are significantly increased in SLE (104, 105, 109). TNFRSF17/BCMA and $CD180^-$ B cell subsets would be ideal targets for SLE treatment.

CD44 engagement could control CD40L-mediated polyclonal B cell activation (88). Cross-linking of CD53 with antibodies against CD53 promotes activation of resting B cells, speeds up the entrance into the G1 phase of cell cycle, and induces Ig production during the incubation with T cell supernatant (47).

Reduction of Protein Abundance With shRNAs or siRNAs Against Certain Tetraspanins or Partners

Interference through shRNAs against CD63 causes redistribution of LMP1, leads to a dramatic increase in LMP1-induced NF-κB activity, and would benefit treatment of EBV infection (49).

Overexpression or Delivery of Certain Tetraspanins or Partners in B Cells

CD9 expression increases exosome production and promotes lentivirus infection (40), thus CD9 could be overexpressed in the engineered cells producing therapeutic exosomes to enhance the yield of exosomes and the delivery efficiency of exosomes.

Interference of Tetraspanins or Partners With Small Molecules, Inhibitors, or Stimulators of Diseases

ADAM10 improves IgE production *via* its sheddase activity on CD23, an IgE receptor with low affinity (70). Adam10 increases in the B cells of allergic patients and Th2 prone mice (71) and

TABLE 4 | Functions of tetraspanin partners on B cells.

Partner[a]	Tetraspanin interacted	Function of partners on B cells
Adam10	TSPAN33	Required for development of T1 B cells to marginal zone B cells (69); increased in allergic patients, sheddase of CD23, and promotes IgE production (70); release of TACI in B cells and reflects systemic and compartmentalized B cell accumulation and activation (71); required for CD23 sorting into B cell-derived exosomes (72)
CD19	CD37, CD82, CD81	Interacts with CD21, CD81, and B cell receptor (BCR) complex to augment signals by the pre-BCR/BCR for transducing signals; modulates B-cell fate decisions at multiple stages of development (37, 73, 74); pivotal for Akt activation that is mediated by BCR (75); intensifies Src-family PTK activation following BCR ligation (76); important for recruitment of Vav, Grb2, PI3K, phospholipase Cγ2, and c-Abl, or SHPI and SHIP phosphatases (77)
CD1d	CD82	Regulates interaction between activated T cells and B cells which is crucial to B cell proliferation and antibody production (78); mediates antigen presentation and augments antibody responses (79); CD1d knockout in mice impairs resistance to *Borrelia burgdorferi* infection due to impaired antibody production (80); CD1d upregulation on Breg cells is induced by chronic intestinal inflammatory conditions (81)
CD2	CD53	Expressed preferentially on fetal thymic B cells, anti-CD2 antibody increases IL-4-dependent Ig production by thymic B cells (82); the interaction of CD2 with LFA-3 enhances B cell responses (83); modulates T cell-dependent B cell activation (84); all peripheral B cell, the majority of bone marrow B cells and half of pre-B cells are CD2 positive (82).
CD36	CD9	Expressed by most resting MZ B cells, has no role in the development of B cells but regulates both primary and secondary phosphoryl choline antibody responses during *S. pneumoniae* infection (85); a target gene of POU2F2 transcription factor (86)
CD44	TSPAN8	Complex of CD44 and CD74 binds macrophage migration inhibitory factor to induce B cell survival (87); CD44 engagement could prevent polyclonal B cell activation by CD40L, while allowing B cell activation by interacting between soluble IgM and CD40L (88); required for interaction between B cells and monocytes independent of the B-cell receptor (89); induces murine B cell activation through hyaluronate-CD44 interactions (90)
CR2/CD21	CD37, CD81	An Epstein–Barr virus receptor on B cells and transduces signals (91); an interferon α receptor on B cells (92); a novel target for depletion of EBV-infected cells (93); binds to gp350 for efficient EBV infection of resting B cells (94); $CD21^{low}$ B cells are apoptosis-prone (95); uncoupling of CD21 and CD19 significantly reduces survival of GC B cells and titers of secondary antibody (96); defective B cell ontogeny and humoral immune response is similar between human CD21 transgenic mice and aging wild-type mice (97); premature expression of human CD21 promotes B cell deletion and reduces auto-antibody titer significantly (98); CD21/CD19-mediated signaling enhances B cell survival in primary immune response (99); forms complex with CD19 and CD81 into signaling-active lipid rafts (100, 101)
MET	CD82	Recruited to CD74/CD44 complex and activated by HGF then leads to a survival cascade of B cells (102); stimulated by HGF/SF and enhances GC B cell adhesion to both VCAM-1 and fibronectin; predominantly expressed on $CD38^{+}CD77^{+}$ tonsillar B cells (103)
TNFRSF17/BCMA	TSPAN6	Reduced BCMA expression on peripheral B cells associates with severe syndrome of systemic lupus erythematosus (SLE) patients (104); preferentially expressed by autoantibody producing $CD180^{-}$ B cells from active SLE patients (105); associates with TNF receptor-associated factors and activates NF-κB, elk-1, c-Jun N-terminal kinase, and p38 MAPK (106); receptor of a TNF homolog and implicated in SLE disease mediated by B cells (107)

[a]*Obtained by searching keywords in title with the partners of tetraspanins in* **Table 4** *and keywords in title/abstract with "B cell," and excludes the literature about cancer, lymphoma, and leukemia.*

would help diagnostically in predicting Th2 disease susceptibility. ADAM10 inhibitors could be used for attenuating allergic diseases.

Immunotherapy With Certain B Cell Subsets Defined by Specific Tetraspanins or Partners

IL-10 secreting Breg defined by CD19 and CD9 in mouse (36) or CD19, CD27, and CD38 in human (4) could be enriched, expanded, and then adoptively transferred for treatments of autoimmune diseases.

AUTHOR CONTRIBUTIONS

FZ, XW, and XH summarized the literature, wrote the manuscript, and prepared figures. GR, SZ, and UB provided critical comments and wrote part of the manuscript. JS supervised all the work and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01606/full#supplementary-material.

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Monocyte Subsets Have Distinct Patterns of Tetraspanin Expression and Different Capacities to Form Multinucleate Giant Cells

Thomas C. Champion[1,2], *Lynda J. Partridge*[3], *Siew-Min Ong*[2], *Benoit Malleret*[2,4], *Siew-Cheng Wong*[2,4†] *and Peter N. Monk*[1*†]

[1] *Department of Infection, Immunity and Cardiovascular Disease, University of Sheffield, Sheffield, United Kingdom,* [2] *Singapore Immunology Network, Agency for Science, Technology and Research, Singapore, Singapore,* [3] *Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, United Kingdom,* [4] *Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University of Singapore, National University Health System, Singapore, Singapore*

Monocytes are able to undergo homotypic fusion to produce different types of multinucleated giant cells, such as Langhans giant cells in response to *M. tuberculosis* infection or foreign body giant cells in response to implanted biomaterials. Monocyte fusion is highly coordinated and complex, with various soluble, intracellular, and cell-surface components mediating different stages of the process. Tetraspanins, such as CD9, CD63, and CD81, are known to be involved in cell:cell fusion and have been suggested to play a role in regulating homotypic monocyte fusion. However, peripheral human monocytes are not homogenous: they exist as a heterogeneous population consisting of three subsets, classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and non-classical ($CD14^{+}CD16^{+}$), at steady state. During infection with mycobacteria, the circulating populations of intermediate and non-classical monocytes increase, suggesting they may play a role in the disease outcome. Human monocytes were separated into subsets and then induced to fuse using concanavalin A. The intermediate monocytes were able to fuse faster and form significantly larger giant cells than the other subsets. When antibodies targeting tetraspanins were added, the intermediate monocytes responded to anti-CD63 by forming smaller giant cells, suggesting an involvement of tetraspanins in fusion for at least this subset. However, the expression of fusion-associated tetraspanins on monocyte subsets did not correlate with the extent of fusion or with the inhibition by tetraspanin antibody. We also identified a $CD9^{High}$ and a $CD9^{Low}$ monocyte population within the classical subset. The $CD9^{High}$ classical monocytes expressed higher levels of tetraspanin CD151 compared to $CD9^{Low}$ classical monocytes but the $CD9^{High}$ classical subset did not exhibit greater potential to fuse and the role of these cells in immunity remains unknown. With the exception of dendrocyte-expressed seven transmembrane protein, which was expressed at higher levels on the intermediate monocyte subset, the expression of fusion-related proteins between the subsets did not clearly correlate with their ability to fuse. We also did not observe any clear correlation between giant cell formation and the expression of pro-inflammatory or fusogenic cytokines. Although tetraspanin expression appears to be important for the fusion of intermediate monocytes, the control of multinucleate giant cell formation remains obscure.

Keywords: monocyte, tetraspanin, cd9, fusion, monocyte subsets

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***Correspondence:**
Peter N. Monk
p.monk@shef.ac.uk

†*Joint principal authors.*

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INTRODUCTION

Human monocytes are able to migrate from the bloodstream into the tissues and differentiate into macrophages and monocyte-derived dendritic cells (1). They are important in defense against various pathogens (2) but are also implicated in autoimmune and inflammatory diseases (3). Blood monocytes are heterogeneous and three subsets have been defined: classical (Cl, $CD14^{++}CD16^{-}$), intermediate (Int, $CD14^{++}CD16^{+}$), and non-classical (NCl, $CD14^{+}CD16^{+}$), comprising ~85, 5, and 10% of the total, respectively (3, 4). Investigation of the maturation and differentiation kinetics of labeled human monocytes *in vivo* suggests that they mature from Cl to Int and then to NCl (5, 6). The subsets differ in their gene expression profiles, cell surface markers, and cytokine secretion (7–11). The blood populations of the Int and NCl have been observed to be increased in patients with tuberculosis (12) and rheumatoid arthritis (13), whereas Int numbers are increased in various other inflammatory conditions, including Crohn's disease (14), sarcoidosis (15), and cardiac disease (16, 17).

Under certain circumstances, monocytes and macrophages are able to fuse to form multinucleated giant cells (MGC), such as the osteoclast MGC that remodel and maintain bone homeostasis (18). Monocytes can form inflammatory MGC, such as Langhans giant cells (LGC), in response to *M. tuberculosis* infections during granuloma formation around infected macrophages (19). Monocytes can also fuse in response to non-phagocytosable foreign material such as medical implants, forming foreign body giant cells (FBGC) (20).

The mechanism of monocyte fusion is still largely unknown and only a handful of essential proteins have been identified (21, 22). Furthermore, LGC and FBGC formation appears to be initiated by different cytokines, IFNγ and IL-4, respectively, which could suggest that they coordinate fusion through multiple signal transduction pathways (23, 24). Monocytes activated by fusogenic stimuli secrete chemokines, such as CCL2 and CCL3, upregulate cell–cell adhesion proteins (LFA-1, ICAM-1, and E-cadherin) (25) and fusion-facilitating proteins, such as CD200 (26), SIRPα/CD172a/MFR (27), CD47 (28), CD36 (29), CD62E (E-selectin) (30), matrix metallopeptidase 9 (MMP9) (31), and dendrocyte-expressed seven transmembrane protein (DC-STAMP) (32, 33).

The tetraspanin family of membrane proteins has been implicated in the regulation of several different types of cell–cell fusion, including CD9, CD81, and CD151 in sperm–egg interactions (34), CD9 and CD81 in muscle cell fusion (35), CD82 in HTLV-1 syncytial formation (36) and CD9 in HIV-1-induced cell fusion (37). Osteoclast formation is known to be regulated by CD9, Tspan-5, and Tspan13 (38, 39). In experimental systems using concanavalin A (ConA)-induced fusion, anti-tetraspanin antibodies against CD9, CD81, CD151, and CD63 have been shown to inhibit or enhance the formation of MGC (40–42). Importantly, many of the fusion regulatory proteins implicated in MGC formation have been shown to be associated with tetraspanins in the plasma membrane (43).

Recently, CD9, CD53, CD63, and CD81 were shown to be expressed differently on the three monocyte subsets (44), indicating that subsets may have different fusion behaviors. In this study, we have investigated the propensities of the monocyte subsets for fusion, and attempted to correlate this with the expression of a group of fusion-related tetraspanins, fusion proteins, and cytokines. Further understanding of the contribution of monocyte subsets to fusion and the role tetraspanins play in the fusion process may help develop treatments for granulomatous diseases such as tuberculosis and inhibit foreign body reactions during medical implant rejection.

Abbreviations: ConA, concanavalin A lectin; MGC, multinucleated giant cell; Cl, classical monocyte sybset; NCl, non-classical monocyte subset; Int, intermediate monocyte subset; LGC, Langhans giant cell; SGC, syncytial giant cell; FBGC, foreign body giant cell; FI, fusion index; MACS, magnet-activated cell sorting; DC-STAMP, dendrocyte-expressed seven transmembrane protein; SEM, scanning electron microscopy; MFI, median fluorescence intensity; MMP9, matrix metallopeptidase 9.

MATERIALS AND METHODS

Cells

All experiments used human blood monocytes collected in EDTA. For experiments using purified monocyte subsets, cells were obtained from apheresis cones donated by anonymous platelet donors in Singapore. Blood samples and experimental procedures were approved by the Institutional Review Board, Singapore, in accordance to guidelines of the Health Science Authority of Singapore (Reference code: NUS-IRB10-250). Informed written consent was obtained from participants for this study in accordance with the Declaration of Helsinki. Apheresis cones contain approximately $400–1{,}200 \times 10^6$ cells per cone, of which ~68% are lymphocytes, ~25% monocytes, ~5% neutrophils, ~2% basophils, and <1% eosinophils (45).

Monocyte Purification

Human blood from apheresis cones was diluted 1:1 in Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} (Lonza). Diluted blood was separated on Ficoll-Paque PLUS (GE Healthcare Life Sciences) by centrifugation. The PBMC layer was removed and washed with saline to remove platelets. Red blood cells were lysed and cell number and viability determined by counting in the presence of Trypan Blue (Sigma-Aldrich). Total monocytes were positively selected using anti-CD14-beads according to manufacturer's instructions (Miltenyi Biotec). The purity as determined by flow cytometry, and viability by Trypan blue exclusion were consistently >90%. In some cases, monocytes were also purified by adherence to plastic, as described previously (41). Monocytes for subsequent subset fractionation were first enriched by depleting non-monocytic cells using magnet-activated cell sorting (MACS) with anti-CD3 and anti-CD19-beads, according to manufacturer's instructions (Miltenyi Biotec). MACS-enriched monocytes contained typically 69% monocytes. For FACS purification of monocyte subsets, a cocktail containing anti-CD14-efluor450 (eBioscience), anti-CD16-FITC (Miltenyi Biotec), and anti-CD56-APC (BD Biosciences) was added to the MACS-enriched total monocytes. Contaminating NK cells were excluded and monocyte subsets: Cl (~80%; $CD14^{++}CD16^{-}$), Int (~8%; $CD14^{++}CD16^{+}$), and NCl (~11%; $CD14^{+}CD16^{++}$)

were gated based on the gating strategy shown in Figure S1 in Supplementary Material. To maintain reproducibility, subsets were always gated with equal sized square gates with perpendicular borders. A post-sort check was conducted in every subset to ensure that the purity of each subset was ≥90%.

Fusion Assays

FACS-purified monocyte subsets were seeded at 1.5×10^5 cells per 31.65 mm^2 well to give a cell density of 4,739 monocytes mm^{-2}. Within an hour of seeding, ConA from *Canavalia ensiformis* (Sigma-Aldrich) was added at 10 μg ml^{-1} in IMDM (Lonza) containing human AB Serum (Innovative Research, Inc., IPLA-SERAB) and penicillin/streptomycin (Biological Industries). Monocyte subsets were incubated for up to 72 h at 37°C in 5% CO_2 for all fusion assays. The supernatant from each well was collected and stored at −80°C for cytokine measurements. Cells were stained with nucleus/actin staining solution containing 3 μg ml^{-1} DAPI (ThermoFisher) and 1 μg ml^{-1} Phalloidin-TRITC (ThermoFisher) overnight at 4°C in the dark. The cells were then fixed and imaged with an Olympus IX83 inverted microscope running MetaMorph for Olympus imaging software (Olympus, UK). MGC were identified from the image Stack on FIJI ImageJ and freehand outlines were drawn around each MGC (defined as cells with ≥3 nuclei) to make Region Of Interest coordinates that could be saved alongside the Stack files. The DAPI stack and ROI list file were loaded in ImageJ before using a selection on user-generated macros to count the nuclei per MGC, MGC area and the total number of nuclei per field. MGC types were designated using the criteria outlined in Figure S2 in Supplementary Material. LGC and FBGC are known types of MGC but a third category was also detected in our studies, which we termed the syncytial giant cell (SGC). SGC are characterized by having no clear organization of nuclei and with patchy staining for polymerized actin (Figure S2 in Supplementary Material). In all cases, the total nuclei counted in single and fused cells were much lower than the number originally plated. The missing nuclei were designated as "Detached Cells" but the fate of these cells was not investigated further.

Measurement of Tetraspanin and Fusion Protein Expression by Flow Cytometry

A 10-marker panel was developed that could identify the three monocyte subsets, quantify the expression of seven tetraspanins and detect cell viability all in one sample (Table S1A in Supplementary Material). The panel consisted of a LIVE/DEAD Blue dye, two monocyte subset markers (anti-CD14- PE-CF594 and anti-CD16- PE-Vio770) and tetraspanins (anti-CD9-Biotin, anti-CD37-APC, anti-CD53-CF405M, anti-CD63-PerCP, anti-CD81-Alexa Fluor 700, anti-CD82-PE, anti-CD151-FITC). Strepavidin-APC-Cy7 was used as the secondary reporter for CD9-Biotin. To detect changes after ConA treatment, adherent monocytes were treated with or without ConA for 4 h (at which point monocyte fusion can be observed), and then harvested by scraping prior to antibody staining, as above. A compensation matrix was generated on FACSDiva software using negative control or capture anti-mouse Fc compensation beads for all fluorophore combinations. In separate experiments, fusion protein antibodies were used individually on freshly isolated monocytes, with a FITC-labeled secondary antibody, using the CD14/CD16 antibody pair to distinguish subsets. In all cases, antibodies were individually titrated to ascertain the concentration for optimum binding and compared to an appropriate isotype control antibody. Flow acquisition was performed on a BD LSR II.

Median Nuclei per MGC

The number of nuclei per MGC had a positive skew whereby smaller (3–8 nuclei) MGC were far more common than larger (≥20 nuclei) MGC and so the median was used to describe the average size of a giant cell in any given condition.

Fusion Index (FI)

Fusion index expresses the fusion of cells as the ratio of nuclei inside fused cells with ≥3 nuclei to the total number of nuclei counted and expressed as a percentage.

Cytokine Assays

The supernatants collected at 24, 48, and 72 h from the fusion studies were stored at −80°C before analysis for CCL2 (MCP-1), CCL3 (MIP-1α), RANTES, IL-1α, IL-1β, TNFα, IL-6, IL-17A, IL-4, IL-10, IL-13, GM-CSF, IL-3, IFNγ, and VEGF, using Luminex® xMAP® technology and customized human 9- and 15-plex kits (Merck Millipore) with DropArray™-bead plates (Curiox).

Scanning Electron Microscopy (SEM)

For imaging by SEM, sorted cells were allowed to adhere for 15 min at room temperature to glass coverslips pretreated with poly-L-lysine (Sigma), then were fixed for 1 h at room temperature in 2.5% (w/v) glutaraldehyde and 0.1 M phosphate buffer (pH 7.4) and were washed twice in PBS. After fixation for 1 h at room temperature with 1% (w/v) osmium tetroxide (Ted Pella), cells were washed in deionized water and dehydrated with a graded series of ethanol immersions from 25 to 100%, and were dried to the critical point (CPD 030; Bal-Tec). The glass coverslip was then laid on adhesive film on a scanning electron microscope sample holder and was firmly touched with an adhesive sample holder. The surface on which the cells were deposited, as well as the adhesive surface, were both coated with 5 nm of gold in a high-vacuum sputtering device (SCD005 sputter coater; Bal-Tec). The coated samples were examined with a field emission scanning electron microscope (JSM-6701F; JEOL) at an acceleration voltage of 8 kV with the in-lens secondary electron detector. Fluorescence and brightfield images were also taken and collaged into larger map images using ImageJ FIJI. The brightfield map was compared with the low magnification SEM images to identify the location of the high magnification SEM images. The appropriate high magnification SEM images and 20× magnification fluorescent images were then matched, cropped, and merged using ImageJ.

Statistical Analyses

All statistical analyses were performed with GraphPad Prism v6.04 and the appropriate tests are noted in the legend of each figure. In all figures the data value represents the number (*n*) of

different donor repeats in the experiment, and the SEM is reported where $n \geq 3$, except where stated. All fluorescence-based values [flow cytometry median fluorescence intensity (MFI)] were log-transformed before statistical analysis. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

RESULTS

Monocyte Subsets and MGC Formation

Human blood monocytes were first negatively selected by removing non-monocytic cells using MACS and then subjected to positive selection for individual subsets using stringent gating based on anti-CD14 and anti-CD16 antibody binding (Figure S1 in Supplementary Material).

Fusion was induced using ConA, a lectin known to stimulate cell fusion in diverse cell types, e.g., *Drosophila* somatic cells (46). The exact mechanism of ConA facilitated fusion is currently unknown. However, it has been shown that ConA triggers a release of fusion initiating cytokines from mouse macrophages, such as IFNγ, TNF-α, IL-1β, and IL-4 (47). The behavior of the different subsets during ConA stimulation was determined by counting stained nuclei to provide a measure of the proportions of single and fused cells where the latter refers to cells with >3 nuclei (**Figure 1**). Interestingly, the majority of the monocytes were lost after 72 h, presumably due to detachment and/or cell death. Int monocytes fused more rapidly than Cl and NCl subsets, but were also significantly more likely to be dead/detached by 48 and 72 h. Cl monocytes were significantly less likely to fuse than either of the other two subsets, although the differences between subsets became less pronounced over time.

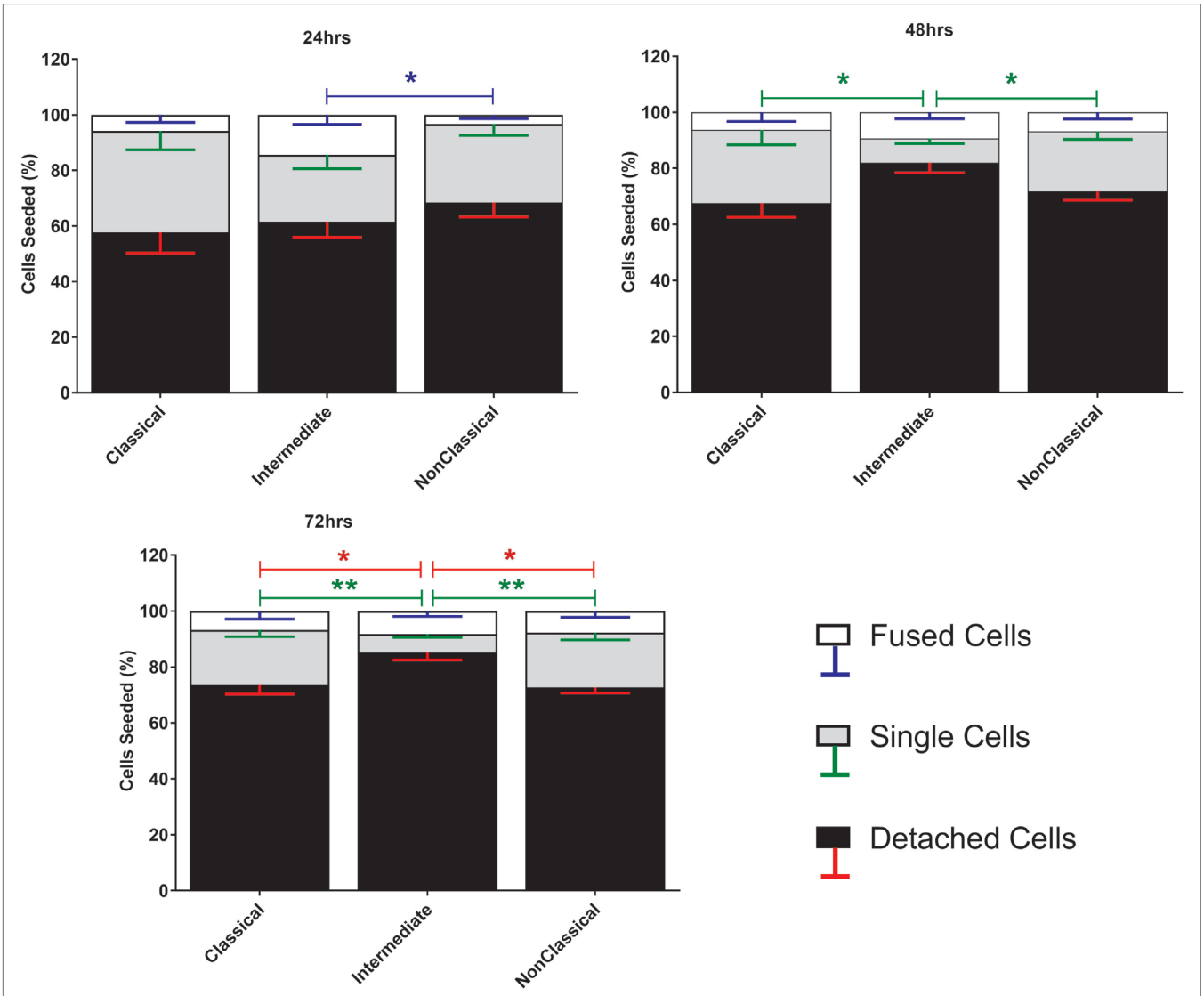

FIGURE 1 | Cell fate during ConA-induced fusion varies between monocyte subsets. The fate of sorted monocyte subsets was determined by counting nuclei at 24, 48, and 72 h and expressed as a percentage of the cell numbers originally plated. Bars represent means ± SEM, $n = 8$. Significance was tested with a Kruskal–Wallis test with a Dunn's multiple comparisons test comparing the means of the same fate and time point against the other subsets. Black bars/red error bars: detached cells, gray bars/green error bars: single cells and white bars/blue error bars: fused cells with >3 nuclei.

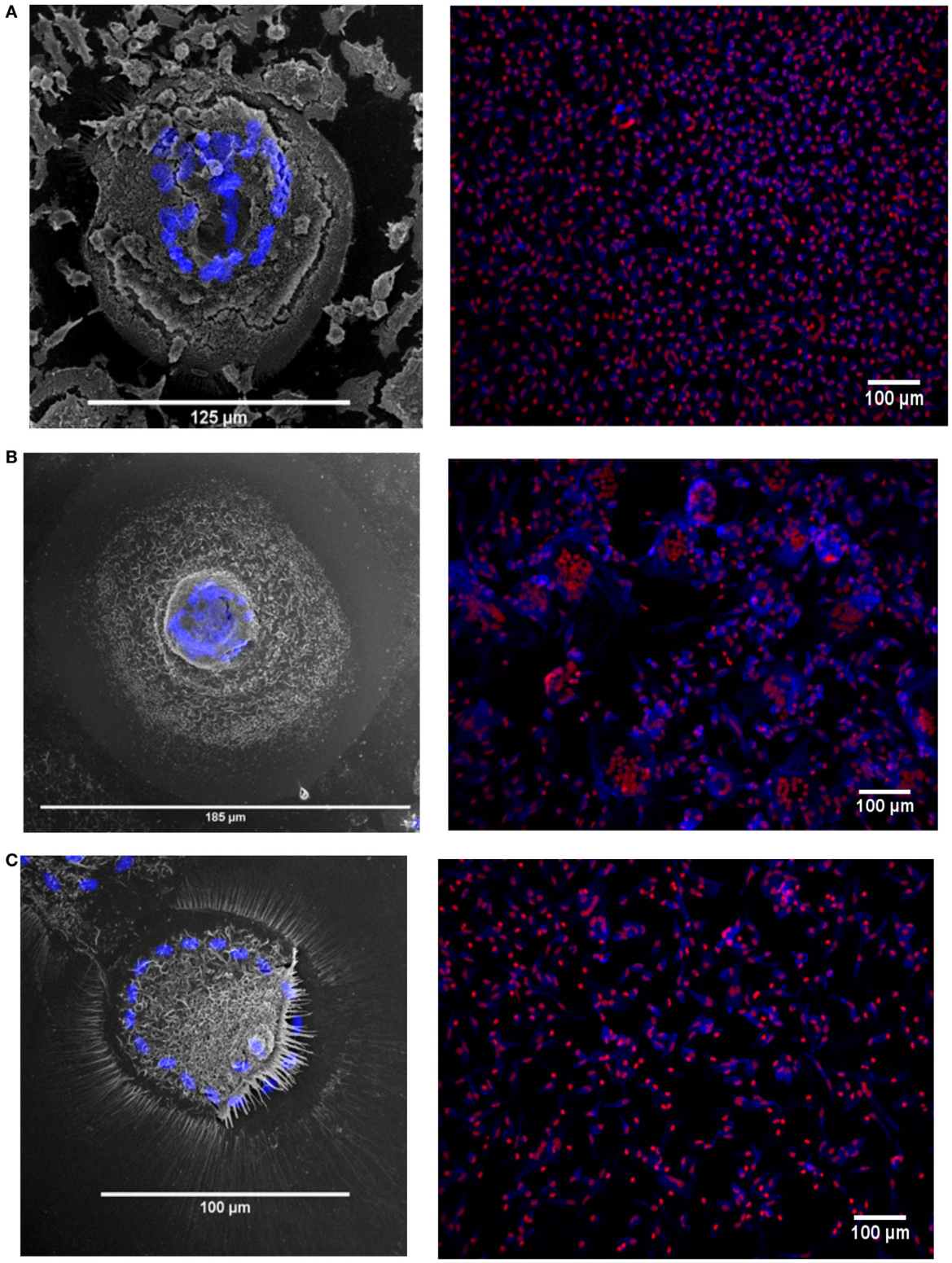

FIGURE 2 | Fusion in different monocyte subsets imaged by tandem fluorescent scanning electron microscopy (SEM) and wide field fluorescence microscopy. **(A–C)** left panels. monocyte-derived giant cell (MGC) were generated by 72-h concanavalin A (ConA) treatment of FACS-sorted monocyte subsets, stained with Hoechst and then raster-scanned so that the MGC imaged in SEM could be located and the nuclear channel overlaid onto the image. Nuclei shown in blue. **(A–C)** right panels. Three representative montages containing images taken of each of the monocyte subsets from one donor after 72 h ConA treatment. Blue = F-actin, Red = nuclei. **(A)** classical, **(B)** intermediate, **(C)** non-classical subset-derived MGC.

The MGC were classified as LGC, FBGC, or SGC based on the arrangement of nuclei within each MGC according to the criteria shown in Figure S2 in Supplementary Material. Representative wide field and tandem fluorescence-SEM images of fused monocytes formed after 72 h are shown for each subset in **Figure 2**. There are distinct differences in the sizes and morphologies of the MGC (**Figure 2**), suggesting subset-specific factors in MGC formation. Interestingly, the monocyte purification method, i.e. adherence or positive purification by MACS using anti-CD14 strongly affected MGC formation in response to ConA. Despite a similar median number of nuclei observed per MGC, MACS-purified monocytes formed significantly larger MGC than

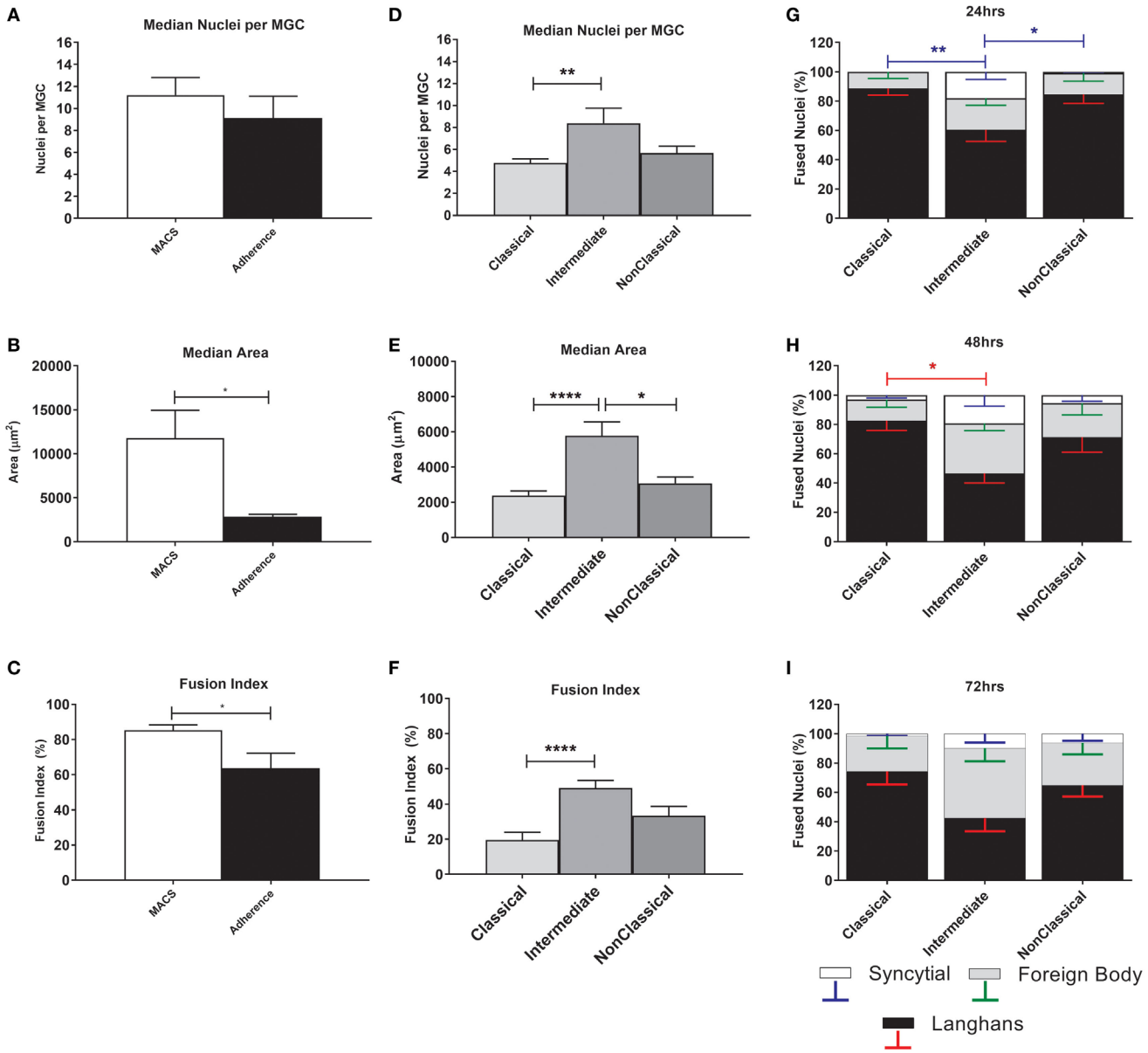

FIGURE 3 | Fusion parameters vary between monocyte purification method and between subsets. **(A–C)** Unfractionated monocytes, purified by magnet-activated cell sorting (MACS) or adherence were incubated for 72 h with concanavalin A (ConA) to induce fusion. After fluorescence imaging, three parameters of monocyte-derived giant cell (MGC) were recorded: median number of nuclei/MGC, fusion index (FI), and median area occupied by each MGC. Significance was tested using an unpaired *t*-test. **(D–F)** Monocytes sorted into subsets by FACS were incubated for 72 h with ConA to induce fusion. After fluorescence imaging, three parameters of MGC were recorded: median number of nuclei/MGC, FI and median area occupied by each MGC. Significance was tested with a Kruskal–Wallis test with a Dunn's multiple comparisons test. **(G–I)** After 24, 48, and 72 h incubation with ConA, the proportions of each type of MGC were recorded and presented as a percentage of the total fused nuclei counted. Bars represent means ± SEM, $n = 8$. Significance was tested with a Kruskal–Wallis test with a Dunn' multiple comparisons test comparing the means of the same MGC type within the same time point against the other subset means. Black bars/red error bars: Langhans giant cell, gray bars/green error bars: FBGC and white bars/blue error bars: syncitial giant cell (SGC).

adherence-purified monocytes, as determined by the median area and FI (**Figures 3A–C**).

The physical parameters of the MGC formed by monocyte subsets were assessed at 72 h. FI is significantly higher for Int relative to Cl monocytes, as is the median number of nuclei per MGC (**Figures 3D–F**), while Int and NCl subsets were similar for both measurements. Interestingly, the median area covered by each MGC is higher for Int monocytes relative to the other two subsets, perhaps related to the higher percentages of FBGC and SGC observed in Int cultures (**Figures 3G,H**). At 24 h, the Int monocytes formed significantly more SGC (**Figure 3G**) whereas at 48 h of ConA stimulation, they were significantly less likely to form LGC than Cl monocytes (**Figure 3H**). By 72 h, no significant difference in the types of MGC formed was observed between the subsets (**Figure 3I**). Thus, there are quantitative differences between the MGC formed by the subsets, in terms of the kinetics of fusion, the sizes, and morphologies of the MGC formed.

Tetraspanin Expression on Monocyte Subsets

Tetraspanins, particularly CD9, CD81, CD151, and CD63, have been associated with cell fusion in monocytes, developing muscle, and during fertilization (43). We therefore measured the plasma membrane expression of seven common tetraspanins in the freshly purified subsets (**Figures 4A,B**). The surface expression of tetraspanins on the monocyte subsets shows wide variation, with CD9 and CD37 significantly more highly expressed in the Int subset, in terms of absolute expression levels (MFI) and CD37 is more widely expressed in Int monocytes when expressed as a percentage of the population of cells. After 4 h ConA treatment of adherent monocytes, the expression of all of some of the tetraspanins (CD53, CD82, and in percent positive cells, CD37) declines (**Figures 4C,D**). In terms of the expression per cell, the MFI values for CD53 and CD82 decrease significantly in all subsets. Interestingly, expression of CD9, often thought to be a negative regulator of fusion and so expected to decrease after ConA treatment, showed no significant changes in any of the subsets (**Figures 4C,D**). We also identified a population of $CD9^{High}$ cells in the unstimulated Cl subset. Typically, ~75% of Cl monocytes were $CD9^{Low}$ and ~23% $CD9^{High}$ (**Figure 5A**). Examining the co-expression of other tetraspanins with CD9, the dot-plots show an apparent degree of correlation and statistical analysis confirms that CD151 is significantly elevated in $CD9^{High}$ Cl monocytes (**Figures 5B,C**). However, when sorted, $CD9^{High}$ Cl monocytes did not show a different ability to fuse compared to $CD9^{Low}$ Cl monocytes (data not shown).

Effects of Anti-Tetraspanin Antibodies on MGC Formation

Anti-tetraspanin antibodies have previously been shown to either positively (anti-CD9, anti-CD81) or negatively (anti-CD63, anti-CD151) affect the size of MGC formed by fusing monocytes (40–42), although the contribution of the different subsets and

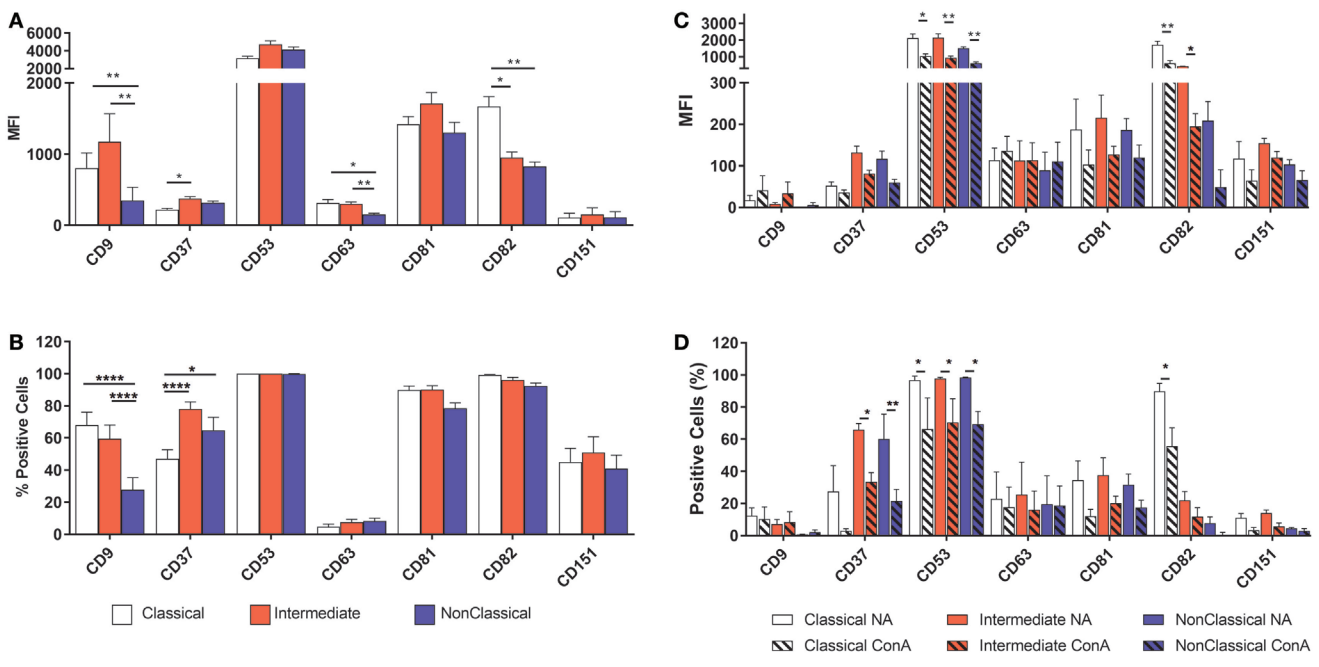

FIGURE 4 | Tetraspanin expression varies between monocyte subsets. Monocytes were either freshly sorted into subsets by FACS, or were purified then allowed to adhere, incubated for 4 h with concanavalin A (ConA) to induce fusion and then harvested, before being tested for the expression of a panel of common myeloid cell tetraspanins using flow cytometry. **(A)** Freshly purified monocyte subsets, expression level per cell (MFI). **(B)** Freshly purified monocyte subsets, percentage of the cell population with expression above isotype control binding levels. **(C)** Adherent, ConA treated monocytes, expression level per cell (MFI). **(D)** Adherent, ConA treated monocytes, percentage of the cell population with expression above isotype control binding levels. The data are the means ± SEM of monocytes from four donors. For **(A,B)**, significance was tested by two-way ANOVA and a Tukey multiple comparison test. For **(C,D)**, significance was tested with multiple *t* tests with Benjami, Krieger, and Yekutieli false discovery rate approach and Holme–Sidak multiple comparisons.

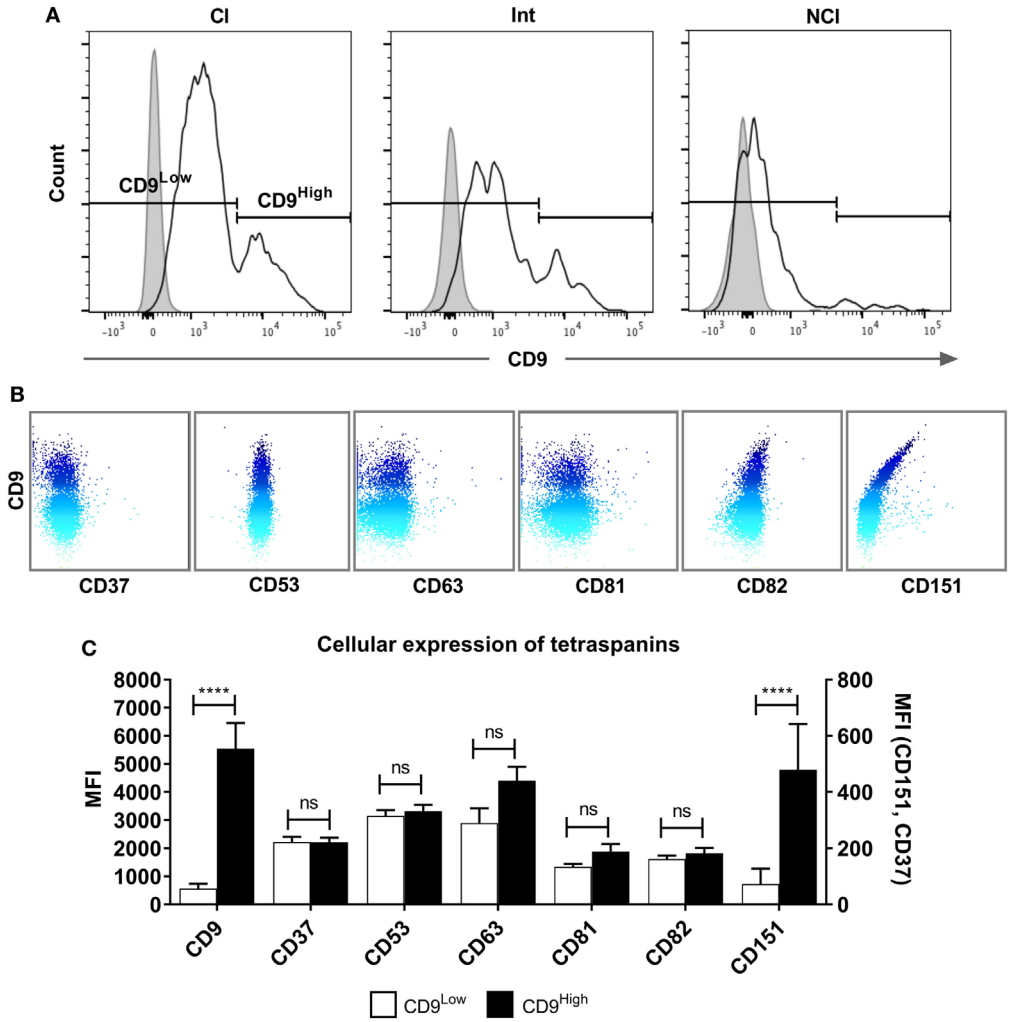

FIGURE 5 | A tetraspanin CD9^{High} subset of classical monocytes. **(A)** Histograms showing the expression of CD9 on freshly isolated classical (Cl), intermediate (Int), and non-classical (NCl) monocyte subsets from a representative donor, with isotype control fluorescence shown as shaded areas. The gating strategy to separate the CD9^{High} and CD9^{Low} populations is indicated by the markers. CD9 was the only tetraspanin in the histograms to show a bimodal peak of expression. **(B)** Dot-plots showing the surface co-expression of tetraspanins on classical subset monocytes with CD9 from a representative donor. Increasing expression of CD9 is indicated by the blue shading. **(C)** Quantification of tetraspanin expression on Cl subset monocytes gated for CD9 high and low expression as shown in panel A. Bars represent the means ± SEM, from 10 different donors. Significance was tested using one-way ANOVA with Sidak's multiple comparisons test for each pair of columns.

the types of MGC formed have not been analyzed before. Here, we have used a range of anti-tetraspanin antibodies to investigate their contribution to subset- and MGC type specific effects during monocyte fusion (Table S1B in Supplementary Material). First, we investigated the effects of antibodies on the fate of seeded cells (**Figure 6**). None of the tetraspanin antibodies caused a significant increase in cell detachment, suggesting that any effects on fusion were not caused by changes in cellular adherence or survival. However, several antibodies (against CD9, CD53, CD63, and CD151) did show a trend toward increased cell detachment.

Strikingly, the proportions of each type of MGC produced by fusion of Int monocytes were changed by the anti-tetraspanin antibodies used here (**Figure 7**) whereas Cl and NCl subsets were not affected. Anti-CD63, in particular caused significant changes in the proportions of MGC formed. It inhibited SGC formation completely and promoted a much higher proportion of LGC to FBGC. Anti-CD9, anti-CD53, and anti-CD151 antibodies exhibited similar effects but the changes did not reach significance. Total (unseparated) MACS-purified monocytes were also significantly affected by only anti-CD63. Anti-CD37 antibody also showed a trend toward the inhibition of SGC formation in Cl and NCl monocytes. Interestingly, the proportions of the various MGC types formed by unfractionated monocytes did not resemble those formed by the isolated subsets, suggesting that interactions between the monocyte subsets can also affect the type of MGC formed (**Figure 7**). The adherence-purified monocytes responded only to anti-CD9 antibodies, with higher proportions of larger FBGC and SGC (Figure S3 in Supplementary Material).

With respect to the sizes of the MGC formed, only Int monocytes were affected by treatment with anti-tetraspanin antibodies.

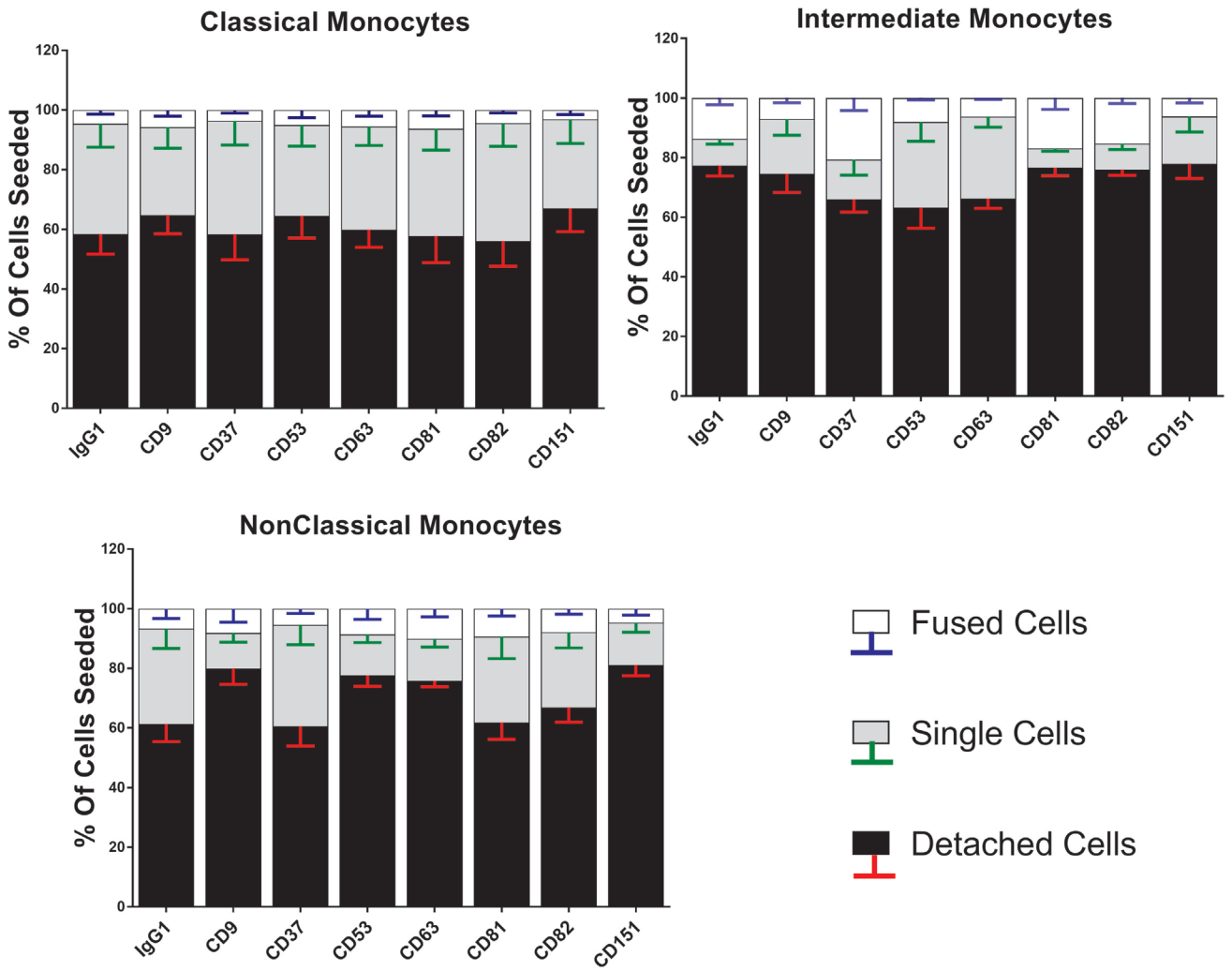

FIGURE 6 | Anti-tetraspanin antibodies do not affect cell fate during concanavalin A (ConA)-induced fusion. The fate of sorted monocyte subsets was determined by counting nuclei at 72 h and expressed as a percentage of the cell numbers originally plated. Bars represent means ± SEM, $n = 3–8$. Significance was tested with a Kruskal–Wallis test with Dunn's multiple comparisons test comparing the means of the same state within the same time point against the other subsets. Black bars/red error bars: detached cells, gray bars/green error bars: single cells and white bars/blue error bars: fused cells with >3 nuclei.

In contrast to previous data on total monocytes purified by adherence (40, 41) and as shown here in Figure S3 in Supplementary Material, anti-CD9 antibodies did not cause an increase in MGC size and have no significant effects on either FI or the number of nuclei per MGC on any of the monocyte subsets. However, anti-CD63 antibodies were found to be consistently inhibitory on all parameters of Int fusion (**Figure 8**). Anti-CD151 antibody also caused a significant decrease in MGC median area whereas anti-CD53 also inhibited MGC formation but this did not reach significance, $p = 0.056$.

Expression of Fusion-Related Molecules on Monocyte Subsets

Many molecules have been associated with monocyte fusion (26–33) and so we examined a panel of 10 membrane proteins for differential expression on the unstimulated subsets. Int monocytes were clearly enriched for a number of these, including DC-STAMP, CD98 CD17a, and CD200 relative to one or both of the other subsets (**Figure 9**). This overall pattern of fusion molecule expression might explain the greater propensity of the Int subset to undergo ConA-stimulated fusion.

Cytokine Expression in Fusing Monocytes

To further investigate the mechanism behind the greater fusogenicity of Int monocytes, we analyzed cytokine production during ConA-mediated fusion to determine if fusogenic cytokine production could contribute to this. ConA-stimulated cytokine production was significantly higher in Int monocytes for IL-1α and IL-1β, confirming the higher pro-inflammatory capacity of this subset (**Figure 10**). However, other cytokines previously identified as being pro-fusogenic, such as CCL2, IL-4 and IL-13, were not elevated in cultures of Int monocytes when compared to

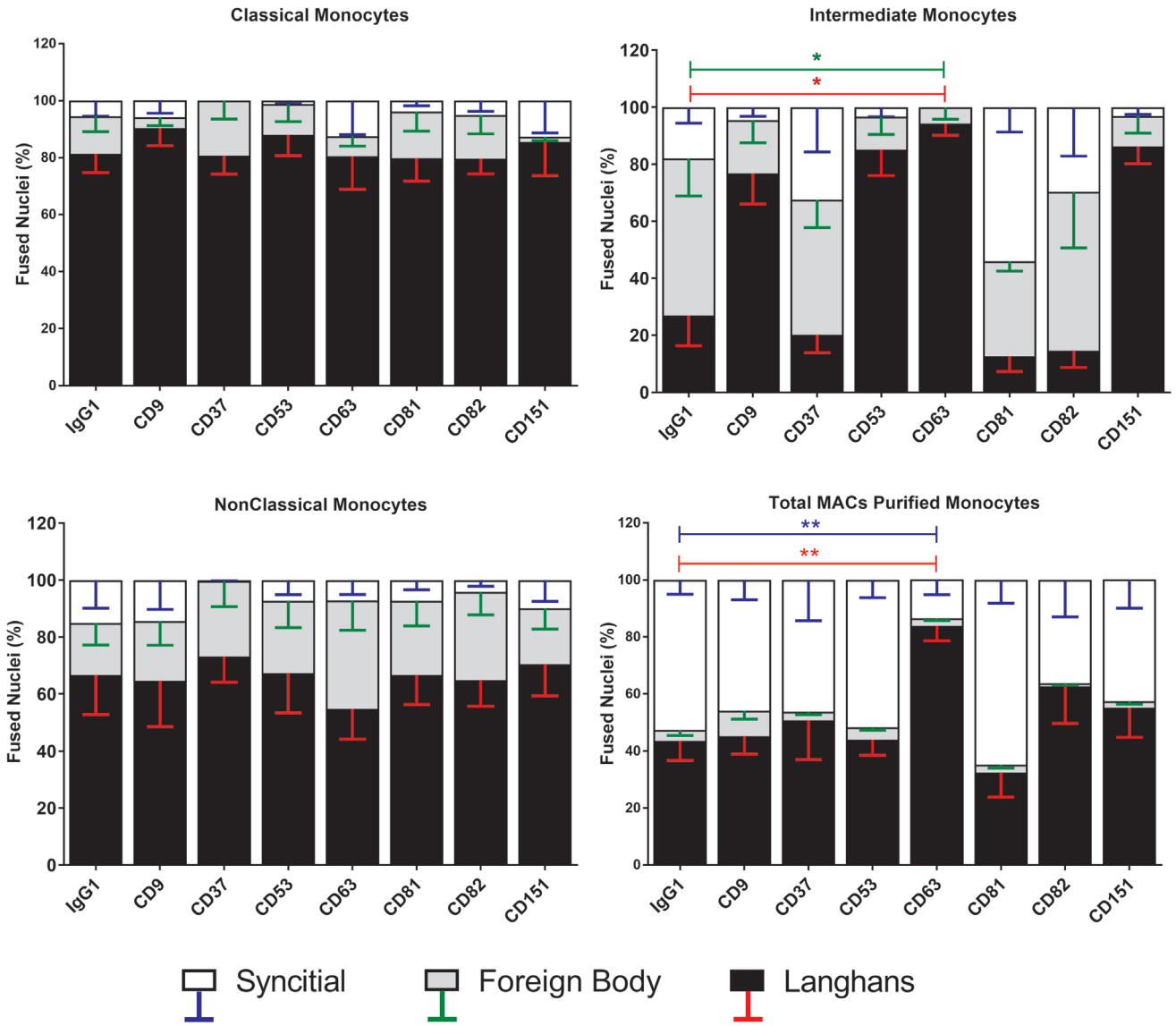

FIGURE 7 | Anti-CD63 antibodies can modulate giant cell morphology only in the intermediate monocyte subset. Purified monocyte subsets were cultured in media containing concanavalin A (ConA) and either an anti-tetraspanin antibody or IgG1 control at 10 μg ml^{-1} for 72 h. Nuclei counted inside each monocyte-derived giant cell (MGC) were ascribed to one of the giant cell types and presented as a percentage of the fused nuclei counted. Bars represent means ± SEM, n = 3–8, tested with a Kruskal–Wallis test with Dunn's multiple comparisons tests comparing the same MGC types between the anti-tetraspanin conditions and the IgG1 control. Black bars/red error bars: Langhans giant cell, gray bars/green error bars: foreign body giant cell and white bars/blue error bars: SGC.

the other two subsets. Thus, the Int monocytes appear to generally secrete higher levels of cytokines but no particular cytokine (of those measured) can be described as playing a pivotal role in the control of MGC formation. IFNγ, IL-10, IL-17A, CCL5, and VEGF were also tested but were either not detected or were not significantly different from unstimulated controls.

DISCUSSION

Here, we demonstrate for the first time that human monocyte subsets show very different propensities to form MGC in response to ConA stimulation. The Int subset fused faster and formed more of the larger FBGC and SGC types while the Cl fused to form mostly the smaller LGC.

Monocyte Purification Methods

Previous studies on the role of tetraspanins in ConA-induced monocyte fusion used cells purified by adherence (40, 41), which have a very different pattern of fusion and anti-tetraspanin antibody sensitivity to the MACS-purified total monocytes used in this report (**Figures 3** and **7**; Figure S3 in Supplementary Material). The MACS technique specifically enriches monocytes whereas the adherence method relies on the ability of cells to rapidly adhere to plastic surfaces and some contaminating T

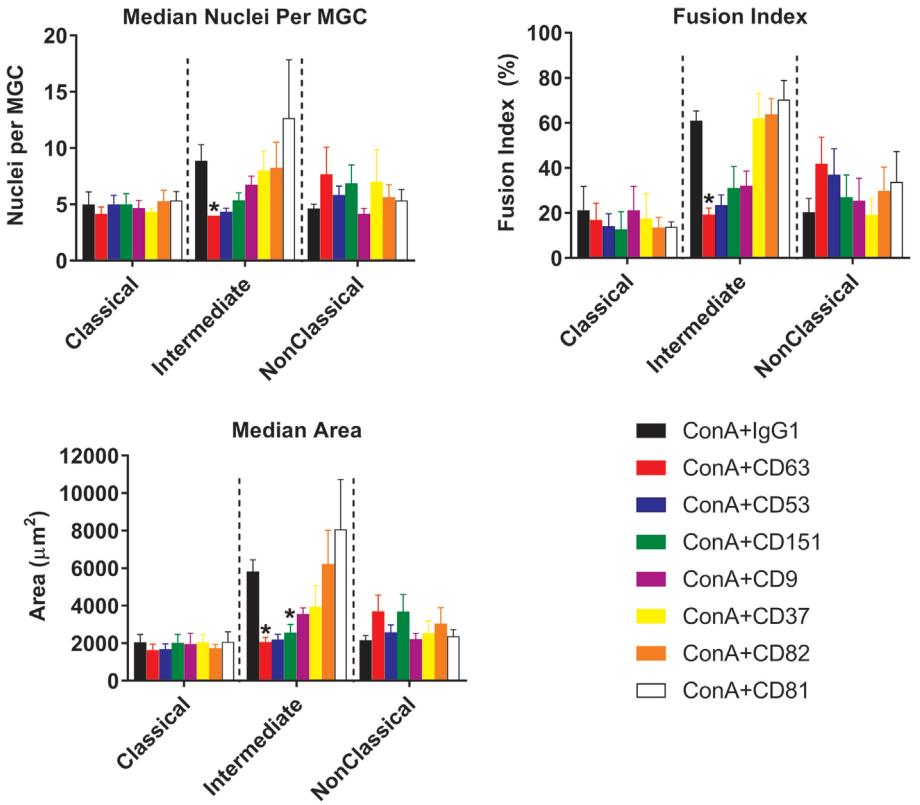

FIGURE 8 | Anti-tetraspanin antibodies inhibit fusion rate and size of giant cells produced by intermediate monocyte subset. Purified monocyte subsets were cultured in media containing (ConA) and either an anti-tetraspanin antibody or IgG1 control at 10 μg ml^{-1} for 72 h. Bars represent means ± SEM, of 3–8 separate experiments. Significance was tested with a Kruskal–Wallis test and a Dunn's multiple comparisons test comparing the anti-tetraspanin antibody means against the IgG1 control within each subset.

and NK cells may be present. In addition, Int and NCl subsets are less adherent than Cl within the first 24 h after isolation, and so in the previous studies more of the initially adherent low-fusing Cl and fewer of the high-fusing Int and NC subsets may have been present. Although Int were observed to be the most fusogenic of the subsets (**Figure 3A**), total MACS-purified monocytes stimulated with ConA showed even higher fusion parameters with many more FBGC and SGC (**Figure 7**). This suggests that fusion potency is increased by interaction between the subsets.

Monocytes Subsets and Fusion

The histological type of MGC formed by each subset has been quantified, with the Int and NCl showing a greater capacity to form the larger FBGC and SGC types. This has implications for the treatment of medical implant rejection as it is clearly the Int and NCl subsets that form the larger MGC associated with foreign body rejection. Interestingly, the Int subset is increased in the blood of sarcoidosis patients (15), a condition characterized by granulomas in which FBGC and LGC are present (48). The increased ability of the Cl subset to form LGC could indicate that they are specialized in responding to mycobacterial infections, as LGC are commonly found in granulomatous infections *in vivo* (19).

Tetraspanin Expression on Subsets

Our data for the expression of CD9, CD53, CD63, and CD81 does not correlate directly with that of Tippett and co-workers (44), who observed higher percentages of cells expressing CD9 and CD63 overall in each subset. Furthermore, they ranked the intensity of surface expression of CD9 on the subsets as Cl > Int > NCl, CD53 as NCl > Int > Cl, and CD81 as NCl > Int > Cl, whereas here all three tetraspanins were found to be highest on the Int subset. However, the CD14/CD16 gating strategy used by Tipett and co-workers may not have been as stringent as here and so the distinction between the subsets may be less clear. In addition, they did not mention any techniques to remove CD16+ NK cells, which overlap with NCl in CD14^{+}/CD16^{+} populations. Overall, we found the Int subset expressed the highest levels of all tetraspanins in freshly purified monocytes except for CD82, which was significantly higher in the Cl subset. The addition of ConA induced significant decreases in the level of CD53 and CD82 and a decrease in the percentage of cells expressing CD37, CD53, and CD82. It appears that ConA induces rapid downregulation of these tetraspanins from the cell surface, although there is no obvious correlation with fusion. Other tetraspanins implicated in fusion, such as Tspan13 and Tspan5, show decreased or increased expression, respectively, in response to RANKL stimulation (39). Tarrant and co-workers (49) showed that

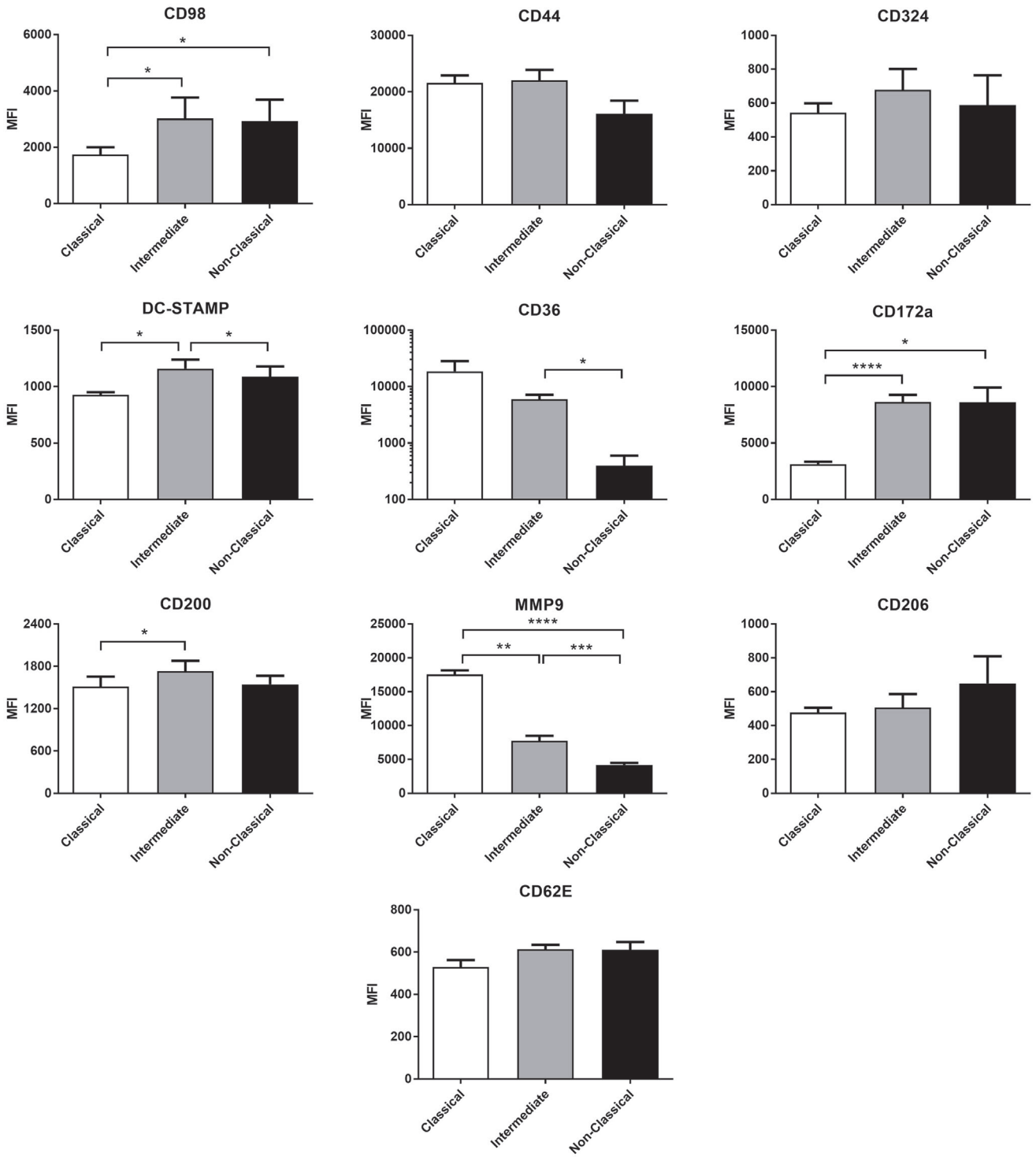

FIGURE 9 | The expression of fusion-related molecules is higher on intermediate monocyte subset. Freshly isolated monocytes were analyzed for fusion protein expression on subsets using flow cytometry. Bars indicate means ± SD from three separate experiments. Significance of difference between subsets was tested with a one-way ANOVA and a Tukey multiple comparisons test.

Tspan32 knockout mice produced T-cells that became hyper-stimulated by ConA. In future studies, it would be interesting to investigate the effects of ConA on further members of the tetraspanin family. The Cl monocyte subset showed an intriguing bimodal expression of CD9, with nearly 25% of this subset (and thus ~20% of total monocytes) having a significantly higher

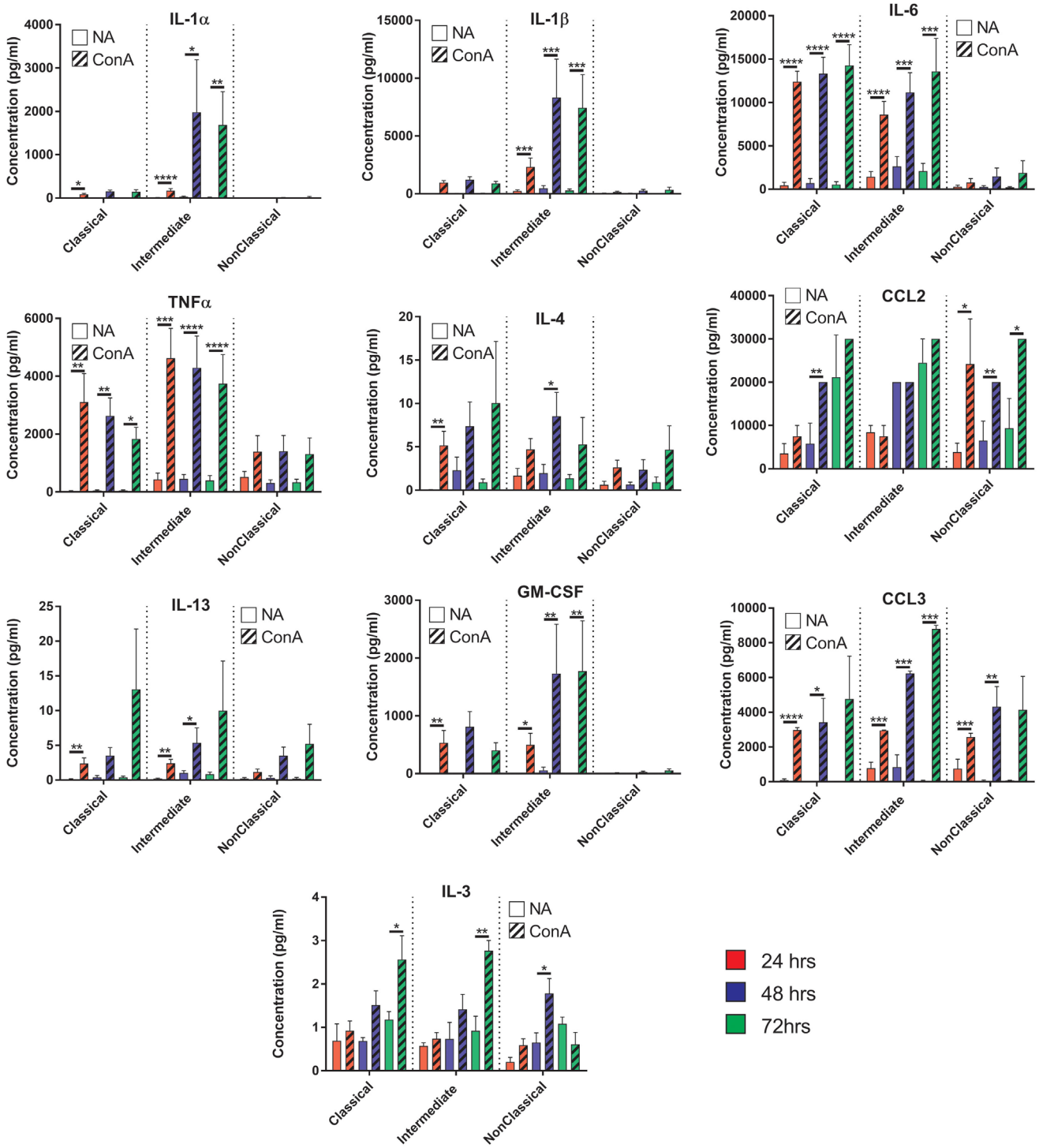

FIGURE 10 | Cytokine production profiles during fusion do not correlate with fusion rate or giant cell morphology. Supernatants from the fusing monocytes were collected and analyzed by ELISA for 15 cytokines relevant to fusion. Clear bars: control (NA), striped bars: concanavalin A (ConA) treated; with each time point [24 (red), 48 (purple), 72 h (green)] presented in adjacent pairs. Bars represent means ± SEM, from eight separate experiments, all tested for significance with a two-way ANOVA with a Sidak's multiple comparison test comparing the means of control (NA) vs ConA treated monocytes at the same time point within each subset.

surface expression of this tetraspanin. Co-expression analysis of the tetraspanins showed a positive correlation between CD9 and CD151 expression on Cl. While $CD9^{High}$ Cl showed no increase in fusion potential, it would be interesting in future work to investigate other functions of these cells, such as their propensity for extravasation.

Anti-Tetraspanin Antibodies and Fusion

The Int subset showed clear significant decreases in fusion parameters and MGC types produced when cultured in the presence of anti-CD63. Anti-CD63 also significantly inhibited MGC formation by total MACS-purified monocytes in response to ConA, in agreement with previous data (41). No inhibition by anti-CD63 was observed for the Cl and NCl subsets, however, suggesting that fusion may be orchestrated differently in the subsets. It is also possible that the lower baseline fusion rates of Cl and the NCl subsets could be masking any notable reductions by these antibodies. It is not clear from the present work if anti-CD63 treatment is directly affecting cell fusion. CD63 knockdown causes arrested motility due to decreased actin polymerization by engaged E-cadherin (50). Therefore, it is possible that the decrease in fusion is a result of arrested mobility and not interference with the fusion mechanism. The lack of a change in the expression level of CD63 during ConA stimulation suggests that antibody might be modulating function by sequestering CD63 away from partner proteins, for example, or by clustering molecules together to activate signaling. Further work is required to distinguish between these possibilities.

Interestingly, antibodies against CD9, CD53, CD63, and CD151 did show a trend toward increased cell detachment in the NCl subset and this pattern was also seen in the effects on Int subset MGC types. This suggests that these tetraspanins might have a role in monocyte behavior but that antibodies are not ideal tools to study this role.

Fusion-Related Membrane Proteins

We hypothesized that increased fusion and sensitivity to anti-CD63 antibody in the Int subset might be due to changes in the expression of membrane proteins known to play a role in fusion, many of which are also known to be partners of tetraspanins. The high-fusing Int monocytes showed generally high levels of the fusion-mediating molecules DC-STAMP, CD172a, CD200, and CD62E and low levels of MMP9 and CD36 relative to the other subsets. DC-STAMP, the only molecule significantly higher in Int than in both of the other subsets, has been shown to be essential for cell–cell fusion in osteoclasts and FBGCs (32, 33). CD200, significantly higher in Int than Cl, is expressed in monocytes after the induction of fusion (26). SIRPα/CD172a/MFR, also higher in Int than Cl, has been shown to be essential for MGC formation (27). MMP9 has been shown to be involved in mouse MGC formation *in vivo* and in response to IL-4 *in vitro* (31) but was found here to be significantly lower in Int monocytes than in Cl. CD36, a phosphatidylserine and lipid binding protein, has been shown to have a role in cytokine-induced MGC but not in osteoclast generation (29). Cl and Int monocytes had higher levels of CD36 than NCl, but as Cl and NCl have similarly low fusogenic potential, this suggests that CD36 expression is not specifically related to higher fusion rates in the Int subset. CD62E (E-selectin) has been implicated in osteoclast formation (51) and MGC formation driven by *B. pseudomallei* infection of U937 cells (30). While it is not significantly more highly expressed in Int monocytes than Cl, CD62E is expressed at a similar level on the lower fusing NCl monocytes. Taken together, however, our data indicate that differences in the expression of multiple fusion-related molecules might be related to the greater fusion capacity of Int monocytes.

Cytokine Production During Fusion

We hypothesized that different levels of cytokine production during ConA stimulation might play a role in the differences in fusion between subsets but the lack of a clear pattern suggests that this is not the primary driver of the variation between monocyte subsets. However, the subsets do show remarkably different cytokine profiles during fusion. The Int and Cl secreted pro-apoptotic cytokines (TNFα) within the first 24 h followed by an increase in pro-inflammatory cytokines (IL-1α, IL-1β, and IL-6) by 48 h. IL-1α and IL-1β have been shown to be released from cells undergoing apoptosis (52) and we also observed at these same time points that a large number of monocytes (57–65%) were dead or detached. This could suggest that many ConA-stimulated monocytes undergo apoptosis and the release of internal IL-1 is a necessary step to generate the fusogenic cytokines. However, the NCl subset achieved greater fusion rates than the Cl monocytes but did not release high levels of IL-1α, IL-1β, or TNFα, suggesting that apoptosis of some cell types is not a pre-requisite for fusion.

In summary, we have shown that the various monocyte subsets differ in their capacity to form MGC in response to ConA, with the Int subset showing greatest propensity for fusion. For this subset, there is evidence that the tetraspanin CD63 may be involved in the process. It is interesting to speculate that the increased fusogenic potential of Int moncytes may relate to their roles in granuloma formation in infectious and inflammatory conditions *in vivo*.

AUTHOR CONTRIBUTIONS

PM, LP, and SW planned experiments and wrote the manuscript; BM and S-MO performed experiments; TC designed and performed experiment and analyzed data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01247/full#supplementary-material.

FIGURE S1 | FACS gating strategy of monocyte subsets from magnet-activated cell sorting-enriched fraction. Pseudocolour/contour plots showing the FACS gating strategy for sorting monocyte subsets. First, monocytes were broadly selected by their forward and side scatter profiles followed by singlet gating. NK cells were removed by selecting for $CD56^-$ cells. Second, $CD14^{++}CD16^-$

(Classical), $CD14^{++}CD16^{+}$ (Intermediate), and $CD14^{+}CD16^{+}$ (non-classical) monocytes were gated as shown and sorted. The purity of the sorted populations was always >90%.

FIGURE S2 | Morphologies of the three monocyte-derived giant cell (MGC) types observed during fusion assays. A representative low magnification image with nuclei in red and F-actin in blue. Langhans giant cells can be identified by their horseshoe or ring-shaped nuclear arrangement and are typically the smallest. Foreign body giant cells (FBGC) are larger and contain more nuclei in a stacked central cluster. Syncytial giant cells (SGC) are the largest, have heterogeneous spreading of the membrane and unevenly distributed nuclei within.

FIGURE S3 | Monocyte-derived giant cell (MGC) types generated from adherence-purified total monocytes. The MGC types generated from total monocytes purified by adhesion cultured for 72 h in concanavalin A (ConA) media and corresponding anti-tetraspanin antibody. Fused nuclei were tallied into either Langhans giant cell, FBGC, or SGC depending on what MGC type they were found in and expressed as a percentage of all fused nuclei. Bars represent the mean ± SEM, with data from four separate experiments. Tested with a Dunn's multiple comparison test; comparing the mean ranks of each MGC type to the IgG1 + ConA control (*$p < 0.05$).

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MINI REVIEW
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Tetraspanin Assemblies in Virus Infection

Luise Florin[1] and Thorsten Lang[2]*

[1] Department of Medical Microbiology and Hygiene, University Medical Center of the Johannes Gutenberg University, Mainz, Germany, [2] Department of Membrane Biochemistry, Life & Medical Sciences Institute, University of Bonn, Bonn, Germany

Tetraspanins (Tspans) are a family of four-span transmembrane proteins, known as plasma membrane "master organizers." They form Tspan-enriched microdomains (TEMs or TERMs) through lateral association with one another and other membrane proteins. If multiple microdomains associate with each other, larger platforms can form. For infection, viruses interact with multiple cell surface components, including receptors, activating proteases, and signaling molecules. It appears that Tspans, such as CD151, CD82, CD81, CD63, CD9, Tspan9, and Tspan7, coordinate these associations by concentrating the interacting partners into Tspan platforms. In addition to mediating viral attachment and entry, these platforms may also be involved in intracellular trafficking of internalized viruses and assist in defining virus assembly and exit sites. In conclusion, Tspans play a role in viral infection at different stages of the virus replication cycle. The present review highlights recently published data on this topic, with a focus on events at the plasma membrane. In light of these findings, we propose a model for how Tspan interactions may organize cofactors for viral infection into distinct molecular platforms.

Keywords: tetraspanin, microdomain, virus, entry, endocytosis, trafficking, budding, receptor

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Emily Whitaker and Menenlaos Symeonides contributed to the review of Markus Thali

****Correspondence:***
Luise Florin
lflorin@uni-mainz.de

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INTRODUCTION

The contents of the cell are protected from the extracellular surroundings by the plasma membrane: a lipid bilayer densely populated with protein (1, 2). These proteins are specifically distributed throughout the membrane, a phenomenon associated with lipid microdomains, rafts, phases, or clusters. Local enrichments can be explained by spontaneous self-organization driven by thermodynamic principles (3). Conversely, the composition and architecture of membrane proteins is also actively remodeled in order to control specific functions.

Viruses are genetic entities that can form particles of sizes up to 200 nm and require multiple steps to overcome the cell barrier during entry and egress. To gain access into the cell, viruses employ different host receptors, proteases, and signaling molecules. After internalization *via* endocytosis, non-enveloped viruses escape the membranous organelle system in order to deliver viral genetic information into the cytoplasm or nucleus (4–6). Entry of enveloped viruses occurs through fusion of the viral and cellular membrane at the plasma membrane or in intracellular compartments (7).

Abbreviations: CD, cluster of differentiation; CDV, canine distemper virus; CLDN1, claudin; CoV, coronavirus; EBV, Epstein–Barr virus; ESCRT, endosomal sorting complexes required for transport; FIV, feline immunodeficiency virus, GFRs, growth factor receptors; HAV, hepatitis A virus; HCMV, human cytomegalovirus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HPV, human papillomavirus; HSV, herpes simplex virus; IAV, influenza A virus; ITGB1, integrin β1; LEL, large extracellular loop; LUJV, Lujo virus; MAPK, mitogen-activated protein kinase; MVB, multivesicular body; Ras, rat sarcoma; SR-BI, scavenger receptor type B class I; TEMs or TERMs, tetraspanin-enriched microdomains; TIRF, total internal reflection fluorescence microscopy; TM, trans-membrane; Tspan, tetraspanin; vDNA, viral DNA.

Each viral entry mechanism involves its own set of unique interactions between the virus and the cellular membrane system. Members of the tetraspanin (Tspan) protein family are localized to membranes and as such, associate directly and indirectly, with multiple steps of viral infection.

TETRASPANINS

Tetraspanins are a family of small transmembrane proteins (8) that function in cell migration, signal-transduction, intracellular trafficking, and are used by several pathogens for infection (9–11). Of the 33 human Tspans, CD151, CD82, CD81, CD63, CD9, Tspan9, and Tspan7 have been associated with viral infections (12–16).

Structure

Structurally, Tspans consist of four transmembrane segments, a small extracellular domain, and a large extracellular loop (LEL). Intracellular domains, including the N- and C-terminal tails, are relatively small and contain palmitoylated cysteines. Homology is highly conserved between isoforms with the exception of a small variable domain located within the LEL (17), which may contribute to differences in functionality between isoforms (18, 19).

To date, structural models are only available for CD81. The first model is based on the LEL crystal structure to which the α-helical transmembrane segments were attached in a theoretical conformation. The transmembrane region was predicted to form a four-stranded coiled-coil with two helices extending vertically into the bulkier LEL (20), resulting into a mushroom-shaped structure. The second model, derived from lipidic cubic phase crystallization of the entire protein, describes an arrangement with two major differences. First, instead of assembling into one bundle the transmembrane segments form two coiled-coils resulting in a cholesterol-binding pocket. Second, two kinks exist between the helical transmembrane segments and the LEL, causing the LEL to fold back toward the membrane (21). When cholesterol is released, the kinks straighten, and the LEL adopts an orientation similar to the proposed first model (21).

Tspan-Enriched Microdomains

Tetraspanins are referred to as master organizers of the plasma membrane, largely due to the fact that they form functional units termed Tspan-enriched microdomains (TEMs or TERMs). Biochemical immunoprecipitation experiments employing detergents of varying strengths revealed two major categories of Tspan interactions: (1) robust interactions between Tspans and non-Tspan binding partners, and (2) weak interactions between Tspan family members (22). Within the second category, certain assemblies of homo–Tspan interactions are preferred over hetero-dimerization/oligomerization (23), and the specificity of oligomerization is mediated by a small segment within the LEL referred to as δ-loop (24, 25). Consistent with these biochemical findings, electron microscopy shows that CD63 and CD9 form distinct clusters (26). Using a more systematic approach, super-resolution light microscopy confirms that single Tspan family members cluster within TEMs (27). Together, these data demonstrate that Tspan isoforms segregate into individual nanoclusters within larger Tspan domains.

In immuno-electron microscopy, Tspan microdomains are highly variable in shape and size with an average surface area of 0.2 μm^2 (26). When assuming a spherical shape, this corresponds to a diameter of ≈500 nm. In contrast, super-resolution light microscopy detects spherically shaped structures with a diameter in the range of 100–150 nm (27, 28). These two methods result in surface area coverage calculations that differ by more than one order of magnitude. This substantial variability is likely due to the description of multiple nanoclusters within TEMs *via* electron microscopy, whereas super-resolution light microscopy identifies individual nanoclusters due to a higher epitope labeling density.

At present, the sequence of events for TEM biogenesis is unknown, though we can build a model on the following observations. First, different Tspans can associate with each other, but Tspans of one type preferentially homo-oligomerize. Second, Tspans form very tight complexes with non-Tspan partners such as integrins (29, 30) and signaling receptors (31). Finally, TEMs are stabilized by weak nonspecific interactions mediated by the aforementioned palmitate residues (32, 33) and glycolipids that promote Tspan assemblies (24, 34). These different interaction modalities likely produce small TEMs (**Figure 1A**). Viral surfaces contain abundant identical binding sites that may crosslink small TEMs to large Tspan trafficking platforms (**Figure 1B**). Evidence for virus-induced large Tspan assemblies has been documented by a number of microscopic studies discussed below.

ROLES OF Tspans IN VIRUS INFECTION

Tetraspanins are essential for specific steps in viral entry and exit (12, 13, 15). As described above, contacts between viruses and proteins on the cell surface can lead to large Tspan cluster networks or trafficking platforms (**Figure 1B**). Similarly, viral envelope proteins accumulate in TEMs during morphogenesis and induce large assemblies of Tspans and viral transmembrane proteins to facilitate efficient budding (**Figure 2**). These platforms enable the coordination of factors required for viral endocytosis, penetration, trafficking, and release. Here, we summarize and discuss the role of CD151, CD82, CD81, CD63, CD9, Tspan9, and Tspan7 in the life cycle of Tspan-facilitated viruses [for a detailed discussion on the role of Tspans in human immunodeficiency virus (HIV) infection see (Suarez et al.; Tspans, another piece in the HIV-1 replication puzzle) in this issue].

Tspan Platforms in Virus Endocytosis and Fusion

Studies investigating different viral systems show common mechanisms for how viruses infiltrate their target cells *via* Tspan platforms. Several microscopic studies confirm that Tspans are enriched at viral entry sites of human papillomaviruses (HPVs) (35, 36), hepatitis C virus (HCV) (37–42), coronavirus (CoV), influenza A virus (IAV) (43–45), and HIV (46, 47), and required for penetration of human cytomegalovirus (HCMV) (48) and alphaviruses (16, 49). These viruses use specific Tspans both as receptors and by compartmentalizing host entry factors.

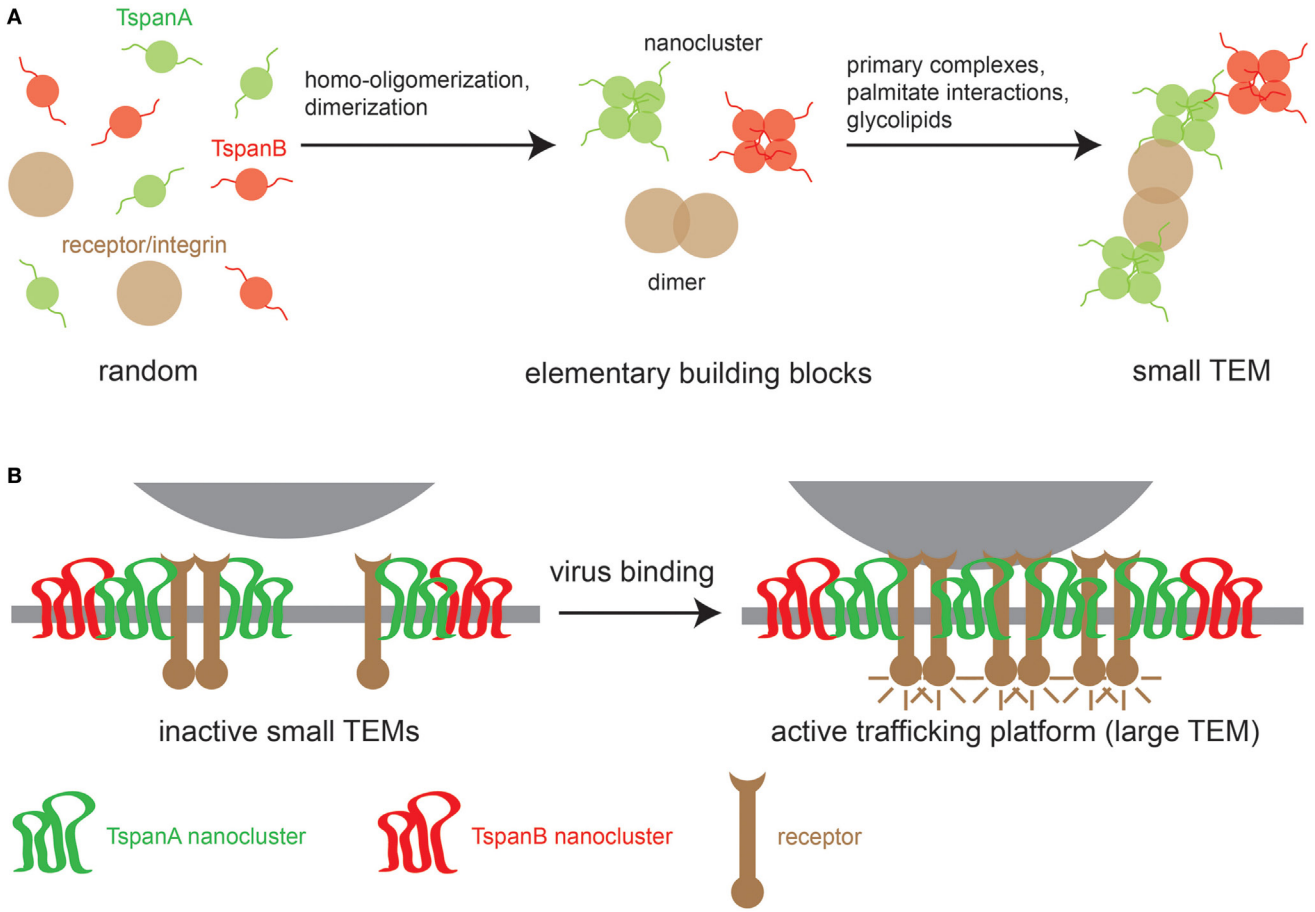

FIGURE 1 | Model of TEM building. **(A)** Left, random distribution of three TEM components: two tetraspanins (TspanA and TspanB) and a primary interaction partner of TspanA [e.g., an integrin or a growth factor receptor (GFR)]. Middle, TEM building blocks; tetraspanins (Tspans) are arranged in homomeric nanoclusters and integrins/GFRs form dimers. Right, building blocks are connected *via* strong specific interactions between TspanA and the receptor/integrin dimer and weak nonspecific interactions between different Tspan nanoclusters. **(B)** When a virus encounters the cellular membrane, virus surface molecules crosslink small TEMs to form larger TEMs, thereby concentrating proteins leading to the activation of intracellular signaling cascades that trigger the uptake of the Tspan trafficking platform.

CD151 in Early Steps of HPV and Cytomegalovirus Entry

Host cell entry of non-enveloped DNA tumor virus HPV16 relies on a fine interplay between the virion and the host cell. HPV16 binding to primary attachment sites triggers cell signaling events and rearrangement of the viral capsid, membrane proteins, and the actin cytoskeleton (15, 50–52). These processes lead to the formation of a virus entry complex and virus uptake *via* a CD151-dependent and clathrin-independent endocytosis pathway (14, 15, 53). In epithelial cells, surface-bound HPV16 particles colocalize with locally enriched CD151 and CD63 on the plasma membrane during invagination and in endosomes (14, 35). Cellular depletion of CD151 and CD63 leads to significant reduction of infections by different oncogenic HPV types, suggesting that these Tspans play a more general role in HPV entry (35, 36, 54, 55). On T-cells, HPV particles are able to trigger the clustering of CD81 which results in the assembly of larger cluster networks required for particle uptake (56). Furthermore, detailed analyses using CD151 mutants revealed that palmitoylation, the δ-loop of the LEL and the C-teminus of CD151 are critical for HPV16 endocytosis (14, 36). These findings indicate that integration of the virus/receptor-complex into larger TEMs and association with cytoplasmic factors (e.g., actin) are required for this process. HPV endocytosis may also involve interactions between multiple receptors and the viral surface, crosslinking smaller TEMs to larger entry platforms (**Figure 1B**). HPV16 receptor-complex components include integrins (36, 57–59), growth factor receptors (GFRs) (60), the annexin A2 heterotetramer (61, 62), and other Tspans (35, 36, 56). CD151 directly interacts with integrins and GFRs (14, 22, 63, 64), and, therefore, positions these HPV receptors within TEMs (11, 65). Through this spatial arrangement of functional proteins, CD151 may control enzymatic activities and signaling pathways required for coordinated assembly of the viral entry platform and endocytosis.

Likewise, entry of the enveloped HCMV depends on CD151 and CD151 partner proteins (e.g., integrins, GFRs) and additional Tspans, such as CD9 (15, 48, 66). CD151 is functionally involved post-binding during viral penetration (48). HCMV membrane

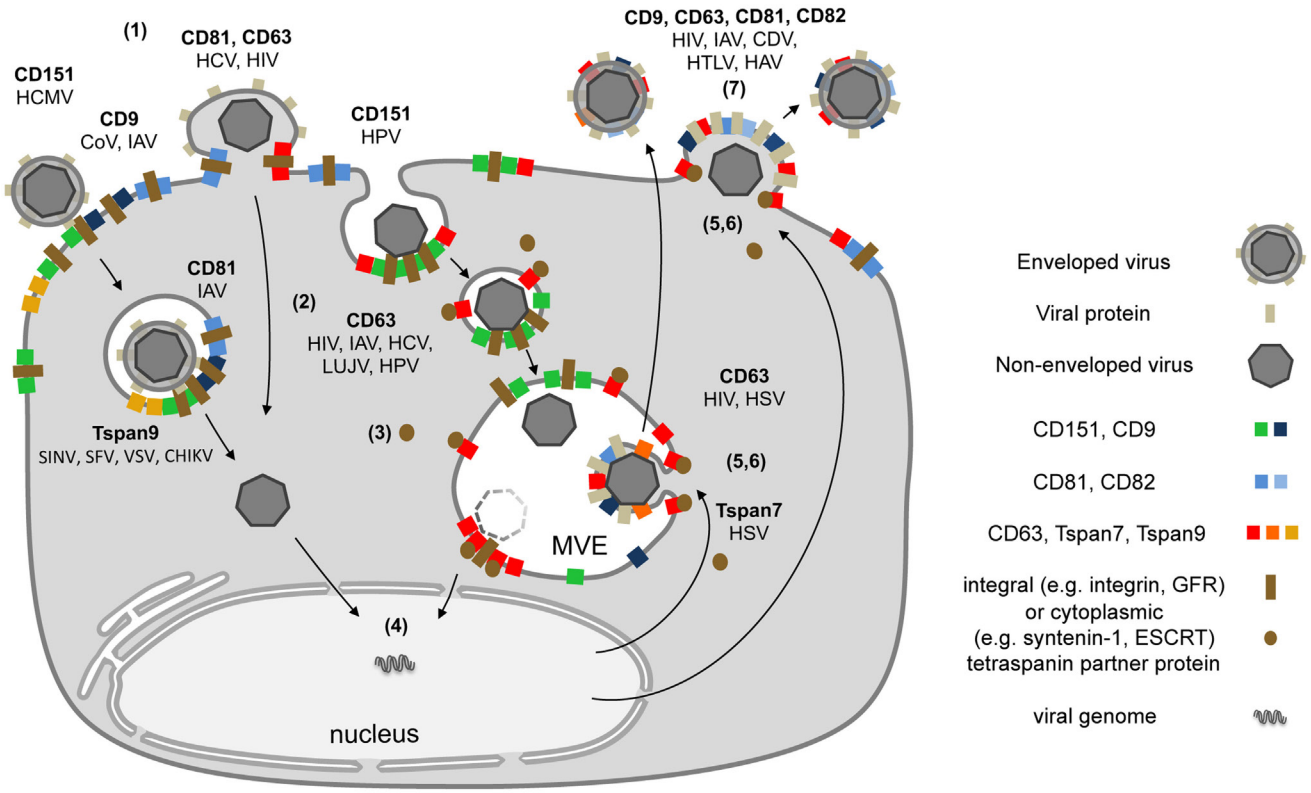

FIGURE 2 | Schematic model depicting the subcellular localization of tetraspanins (Tspans) during virus infection. (1) Interactions between viral particles and entry receptors at Tspan clusters trigger the formation of larger cluster networks. (2) Tspan assemblies promote viral internalization by endocytosis and/or fusion. (3) Endocytosis is followed by intracellular trafficking of virus particles in transport vesicles. During this stage, Tspans mediate and organize interactions with cytoplasmic trafficking molecules. (4) These steps lead to delivery of viral genomes into the cytoplasm or the nucleus and successful infection. (5) Morphogenesis of enveloped viruses on Tspan-enriched microdomains. (6) Integration of viral proteins into Tspan clusters induces spatial enrichment of Tspans and viral proteins. (7) The resulting high concentration of virus envelope components enables efficient budding and release.

fusion occurs after clathrin-independent endocytosis in many cell lines (67–69). Together, these studies suggest that CD151-mediated endocytosis might be a prerequisite for efficient HCMV and HPV infection. Mechanistically, virus-receptor, virus-Tspan, and Tspan-Tspan interactions play a vital role in organizing large Tspan platforms, which facilitate coordinated or simultaneous interactions between virus and host to induce membrane invagination by a mechanism yet to be determined [for detailed review see Ref. (15)].

CD81 and CD9 in HCV, Corona-, and Influenza-Virus Entry

Similar to HPV and HCMV, HCV entry into hepatocytes is a multistep process involving attachment to cell surface heparan sulfate proteoglycans, conformational changes, and transfer of viral particles to secondary receptors (38, 40, 70, 71). These secondary HCV binding molecules also include integrins, the epidermal GFR (EGFR), the ephrin receptor A2 and Tspans as well as claudin-1 (CLDN1), occludin, the scavenger receptor type B class I (SR-BI), and the serum response factor binding protein 1 (SRFBP1) (72–74). Tspan CD81 plays a multifunctional role in HCV entry. CD81 acts as a virus receptor by directly interacting with the HCV glycoprotein E2 (41, 42). CD81 modulates Tspan interactions after HCV binding (75) by triggering EGFR signaling pathways, which enables Tspan/receptor complex-assembly (76, 77) and promotes CD81-EGFR or CD81-CLDN1 complex formation (74, 76). These events are prerequisite for the endocytosis of CD81-HCV clusters and viral glycoprotein-dependent membrane fusion. Proteomic approaches confirmed complex formation of CLDN1, SR-BI, and SRFBP1 with CD81, and demonstrate the functional requirement of integrin β1 (ITGB1) and SRFBP1 for HCV infection and the physical interaction of the Tspan coreceptor-complex with the signaling molecule HRas (73, 77). The rat sarcoma/mitogen-activated protein kinase signaling pathways and EGFR or EphA2 activity trigger lateral diffusion of CD81 for assembly of the viral entry complex consisting of CD81- CLDN1, HRas, and ITGB1 (74, 77). Because GFRs support the uptake of multiple viruses (78), it is probable that activation of their downstream signaling cascades could trigger Tspan receptor clustering accompanied by cytoskeletal network rearrangement required for the entry of other virus families.

Influenza A-viruses and CoVs are enveloped RNA viruses (79). Tspan microdomains, especially CD81 and CD9 enriched microdomains, are preferred IAV and CoV entry sites as they

are required for fusion of viral and host cell membranes in pathogenic infections by both viruses (43–45, 80, 81). IAV and CoV use a variety of coreceptors for this glycoprotein-catalyzed process (82). IAV is routed to CD81-positive endosomes and CD81 is functionally required for the fusion of the viral and the endosomal membrane (45). Here, CD81 may help organize the endosomal membrane and cofactors assisting influenza viral fusion. CoV membrane fusion is mediated by the viral spike glycoprotein (S) and depends on multiple events including proteolytic processing and conformational change of the S protein. Experiments utilizing Tspan knockout cell lines and mice revealed that infection by the human CoV strain 229E requires the Tspan CD9 (43, 44). Pulldown and proximity ligation assays uncovered the four known CoV receptors and a fusion-activating protease within CD9 microdomains. These studies also demonstrated that even in the absence of the virus, CD9 is responsible for the local accumulation of the identified entry receptors. Together, this evidence suggests that CoV uses pre-existing clusters of receptors, proteases, and Tspans for entry. Whether these viruses induce local accumulation of the pre-formed nanoclusters to enable efficient priming of the viral spike proteins during viral egress requires further investigation.

CD63 and Tspan9 as Regulator of Virus Trafficking and Fusion in Infections by HIV, IAV, HPV, and Lujo Virus (LUJV)

Tetraspanins not only organize plasma membrane molecules but also regulate the trafficking of cellular proteins and the transport of endocytosed viruses (11, 15, 83). Many viruses, including HIV, IAV, HPV, and LUJV, localize to CD63-positive endosomes during entry (35, 46, 55, 81, 84). CD63 is most abundant in late endosomes or multivesicular bodies (MVBs) (85) and involved in the membrane organization and trafficking of cellular transmembrane proteins that interact with viruses such as HIV-receptor component CXCR4 (83, 86, 87). Therefore, a functional involvement of CD63 in viral fusion and transport is conceivable. Cellular depletion of CD63 or treatment of cells with CD63 antibodies leads to decreased infectivity of HIV-1, HCV, LUJV, and oncogenic HPV types presenting CD63 as a more general mediator of virus infection (46, 55, 84, 88–90). In contrast to the proviral role of CD63, it is believed that CD9 and CD81 negatively regulate HIV-1 entry by interfering with the formation of the entry receptor complex (47).

Tetraspanin CD63 forms complexes with HPV16 capsid protein L1 (55). As CD63 is involved in the transport of proteins to multiple subcellular locations, it is plausible that different adaptors are required for regulating its trafficking and sorting. For example, syntenin-1 modulates trafficking of CD63 by binding to its C-terminus (91). Consequently, ultrastructural analyses demonstrated the importance of the CD63/syntenin-1 complex for HPV trafficking to MVBs, a process that is required for capsid disassembly (55). The complexity of CD63-mediated viral trafficking is highlighted by the finding that components of the cellular endosomal sorting complex required for transport (ESCRT) are also integrated into the HPV transport platform (55, 92, 93). ESCRT proteins are able to interact with both, syntenin-1 and viral proteins like the HPV16 capsid protein L2 (55, 92–97). Therefore, both viral and cytoplasmic proteins may be targeted to CD63 platforms in a virus-modulated endosomal trafficking pathway.

In addition to its role in trafficking, CD63 facilitates membrane fusion of enveloped viruses. For example, LUJV glycoprotein-mediated membrane fusion is dependent on CD63 and low pH (84), highlighting the importance of the endo/lysosomal system in cell entry. Similarly, Tspan TSPAN9 promotes membrane penetration in early endosomes by the alphaviruses Sindbis virus, Semliki Forest virus, vesicular stomatitis virus, and chikungunya virus (16, 49). Together, CD63 and TSPAN9 may modulate the endosomal compartment to be more permissive for the fusion of viral and cellular membranes.

Tspans in Virus Exit

Morphogenesis of enveloped viruses occurs on membranes of intracellular compartments or at the plasma membrane. Like virus entry, virus exit is a multi-step process driven by viral proteins. This process includes the targeting of viral proteins to specific membrane domains, local concentration of these proteins, virus budding, and release of virus particles. During these processes, Tspans are incorporated into the enveloping membrane of virions, such as HIV, feline immunodeficiency virus, canine distemper virus (CDV), HCMV, influenza, or hepatitis A virus (HAV) (98), implicating TEMs at the site of virus budding.

Earlier reports support this hypothesis using electron and fluorescence microscopy to demonstrate that the HIV core (Gag) and envelope (Env) proteins (26, 99–101), the HTLV-1 Gag protein (102, 103), the Marburgvirus matrix protein VP40 (104, 105), and influenza proteins (45) accumulate in CD9, CD63, CD81, and/or CD82 containing TEMs.

Studies investigating Tspan dynamics in virus budding have shown that the herpes simplex virus (HSV)-1 capsid protein VP26 physically interacts with Tspan7 (earlier known as CTMP-7) (106), and that formation of this complex supports viral egress. Moreover, influenza infection induced redistribution of CD81 on the plasma membrane into concentrated patches of viral budding sites which also contain different viral proteins (45). Likewise, CD63 coordinates sorting of specific viral proteins into extracellular vesicles, such as the major oncoprotein latent membrane protein 1 of the Epstein–Barr virus (107, 108). Comparable to influenza budding, HIV Gag insertion into the plasma membrane induces recruitment of CD81 and CD9 and the coalescence of different membrane microdomains (100, 101, 109–111). Co-immunoprecipitation experiments revealed that Gag proteins interact, directly or indirectly, with CD81 (100). The process of Gag accumulation in Tspan assemblies leads to the formation of larger membrane domains that extend over a few hundred nanometers (109, 112) and contain up to 2,500 tightly packed Gag molecules (113). In addition, multiple studies have shown that modulation of Tspan expression levels and redistribution *via* anti-Tspan antibody treatment in viral or cellular membranes interferes with different steps of the HIV and CDV life cycle including virus-to-cell fusion, reverse transcription, release, and virus-induced cell–cell fusion (114–121). Thus, Tspans can regulate, for example, viral release and cell–cell fusion by controlling

the access of the required cellular machineries to the specific areas.

In addition to Tspans and viral proteins, HIV and HAV exit platforms accumulate cytoplasmic factors, such as components of the ESCRT machinery, which are required for the budding process (113, 122–127). This Tspan-mediated pre-assembly of viral and cellular proteins enables the formation of large budding platforms, a precondition for coordinated viral morphogenesis.

CONCLUSION

At present, the various interaction modalities between viral and cellular proteins preclude the development of a simple model for viral entry. Common molecular mechanisms in viral infection may be revealed by characterizing Tspan platforms in different systems, from their initial involvement at the plasma membrane to their roles in intracellular trafficking and viral egress (**Figure 2**). We hypothesize that active accumulation of molecules into Tspan platforms drives viral infection forward in a defined step-wise sequence.

AUTHOR CONTRIBUTIONS

LF and TL wrote the manuscript and designed the figures.

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Tetraspanins, Another Piece in the HIV-1 Replication Puzzle

*Henar Suárez[1], Vera Rocha-Perugini[2,3], Susana Álvarez[4] and María Yáñez-Mó[1,5]**

[1] *Departamento de Biología Molecular, Universidad Autónoma de Madrid, Madrid, Spain,* [2] *Servicio de Inmunología, Hospital de la Princesa, Instituto de Investigación Sanitaria La Princesa (IIS-IP), Madrid, Spain,* [3] *Vascular Pathophysiology Research Area, Centro Nacional de Investigaciones Cardiovasculares Carlos III, Madrid, Spain,* [4] *Servicio de Inmunobiología Molecular, Hospital General Universitario Gregorio Marañón, Madrid, Spain,* [5] *Centro de Biología Molecular Severo Ochoa, Instituto de Investigación Sanitaria La Princesa (IIS-IP), Madrid, Spain*

Despite the great research effort placed during the last decades in HIV-1 study, still some aspects of its replication cycle remain unknown. All this powerful research has succeeded in developing different drugs for AIDS treatment, but none of them can completely remove the virus from infected patients, who require life-long medication. The classical approach was focused on the study of virus particles as the main target, but increasing evidence highlights the importance of host cell proteins in HIV-1 cycle. In this context, tetraspanins have emerged as critical players in different steps of the viral infection cycle. Through their association with other molecules, including membrane receptors, cytoskeletal proteins, and signaling molecules, tetraspanins organize specialized membrane microdomains called tetraspanin-enriched microdomains (TEMs). Within these microdomains, several tetraspanins have been described to regulate HIV-1 entry, assembly, and transfer between cells. Interestingly, the importance of tetraspanins CD81 and CD63 in the early steps of viral replication has been recently pointed out. Indeed, CD81 can control the turnover of the HIV-1 restriction factor SAMHD1. This deoxynucleoside triphosphate triphosphohydrolase counteracts HIV-1 reverse transcription (RT) in resting cells *via* its dual function as dNTPase, catalyzing deoxynucleotide triphosphates into deoxynucleosides and inorganic triphosphate, and as exonuclease able to degrade single-stranded RNAs. SAMHD1 has also been related with the detection of viral nucleic acids, regulating the innate immune response and would promote viral latency. New evidences demonstrating the ability of CD81 to control SAMHD1 expression, and as a consequence, HIV-1 RT activity, highlight the importance of TEMs for viral replication. Here, we will briefly review how tetraspanins modulate HIV-1 infection, focusing on the latest findings that link TEMs to viral replication.

Keywords: tetraspanins, HIV, entry, assembly, budding, reverse transcription

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****Correspondence:***
María Yáñez-Mó
maria.yannez@uam.es

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THE CELLULAR PLASMA MEMBRANE AS THE FIRST MODULATOR OF HIV-1 INFECTION

HIV-1 virus belongs to Lentivirus within the RNA family *Retroviridae*. It carries two identical molecules of positive ssRNA that are converted to dsRNA intermediate by viral RNA-dependent DNA polymerase (reverse transcriptase). HIV genome encodes for 16 proteins participating in several phases during the HIV life cycle, the structural polyproteins Gag [consisting of matrix, capsid,

Abbreviations: ADAM, a disintegrin and metalloprotease; CA, capsid; dNTPs, deoxynucleotide triphosphates; ERMs, ezrin, moesin, and radixin; ICD, intracellular domain; MA, matrix MA; NC, nucleocapsid; NLS, nuclear localization signal; NPC, nuclear pore complex; PIC, pre-integration complex; PIP2, phosphatidylinositol 4,5-biphosphate; RT, reverse transcription; RTC, reverse transcription complex; TEMs, tetraspanin-enriched microdomains.

nucleocapsid (NC), and p6 proteins], Pol (consisting of protease, reverse transcriptase, and integrase), and envelope (Env; gp120 and gp41); regulatory proteins (Tat and Rev); and accessory proteins (Vif, Vpr, Vpu/Vpx, and Nef) (1).

The HIV-1 envelope glycoprotein (Env) facilitates viral attachment and entry into host cells (2). Three spikes form the Env trimeric complex, each spike consisting of the association of a gp120 subunit on the surface and a transmembrane gp41 molecule (3). Gp120 interacts with CD4, the cellular transmembrane receptor expressed on the membrane of the target cell; and this induces a conformational change in gp120 that exposes new sites for co-receptor binding. There are two types of HIV-1 viruses regarding co-receptor preference, either CCR5 and/or CXCR4. After this second interaction, a hydrophobic region in gp41 is exposed and inserted into the plasma membrane, so that viral and cellular membranes get close enough to create the fusion pore (2, 4). Besides the receptor and co-receptor, other cell surface molecules expressed on dendritic cells (DC) can act as attachment factors, although they do not trigger viral fusion. Most attachment factors are C-type lectins, or calcium-dependent glycan-binding proteins such as DC-SIGN, Siglec-1, mannose, langerin, or DCIR (5–7).

The plasma membrane is not a homogeneous surface but contains specialized microdomains that can be differentiated by their composition and function: lipid rafts, tetraspanin-enriched microdomains (TEMs), caveolae, and clathrin-coated pits (8, 9). Lipid rafts, enriched in cholesterol and saturated lipids with long hydrocarbon chains and hydroxylated ceramide backbones (10–12), provide an environment that favors the inclusion of oligomeric proteins such as flotillins and caveolins, or proteins with lipid modifications such as palmitoylation or GPI anchors (13–15). While lipid rafts properties rely mainly on their lipid content, TEMs are organized around protein–protein interactions nucleated by tetraspanins (9). Tetraspanins, a superfamily of ubiquitous four transmembrane proteins, laterally interact with other membrane molecules stablishing specialized domains or platforms called TEMs. The most common partners of tetraspanins are integrins, proteins of the immunoglobulin superfamily, metalloproteinases, membrane receptors, and other tetraspanins (9). TEMs also include cholesterol and gangliosides. Lipid–protein and protein–protein interactions are facilitated by multiple palmitoylation sites in both tetraspanins and their partners (16).

Given the complex structure of the plasma membrane, it is not surprising that the CD4 receptor and CCR5/CXCR4 co-receptors are not randomly distributed on the cell surface, but show a controlled segregation pattern into defined membrane clusters (17). This enrichment in specialized microdomains has been also reported for attachment factors such as DC-SIGN, in the surface of DCs (18). Inclusion of HIV-1 receptors and co-receptors in lipid rafts, caveolae microdomains, or TEMs tightly regulate viral entry. Since the presence of cholesterol is a common feature of these different microdomains, its depletion or the use of antibodies that specifically recognize clustered cholesterol on the cell surface induces a reorganization of the plasma membrane, disrupts receptor clustering and membrane dynamics, and inhibits virus entry (19, 20). These antibodies do not appear to mask CD4 and CXCR4 interaction sites, but rather seem to affect CXCR4 membrane diffusion, triggering an excess of CD4-CXCR4 clustering, which prevents proper attachment of the viral envelope proteins (19). CD4 and CCR5 co-receptor interact with each other under basal conditions, and addition of gp120 protein bring them closer (17, 21). Tetraspanins CD81 and CD82 also associate with the CD4 receptor on T-cells (22, 23), and gp120 attachment to CD4 induce co-clustering of CD81 (24). CD81 modulates CD4 dimerization and clustering, and it decreases CD4 ability to bind to gp120 (25). All these results support the notion that membrane microdomains are critical regulators of HIV-1 receptors diffusion, allowing proper clustering and efficient protein–protein interactions required for viral entry (26) (**Figure 1B**). Under resting conditions, lipid rafts and TEMs are mainly independent domains at the cell surface, recognized by the presence of specific markers. However, after viral infection, Gag can induce the coalescence of the two types of domains (27).

Other studies suggest that these microdomains may also be important to regulate receptor recycling and trafficking to the plasma membrane. Thus, the tetraspanin CD63 regulates CXCR4 expression on the plasma membrane of T-lymphocytes and activated B cells. Moreover, CD63 glycosylation sites are critical for the interaction with CXCR4 (28) and promote CXCR4 trafficking from the Golgi apparatus to late endosomes and lysosomes for its degradation (29, 30) (**Figure 1B**).

CYTOSKELETON, A SECOND BARRIER FOR THE VIRUS?

Successful HIV-1 entry and infection depends on two sequential events, proper clusterization of the CD4 receptor and co-receptors after viral attachment, and subsequent polymerization and depolimerization of the cortical F-actin meshwork beneath the plasma membrane.

Although the cortical actin web was first described as a barrier for viral entry (21) (**Figure 1A**), inhibition of the actin nucleation regulator ARP2/3 was shown to inhibit viral Env-induced fusion, highlighting the importance of an early actin polymerization phase that stabilizes viral attachment and subsequent fusion with the plasma membrane (31). In addition, the tetraspanin TSPAN7 has been recently identified as an effector of actin nucleation (32), necessary for the formation of actin-rich dendrites in DCs that capture, present, and transfer viruses to T-lymphocytes (33), in the process called trans-enhancement or trans-infection (**Figure 1A**).

Gp120 binding to CXCR4 regulates actin dynamics through the switch off and on of the actin-binding protein cofilin (21), which is inactivated by LIMK-1-dependent phosphorylation, promoting actin polymerization and receptor clustering (34). LIMK-1 is activated by CXCR4 *via* two different pathways: the Rac1/PAK and the RhoA/ROCK pathways. The activation of the latter depends on filamin-A, an actin adaptor protein that binds to CD4, CXCR4, and CCR5 (35). Although the primary activator of both pathways has not been addressed yet, tetraspanins CD82 or CD81 could be good candidates.

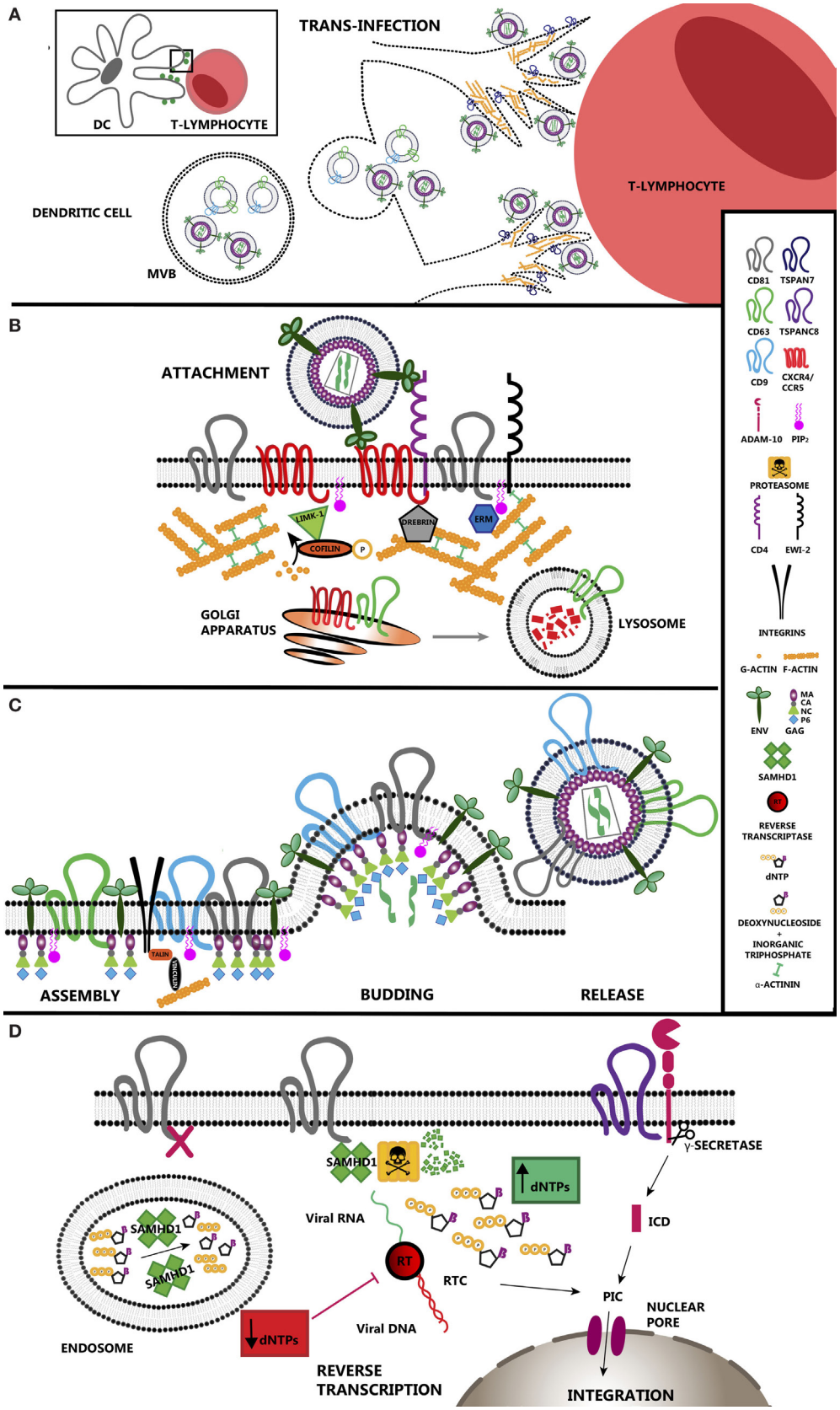

FIGURE 1 | Continued

FIGURE 1 | Tetraspanin roles during HIV-1 infection. **(A)** Tetraspanins regulate transinfection of T-lymphocytes. Dendritic cells stablish contacts with T-lymphocytes during antigen presentation. HIV-1 takes advantage of immune synapses to enhance the infection of T-lymphocytes, the main target cells for the virus. This strategy is called trans-enhancement or transinfection and takes place through two different pathways. One involves the endocytosis of viral particles by DCs, which gives them access to endosomal compartments. As happens with exosomes, viral particles accumulate in multivesicular bodies that finally fuse with the plasma membrane releasing those particles together with exosomes into the intercellular space. The second pathway involves TSPAN7, which inhibits viral endocytosis and promotes formation of actin rich protrusions in DCs. In this scenario, viral particles are sequestered on the surface of these cells, allowing virus exposure and transfer to T-lymphocytes. **(B)** TEM regulation of HIV-1 entry. CD4 and co-receptors CCR5/CXCR4 segregate within tetraspanin-enriched microdomains (TEMs), which control their proper distribution and dynamics enhancing HIV-1 attachment efficiency and subsequent entry. CD63 regulates the expression of CXCR4 on the cell surface by stimulating its degradation through the lysosomal pathway. Env binding to its receptor and co-receptor brings them closer and triggers several intracellular pathways where actin polymerization is the main response. Active LIMK1 phosphorylates and inactivates cofilin, stimulating actin polymerization. Proteins such as moesin or α-actinin have a structural function as they link receptors and tetraspanins to the subcortical actin network. Other proteins such as drebrin control the stability of the actin web. TSPAN7 is also a positive regulator of actin polymerization, although the effectors downstream have not been addressed yet. **(C)** HIV-1 assembly occurs at TEMs. Viral protein Gag interacts with the inner leaflet of the plasma membrane *via* its myristoylation, which increases the affinity for cholesterol-enriched areas. Gag also interacts with the positively charged PIP2 and the inner loop of different tetraspanins such as CD81 and CD82. Gag induces CD9 clusterization. However, there is no direct evidence indicating an essential requirement for tetraspanins during HIV-1 budding. Recruitment of all these components into restricted areas may involve the presence of the subcortical actin web for their stabilization, where talin and vinculin would act as a link. **(D)** HIV-1 reverse transcription (RT) is regulated by tetraspanins. SAMHD1 is a negative regulator of viral RT as it decreases the concentration of deoxynucleotide triphosphates available in the cell. CD81 regulates SAMHD1 activity by stimulating its degradation *via* proteasome. CD81 depletion induces the relocalization of SAMHD1 inside early endosomes. ADAM-10 activity is regulated by tetraspanin TSPANC8 subfamily. The resulting intracellular domain when cleaved by a γ-secretase has been identified recently as a component of the PIC. When RT is completed, viral DNA is transported into the nucleus where it integrates in the cell genome.

CD82 can interact with CD4 and regulates actin dynamics in both T-lymphocytes and cancer cells through the modulation of RhoA and Rac1 signaling (36, 37), while CD81 regulates Rac activity turnover (38).

Besides Rho GTPase activity, the membrane lipid phosphatidylinositol 4,5-biphosphate (PIP_2) facilitates viral infection by controlling the activity of several actin-binding proteins (31). Among them, ERMs (ezrin, moesin, and radixin), whose activation requires the interaction with PIP_2 at the plasma membrane (39). Gp120 binding to CD4 receptor activates moesin, which triggers the reorganization of subcortical F-actin and stimulates CD4-CXCR4 clustering in T-lymphocytes (40) (**Figure 1B**). However, other studies performed in Hela cells described moesin as a negative regulator of viral infection (41), through the control of microtubule stability that could affect viral transport to the nucleus (42, 43). Moesin also interacts directly with CD81, or indirectly with either CD9 or CD81 through EWI-2, a TEM component member of the immunoglobulin family (44). EWI-2 is also linked to the actin cytoskeleton *via* α-actinin, an actin-binding protein negatively regulated by PIP2 that induces a restrictive conformation for HIV entry on the cortical actin network (45, 46). Another CXCR4 interactor, drebrin, stabilizes actin in a process dependent on the viral envelope, so that drebrin silencing increases HIV-1 entry again supporting the idea of a need for a later actin depolymerization step for viral access into the cell (47) (**Figure 1B**).

This later step of depolymerization is also promoted by CXCR4 through the activation of cofilin *via* Gαi signaling (48). Alteration in the levels of gelsolin, another actin regulatory molecule, also impairs HIV-1 infection (49). In this stage, destabilization of the actin network at later phases of viral entry would provide access of the virus to microtubules, which will transport the RTC [reverse transcription (RT) complex] toward the nucleus (31, 50). Studies using nocodazole (an inhibitor of microtubule polymerization), or kinesin and dynein inhibitors delay HIV-1 uncoating and promote the accumulation of viral particles far away from the nucleus. Kinesin and dynein may contribute to the uncoating process by applying opposite forces that could destabilize and disrupt the structure of the capsid while it travels through the cytoplasm (51).

Tetraspanins and the actin cytoskeleton are also crucial for DC-mediated trans-infection by which the virus is retained at or near the cell surface of a DC and transmitted to a T-lymphocyte *via* the close contact of both cells. TSPAN7 expressed in DCs is important for the formation of actin rich spikes that are able to retain viral particles on their surface (32) (**Figure 1A**). In addition, DCs can trap viral particles in large intracellular vesicles staining for tetraspanins CD81 and CD63 (52), although these structures may not be completely closed and remain connected with the extracellular space allowing a quick release of viral particles (53) during DC–T cell contacts. The exosome secretion pathway has been proposed as an alternative transmission route between cells without fusion events. Indeed, HIV-1 can directly use the endosomal pathway to enter DCs and be thereafter released together with exosomes after the fusion of multivesicular bodies with the plasma membrane (54, 55) (**Figure 1A**). In addition, recent studies suggest that in top of that, exosomes from DC are loaded with molecules that could enhance viral replication and release, such as CCR5 or CXCR4, which facilitate T-lymphocyte infection, miRNAs or viral proteins, such as Nef (56).

HIV-1 PROMOTES PLASMA MEMBRANE REMODELING

Upon successful infection, HIV-1 virus can modify the cell surface of infected cells to facilitate the release of new viral particles. Vpu and Nef are the viral proteins involved in this modulation. Both of them can control CD4 expression at the cell surface by different mechanisms. Nef is synthesized during the early steps of the infection, interacting with the plasma membrane through myristoylation modifications and

with the C-terminal domain of the CD4 receptor (57, 58). Nef forms a complex with AP-2, promoting CD4 endocytosis and subsequent transport to the lysosomal pathway for its degradation (59). Nef can also control MHC-I levels to protect the infected cell from the immune system, by stimulating its endocytosis from the cell surface and by inducing its accumulation at the trans-Golgi network (60). In contrast, Vpu is a transmembrane viral protein that is transcribed during the late steps of the viral cycle, blocking CD4 transport from the endoplasmic reticulum to the membrane and stimulating CD4 degradation by the endoplasmic-reticulum-associated protein degradation pathway (61).

Tetraspanins CD9, CD81, CD82, CD63, and CD231 are included in HIV-1 particles negatively regulating viral infectivity (62). How the virus regulates their inclusion into virions remains unknown, but it does not seem to be an uncontrolled process since L6, a transmembrane protein with similar topology is excluded (62). Remarkably, HIV-1 viral proteins also control tetraspanin expression on the plasma membrane. Vpu and Nef downregulate a wide variety of tetraspanins inducing their enrichment at the perinuclear region of the cell (63). T-lymphocytes from HIV-1 patients showed a reduced expression of CD82 and CD81 (64), while the expression of the latter was increased in B-lymphocytes (65). CD81 and CD82 downregulation was attributed to Vpu, and to a lesser extent to Nef. Vpu was shown to directly bind CD81, stimulating its degradation by either the proteasome or the lysosomal pathways. Although CD82 does not directly interact with Vpu, the viral protein also drives its degradation, probably through the association with CD81 (63). Therefore, downregulation of tetraspanin expression seems to be essential for virus spread. In addition, CD81 and CD9 play a negative role in viral-induced syncytia formation (24).

Viral assembly and budding is driven by Gag polyprotein, which is formed by matrix (MA), capsid (CA), NC, p6 domains, and two spacer peptides, named SP1 and SP2 (66) (**Figure 1C**). The initial evidence that suggested that assembly takes place in specialized microdomains came from the presence of high levels of cholesterol and sphingolipids in the HIV-1 envelope (67–69). After synthesis in the cytoplasm, Gag interacts with two molecules of viral RNA through its NC domain (70). Gag association with the cell surface then is driven by a cluster of positive amino acids in the MA domain, with affinity for negatively charged PIP_2 in the inner leaflet of the plasma membrane. Myristoylation of the N-terminal region of the MA domain contributes to its association to membrane areas enriched in cholesterol and sphingolipids, like lipid rafts or TEMs (26, 70). Env and Gag colocalize with tetraspanins CD63, CD81, and CD9 at the plasma membrane of T-cells and direct coimmunoprecipitation of CD81 with Gag has been reported (68, 71) (**Figure 1C**). Moreover, in both T-cells and macrophages, there is a relocalization of CD63 from intracellular compartments to viral assembly sites; however, its depletion does not affect viral release (72) (**Figure 1C**). In macrophages, viral assembly takes place in vacuoles that originate from invaginations of the plasma membrane (73), and present focal-adhesion-like domains more abundant in cells infected with the virus (74). These domains are enriched in integrin β2, focal adhesion components, tetraspanins CD9, CD53, CD81 and CD82, and in PIP_2 and AP-2, common components of clathrin-coated pits (75). After the budding of new virions, Gag is processed by the viral protease into the mature proteins enabling the formation of the capsid that contains the viral RNA genome and the enzymes needed for its replication (10, 70).

Further studies will be required to clarify the specific role of tetraspanins during the assembly and budding of new virions. All existing evidences support that tetraspanins are located at the exit sites and are incorporated in newly formed virions; however, future research should decipher whether they are functionally important for the organization and recruitment of all the components needed for budding (10).

INTRACELLULAR EVENTS OF HIV-1 INFECTION ARE SURPRISINGLY ALSO DEPENDANT ON MEMBRANE MICRODOMAINS

The binding to the viral receptor and co-receptor triggers an intracellular response that prepares the host cell for HIV-1 RT. After the fusion of the viral and the cell membranes, the capsid of the virus is released into the cytoplasm. RT occurs in a complex called RTC (RT complex), which is formed by viral proteins (reverse transcriptase, integrase, matrix protein and Vpr), the RNA genome, and host proteins needed to complete the cDNA synthesis (3, 76, 77). When the RNA genome is completely transformed into cDNA, this complex, still composed by a combination of viral and cellular proteins, is named PIC (pre-integration complex) (78). One surprising component of the HIV-1 PIC is the intracellular domain of the transmembrane A Disintegrin And Metalloprotease-10 (ADAM10) (79); so, when ADAM10 expression is inhibited, a decrease in HIV-1 RT has been reported (**Figure 1D**). Tetraspanins could be also involved in this event, since ADAM10 localization, trafficking, and substrate specificity is regulated by a subfamily of tetraspanins characterized for having eight Cys residues in their large extracellular loops (TspanC8) (80) (**Figure 1D**).

The role of the RTC in the RT process and how the uncoating process takes place is still a matter of debate. The first theory, no longer accepted, proposed that the capsid was lost immediately after membrane fusion and viral entry (81). Many studies have proven that the capsid is required for the RT process since it may provide protection against the host cell defense, as well as anchorage for the needed host factors (76). Another theory proposes that uncoating takes place while the RTC gets to the nucleus. The third one, however, claims that the whole capsid might travel along the cytoplasm until it reaches the nuclear pore complex where it is disassembled (77). There are different pieces of evidence that show that the capsid remains stable for some time after viral entry, and mutations that increase or decrease the stability of the capsid all have a negative effect on HIV-1 infection (81). Most results suggest that uncoating may occur during HIV-1 RT (82), and it should not involve a complete breakdown of the capsid but

a progressive disassembly along the trip through the cytoplasm (77). Destabilization of its structure would allow the access of the nucleotides, and host proteins needed for the viral RT (83). Before nuclear entry, however, the core has to be disrupted as it is too large to cross the nuclear pore (82).

Interestingly, some data suggest that membrane-bound tetraspanins also regulate after-entry events in HIV-1 infection. CD63 has been shown to regulate HIV-1 RT, nuclear transport, and integration; however, the mechanisms involved remain unsolved (84–86). RT is also modulated by tetraspanin CD81, *via* the regulation of SAMHD1 expression (87). SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase that controls the availability of deoxynucleotide triphosphates (dNTPs) through their conversion into deoxynucleoside and inorganic triphosphate (88). Recent studies have identified an additional role of SAMHD1 in DNA repair and genome stability (89, 90). Others suggest that it may also have RNase activity over ssRNA or DNA/RNA duplexes (91), although these later results remain controversial (92). Because of its relevance, the cell has developed several mechanisms for SAMHD1 regulation. Related to its quaternary structure (93), SAMHD1 monomers associate in dimers, and these dimers organize in tetramers. The organization of SAMHD1 monomers into the active tetrameric form depends on the presence of dNTPs for its stabilization (94). Regarding posttranslational modifications, SAMHD1 can be phosphorylated by cyclin A2/CDK1 at T592 after T cell activation (95), or by tyrosine kinases downstream IL-2 and IL-7 stimulation of CD4+ T cells (96). These modifications decrease its dNTPase activity, increasing viral RT (96). Acetylation at K405 has the opposite effect, stimulating SAMHD1 dNTPase activity, and promoting the transition from G1 into S phase in cancer cells (97). SAMHD1 oxidation status is another important regulatory mechanism. Three different cysteines of the enzyme can be oxidized, changing the nucleotide binding site conformation, preventing its tetramerization and subsequent activation (98). SAMHD1 has a nuclear and cytoplasmic distribution (87, 99). Nuclear localization is mainly determined by its NLS sequence (100), and the oxidation status seems to be critical for its accumulation in the cytoplasm (98). Once in the cytoplasm, tetraspanin CD81 seems to regulate the enzyme subcellular localization into endosomes (87) (**Figure 1D**).

SAMHD1 expression levels are also tightly regulated. Reduced levels of SAMHD1 increase the amount of dNTPs available for viral RT. Thus, SAMHD1 is a major regulator of HIV-1 infection as it restricts the availability of dNTPs necessary for HIV-1 RT in resting monocytes, macrophages, CD4+ T cells, and DC. HIV-2 virus, but not HIV-1, expresses an accessory protein called Vpx that tags SAMHD1 for its degradation by the proteasome (93, 101). SAMHD1 interaction with the C-terminal domain of the tetraspanin CD81 also stimulates its proteasomal degradation. Depletion of CD81 abolishes SAMHD1 degradation, which is translocated into early endosomal compartments where it exerts its dNTPase activity (87) (**Figure 1D**). Although it is reported that HIV-1 downregulates CD81 expression at the cell surface (64), this event might only occur late in the viral cycle, after RT has been completed.

CONCLUDING REMARKS

Tetraspanins are important regulators of HIV-1 cycle. They would have a dual role in HIV-1 infection. Tetraspanins would inhibit infectivity by actively participating in viral entry. They would modulate cell surface dynamics and the proper distribution of receptors and co-receptors, both in the host cell and in the viral membrane inhibiting viral entry and induced membrane fusion (24, 62). In contrast, CD81 can enhance viral RT by promoting SAMHD1 degradation through the proteasome, thus increasing the availability of dNTPs in the host cell (87). These opposite functions concur with a first round of active viral entry and replication to produce new viral particles in the cell, followed by a second round of viral latency to avoid recognition by the immune system so it can persist within the organism (102). HIV-1 possesses the tools to control tetraspanin expression by the host cell, avoiding undesirable effects on viral infection. As important membrane organizers, tetraspanins regulate multiple cellular proteins that control the different steps of HIV-1 infection cycle, and thus represent an interesting target for the development of new drugs against viral infection. Finally, it has been reported that peptides against the intracellular region of CD81 can block its activity over SAMHD1 and reduce viral RT (87). This result leaves open the possibility of using specific peptides against tetraspanins as an interesting strategy to restrict HIV-1 infection.

AUTHOR CONTRIBUTIONS

HS wrote the manuscript and designed the figures. VR-P conceived and edited the manuscript. SA commented and edited the manuscript. MY-M conceived and edited the manuscript. All authors read and approved the final manuscript.

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Antitumor Immunity Is Controlled by Tetraspanin Proteins

*Fleur Schaper and Annemiek B. van Spriel**

Department of Tumor Immunology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, Netherlands

Antitumor immunity is shaped by the different types of immune cells that are present in the tumor microenvironment (TME). In particular, environmental signals (for instance, soluble factors or cell–cell contact) transmitted through the plasma membrane determine whether immune cells are activated or inhibited. Tetraspanin proteins are emerging as central building blocks of the plasma membrane by their capacity to cluster immune receptors, enzymes, and signaling molecules into the tetraspanin web. Whereas some tetraspanins (CD81, CD151, CD9) are widely and broadly expressed, others (CD53, CD37, Tssc6) have an expression pattern restricted to hematopoietic cells. Studies using genetic mouse models have identified important immunological functions of these tetraspanins on different leukocyte subsets, and as such, may be involved in the immune response against tumors. While multiple studies have been performed with regards to deciphering the function of tetraspanins on cancer cells, the effect of tetraspanins on immune cells in the antitumor response remains understudied. In this review, we will focus on tetraspanins expressed by immune cells and discuss their potential role in anti-tumor immunity. New insights in tetraspanin function in the TME and possible prognostic and therapeutic roles of tetraspanins will be discussed.

Keywords: tetraspanins, antitumor immunity, tumor microenvironment, adaptive immunity, innate immunity

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***Correspondence:**
Annemiek B. van Spriel
annemiek.vanspriel@radboudumc.nl

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INTRODUCTION

It is now well known that the immune system plays an important role in preventing tumor formation, growth, and metastasis. This is exemplified by the increased susceptibility of immunocompromised patients to develop cancer, and by the recent success of novel cancer immunotherapies including checkpoint inhibitors, dendritic cell (DC) vaccination, and chimeric antigen receptor T cells, which demonstrate that the immune system can be harnessed against cancer.

Antitumor immunity is dependent on tumor cell uptake by antigen-presenting cells (APCs) (DCs, macrophages) that subsequently migrate to nearby lymph nodes to activate T and B cells. After clonal expansion, antigen-specific CD8 T cells can migrate toward the tumor, aiming to destroy tumor cells, a process called tumor immunosurveillance. However, this process is far from perfect as non-immunogenic variants of the tumor escape, resulting in tumor recurrence (1, 2). Escape from the immune system can also occur in other ways, for instance, by inducing an immunosuppressive state in the tumor microenvironment (TME) (3, 4). Here, tumors create a niche where they recruit different cell types to create a specific microenvironment, which favors tumor growth and metastasis (5). These cells include immune cells, which often have acquired immunosuppressive properties, such as regulatory T cells (Tregs), tumor-associated macrophages, and myeloid-derived suppressor cells (MDSCs) (6, 7) (**Figure 1**). Tumor-associated macrophages are very plastic cells that can adapt their phenotype in response to different tumor cell products or hypoxia (8). Alterations in cellular

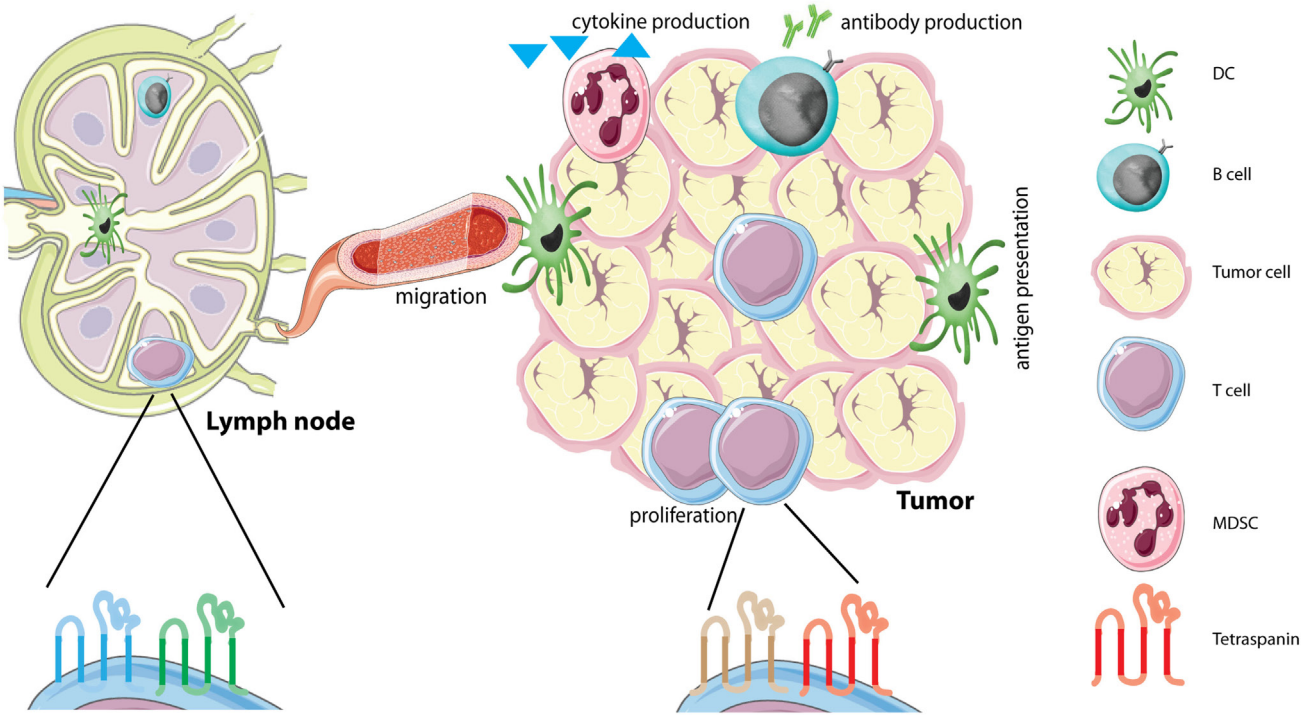

FIGURE 1 | Tetraspanins on immune cells in the tumor microenvironment (TME). The TME is comprised of several different cells, including tumor cells and different immune infiltrating cells. Immune cells in the TME produce different soluble factors, including cytokines or antibodies. Each immune cell subset contains its own distinct tetraspanin web composed of tetraspanins that can have either stimulating or inhibitory functions (see **Table 1**). We hypothesize that immune cells alter the composition of the tetraspanin web when they migrate from the lymph node to the tumor caused by the immunesuppressive environment of tumors. Abbreviations: DC, dendritic cell; MDSC, myeloid-derived suppressor cell.

phenotypes are often accompanied by membrane protein reorganization, as different membrane receptors will be upregulated or internalized.

Tetraspanins, or transmembrane-four superfamily proteins, are evolutionary conserved membrane organizers that regulate protein trafficking, adhesion, migration, fusion, and signaling (9–12). While many tetraspanins (CD9, CD81, CD151) are widely expressed, others are restricted to hematopoietic cells (CD37, CD53, Tssc6). Tetraspanins are not constitutively expressed on all cell types and can differ between effector fates (13–15). Tetraspanins can interact with each other and with partner proteins on the same cell whereby they form "tetraspanin-enriched microdomains" (TEMs) or "tetraspanin web" (9, 11, 16). The overall view is that tetraspanins are modulators of signal transduction, providing organization to membrane domains through lateral interaction with their partners (11, 17–23), including integrins [reviewed in Ref. (24)] and immunoreceptors [CD19 (25), MHC class molecules (17, 18)]. The immunological importance of these interactions has been demonstrated in multiple tetraspanin-deficient (−/−) mice (CD37, CD53, CD81, CD82, Tssc6, CD151) that have defects in humoral and/or cellular immune responses (14, 26–29). These defects include cell migration, T cell proliferation (27, 30–32), antibody production (25, 33), and antigen presentation (14, 34–36). As such, it is likely that they also control antitumor immunity. While studies have clearly demonstrated effects of tetraspanins in primary tumor progression [reviewed in Ref. (37)] or metastasis [reviewed in Ref. (38)], detailed analyses of antitumor immunity in tetraspanin −/− mice is still scarce.

Studies on human tumor cells reported associations between tetraspanin expression and tumor progression showing both reduced (CD82, CD9) and increased expression (CD151, Tspan8) in various cancer types (12, 15, 37–47). In patients with invasive breast cancer, it was shown that CD9 on immune cells was associated with a longer disease free survival, while CD9 expression on the tumor cells showed the opposite effect (48). The relevance of CD37 in tumor suppression has been recently shown in $CD37^{-/-}$ mice that spontaneously develop B-cell lymphoma, and in patients with CD37-negative B-cell lymphoma that have poor survival (45, 49). These results are in line with studies that report tetraspanin expression to serve as a prognostic marker for cancer patients (50). In addition, these findings indicate that tetraspanins not only influence immune cell signaling but also directly protect from tumor formation. This review focuses on tetraspanins expressed on immune cells, and their possible role in antitumor responses and the TME.

TETRASPANINS AND ANTIGEN PRESENTATION

The first steps in an antitumor immune response are the uptake, processing, and presentation of tumor antigens by APCs. DCs are the most professional APCs, and infiltration of mature

activated DCs into tumors has been associated with increased patient survival. There is also ample evidence that tumors can inhibit APCs, leading to escape from antitumor responses (51). CD53, CD81, CD82, and CD37 have been shown to associate with MHC class II complexes (17, 52–54). Recently, CD9 was reported to be involved in MHC class II trafficking in human monocyte-derived DCs (35). The functional consequences of these interactions are demonstrated by altered antigen presentation capacity of tetraspanin$^{-/-}$ DCs. Both CD37$^{-/-}$ and CD151$^{-/-}$ DCs were hyperstimulatory to CD4 and CD8 T cells, although by different underlying mechanisms. CD151 was involved in inhibiting co-stimulation, while absence of CD37 led to increased peptide presentation (34). In Tccs6xCD37 double knock-out mice, an exaggerated hyperstimulatory phenotype of DCs was observed compared to DCs of single knock-out mice (55). This study indicates complementary functions for these two tetraspanins. In addition, DCs lacking CD82 had defects in processing MHC class II (14). Tetraspanin function in cross-presentation (the presentation of extracellular antigens in the context of MHC class I) by DCs has not been investigated, but is not unlikely considering that CD53, CD81, and CD82 interact with MHC class I molecules [(17), and own unpublished data]. Moreover, DCs lacking CD82 showed defective DC–T conjugate formation (14), and CD81 was found enriched in the contact area between APCs and T cells (56), supporting a function for tetraspanins in immunological synapse formation. Finally, tetraspanins (CD63, CD9, CD81, CD37) on exosomes may influence antigen presentation possibly *via* transfer of MHC–peptide complexes (57, 58). CD63 has been reported to inhibit antigen presentation as CD63 knockdown in APCs demonstrated increased secretion of exosomes containing MHCII (59). Together, these studies show that tetraspanins control antigen presentation either at the level of MHC–T cell receptor (TCR) interactions, at the level of co-stimulation, or *via* exosomes, which likely has implications for antitumor responses.

TETRASPANINS AND IMMUNE CELL MOTILITY

To mount an adequate immune response, immune cells need to migrate from peripheral tissues to draining lymph nodes and to the site of the tumor. It is well known that tetraspanins interact with multiple different integrins and as such influence the migratory capacity of cells (60). In the immune system, absence of CD151 was found to decrease T cell motility, leading to reduced inflammation in a model for inflammatory bowel disease (61). Trafficking of DCs to lymph nodes has been studied in different tetraspanin-deficient mice. CD37$^{-/-}$ mice challenged with two different doses of an immunogenic tumor showed defective tumor rejection compared to wild-type (WT) mice, indicating that CD37 is directly involved in antitumor immunity (62). Using irradiated tumor cells, it was shown that T cell responses were impaired, which was due to impaired DC migration to the draining lymph nodes (62). A different study confirmed the decreased motility of CD37$^{-/-}$ DCs (14) and neutrophils (63), and increased motility of CD82$^{-/-}$ DCs (14). Interestingly, the functional effects of CD82 are opposite to those of CD37 indicating that these tetraspanins counteract each other (14). Furthermore, CD81 was reported to be important in DC migration and formation of membrane protrusions *in vitro* (64). The underlying molecular mechanism involved cytoskeleton rearrangements *via* regulation of Rac-1 and RhoA, small GTPases that regulate the actin network. CD81 was required for Rac-1 activation (65), CD82 negatively regulated RhoA, and CD37 promoted activation of Rac-1 (27). Moreover, CD37, CD81, and CD82 have all been reported to interact with integrins (24, 33, 52, 63, 66), and although leukocytes are not dependent on integrins for migration in 3D environments (67), this may provide an additional mechanism for tetraspanin involvement in 2D migration. These studies show that tetraspanins are important in immune cell migration, thus making it likely they are also involved in leukocyte migration into the TME.

T AND B CELL ACTIVATION AND PROLIFERATION

Activation of T cells depends on antigen recognition presented in MHC–peptide complexes on the surface of APCs during immunological synapse formation. Recently, it was determined that CD9 and CD151 support integrin-mediated signaling at the immunological synapse in T cells (68). Accordingly, CD81 in T cells was involved in the organization of the immunological synapse by interacting with ICAM-1 and CD3 (69).

Antigen-presenting cell–T cell interaction and subsequent engagement of TCR and co-stimulatory molecules leads to naive T cell activation and proliferation (70), which can occur in the nearby lymph nodes or the TME. It is well-known that tumor-infiltrating lymphocytes can be an important prognostic factor for cancer (71). More specifically, CD8 cytotoxic T cells are associated with favorable patient outcome while Tregs are associated with decreased survival (6, 72). Furthermore, the positive effect of immune checkpoint inhibitors on clinical outcome of patients with melanoma or lung carcinoma shows that exhausted/dysfunctional T cells in the TME may be reactivated by anti-PD-1 therapy (73, 74). Taken together, these studies underline the importance of T cells in anti-tumor immunity.

Different tetraspanins have been linked to T cell proliferation, as CD37$^{-/-}$, CD151$^{-/-}$, Tssc6$^{-/-}$, and CD8$^{-/-}$ T cells were all hyperproliferative upon TCR stimulation (27, 30–32). Moreover, double CD37$^{-/-}$ Tssc6$^{-/-}$ mice displayed an exaggerated hyperproliferative T cell response, and impaired formation of antigen-specific CD8^{+} T cells after infection (55). In contrast, CD151-positive human T cells exhibited increased proliferation compared to CD151-negative T cells (13).

Another important facet of T cell biology is differentiation into different sub-types. CD81$^{-/-}$ mice displayed impaired Th2 responses, possibly linking tetraspanins to T cell differentiation (32, 75–77). These mice fail to develop Th2-dependent allergic airway hyperreactivity (77). *In vitro* studies revealed that altered B–T cell interactions were responsible for the deficient Th2 response (76). These studies should be further expanded to investigate different T cell subsets, Th1-Th2 balance, and especially T cells in the TME (such as Tregs or Th17 cells) to unravel

tetraspanin function in T cell differentiation during antitumor immunity.

A recent study with CD81$^{-/-}$ mice elegantly demonstrated that tumor growth and metastasis were severely impaired in CD81$^{-/-}$ mice compared to WT mice. Both Tregs and MDSCs lacking CD81 were observed to be deficient in their suppressive ability (46). This is one of the first studies investigating the effect of tetraspanins on regulatory immune cells.

B cells have a dual role in tumor immunity as they can both inhibit and stimulate tumor growth. Tumor-specific antibodies produced by B cells can opsonize tumor cells and lead to antibody-dependent cytotoxicity by natural killer (NK) cells and phagocytes. On the other hand, regulatory B cells (Bregs) secrete IL-10 and TGFβ, which directly inhibit effector immune cells, thus suppressing antitumor immunity (78). Tetraspanins function in humoral immunity has been evidenced by different studies in tetraspanin-deficient mice and the first documentation of a CD81-deficient patient. CD81 is important in the trafficking of CD19, part of the co-receptor complex of the B cell receptor (BCR), to the surface of B cells (25). BCR signaling occurs when B cells encounter their antigen and is important for B cell proliferation, survival, and antibody production. Absence of CD81 led to CD19-deficiency resulting in antibody defects in mice (32, 76) and humans (25). CD81$^{-/-}$ mice show impaired B cell proliferation, decreased responses to Th2 stimuli, decreased antibody production (32), and impaired B cell proliferation after BCR activation (26). CD53, although not required for CD19 expression, is also important for B cell function, as we recently discovered that CD53 promoted BCR-dependent protein kinase C (PKC) signaling (19). Both human and murine CD53$^{-/-}$ B cells have defects in translocation of PKC to the plasma membrane, consistent with an elegant study demonstrating that CD82 stabilizes PKC activation at the surface of leukemia cells (60). These findings indicate that tetraspanins can directly influence immune cell signaling. CD37 is highly expressed by B cells (79) and controls antibody production as shown in CD37$^{-/-}$ mice that display decreased IgG and increased IgA levels, which is a B-cell intrinsic phenotype (29, 80). The underlying mechanism involved CD37 regulation of α4β1 integrin-Akt signaling, which is required during follicular DC–B cell interactions and supports survival of IgG1-secreting cells (33). The importance of CD37 in B cell survival has been confirmed in an independent study demonstrating that the cytoplasmic domains of CD37 couple to the Pi3K–Akt survival pathway (81). Tetraspanin function in anti-tumor immunity is further supported by our unpublished findings showing increased tumor growth in CD53$^{-/-}$ mice compared to WT mice using a syngeneic immunogenic tumor model (F. Schaper et al., in preparation), which is in accordance with the impaired anti-tumor immunity observed in CD37$^{-/-}$ mice (62).

To conclude, tetraspanins have crucial functions in both T and B cell proliferation, survival, and signaling. It is interesting that these functions are non-redundant (deficiency of one tetraspanin results in a certain phenotype) and specific (for example CD81 controls CD19, whereas CD37 controls α4β1 integrin on B cells). Given these specific functions of tetraspanins and their partner molecules, it stands to reason that this can result in either an antitumor or pro-tumor response mediated by immune cells in the TME.

CYTOKINE PRODUCTION AND OTHER EFFECTOR FUNCTIONS

Cytokines are a central part of cellular communication and stimulate cell migration to sides of inflammation. These cytokines can be either immunosuppressive (IL-10, TGFβ, or IL-35) or proinflammatory (IL-12, γIFN, TNFα). Several studies demonstrated that tetraspanins can influence cytokine production. CD37 has been reported to inhibit IL-6 signaling upon infection (80, 82, 83), and during lymphomageneses (45). CD53 has been implicated as negative regulator of IL-6, TNFα, and IL-1β in a population study of house dust mite (84) and linked to TNF-α by genome-wide association studies (85). In line with this, CD81$^{-/-}$ DCs produced more TNF-α compared to WT DCs upon *Listeria* infection (86). Additionally, TNF-α production was increased by CD9$^{-/-}$ macrophages compared to WT macrophages after stimulation with LPS (87). CD9 has also been linked to production of TGFβ and IL-10. Bregs are known to produce large amounts of IL-10 and two independent mouse studies discovered that CD9 could serve as novel phenotypic marker for Bregs (88, 89). Although CD9$^{-/-}$ mice do not have aberrant B cell development or humoral immunity (90), Breg presence and function in CD9$^{-/-}$ mice has not been investigated to date.

In a small study of patients with metastatic melanoma, CD9 expression on NK cells was observed to correlate strongly with serum levels of TGFβ (91). Interestingly, CD9 was absent on NK cells in healthy controls, but upregulated after incubation with TGFβ (91, 92). These CD9-positive NK cells are normally found in the maternal part of the placenta (93), where they exert immunosuppressive actions. These data indicate that suppressive factors, which are also found in the TME, can alter tetraspanin expression on immune cells, which has immunological consequences.

FUTURE DIRECTIVES: TETRASPANINS ON IMMUNE CELLS IN THE TME

Tetraspanins are emerging as important organizing proteins on immune cells that control both humoral and cellular immune responses (**Table 1**), by either stimulating or inhibiting immune cell function. However, more insight is needed into understanding tetraspanins on immune cells in the TME including regulatory lymphocytes, MDSCs, and tumor-associated macrophages. Recently, CD81 has been demonstrated to control MDSC and Treg function in a murine tumor model, which is the first *in vivo* evidence of tetraspanin function in antitumor immunity (46). This, together with the finding that CD37$^{-/-}$ mice have impaired antitumor responses (62) indicates that tetraspanins directly contribute to antitumor immunity. However, tetraspanin expression has thus far only been studied on immune cells from blood or bone marrow (79), and not yet in the TME. In this TME, both stimulating and suppressive immune cells are present, each with their own distinct tetraspanin web. We

TABLE 1 | Key functions of individual tetraspanins on immune cell subsets.

Tetraspanin	Function on immune cells
CD37	– T cell proliferation, peptide MHC presentation, antibody production, IL-6 signaling by B cells
	+ Dendritic cell (DC) migration, B cell survival
CD53	+ B cell receptor-dependent protein kinase C signaling
	– IL-6, TNFα production
Tssc6	– T cell proliferation
CD9	– TNF-α production
	+ MHC class II trafficking
CD81	– T cell proliferation, TNF-α production
	+ DC motility, immunological synapse organization, Th2 response, antibody production
CD82	– DC migration
	+ DC–T cell conjugation
	+ Antigen presentation
CD151	– T cell proliferation, T cell motility, co-stimulation in DCs
CD63	– exosome secretion

–, inhibits; +, stimulates.

propose a model in which tumor cells have the ability to alter the composition of the tetraspanin web on immune cells that enter the TME from the circulation (**Figure 1**). Tumor environmental factors that may influence tetraspanin expression on immune cells include suppressive cytokines, low oxygen levels (hypoxia), growth factors, damage-associated molecular patterns (DAMPs) and tumor-immune cell contact. These tumor environmental factors may either increase or decrease the expression of different tetraspanins on immune cells. Plasticity of immune cells plays an important role in the TME, and it is well established that immune cells can change their phenotype by altering membrane protein expression. Since altered (increased or decreased) tetraspanin expression affects immune cell function, we hypothesize that a changed tetraspanin web supports immune cell plasticity through altered membrane protein organization. This model is supported by (1) multiple studies demonstrating that tetraspanin-deficiency affects proliferation, migration, cytokine production, and antigen presentation, (2) CD9 upregulation on NK cells after TGFβ incubation leading to immunosuppressive NK cells (91), (3) regulation of TGFβ by tetraspanins (94, 95), and (4) own unpublished observations demonstrating that human lymphocytes cultured with tumor cells change expression of multiple tetraspanins. We are only at the beginning of understanding the dynamic nature of the tetraspanin web on immune cells, and we envisage that its composition will change depending on the tumor state (elimination, equilibrium, or escape). Using multispectral imaging (79, 96) on resected tumor material from patients, effects on tetraspanin expression can be investigated in the future. We anticipate that targeting tetraspanins on specific immune subsets (such as CD9 on Bregs) in the TME may have therapeutic potential. Hereby, it should be taken into account that the same tetraspanin can stimulate or inhibit immune cell function, depending on the immune cell type it is expressed on. Research investigating tetraspanins as therapeutic targets in cancer is already ongoing (50), exemplified by targeting CD37 in clinical trials for B cell malignancies (97, 98).

Taken together, further investigation into tetraspanin function on immune cells will add to our understanding of the role that these membrane proteins play in antitumor immunity and the possibility to target the tetraspanin web on immune cells in the TME.

AUTHOR CONTRIBUTIONS

AS supervised the study and FS and AS both wrote the manuscript.

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Immune Targeting of Tetraspanins Involved in Cell Invasion and Metastasis

*Felipe Vences-Catalán and Shoshana Levy**

Division of Oncology, Department of Medicine, Stanford University School of Medicine, Stanford, CA, United States

Metastasis is the ultimate consequence of cancer progression and the cause of patients' death across different cancer types. Patients with initial diagnosis of distant disease have a worst 5-year survival compared to patients with localized disease. Therapies that target primary tumors fail to eradicate distant dissemination of cancer. Recently, immunotherapies have improved the survival of patients with metastatic disease, such as melanoma and lung cancer. However, only a fraction of patients responds to immunotherapy modalities that target the host immune system. The need to identify new druggable targets that inhibit or prevent metastasis is, therefore, much needed. Tetraspanins have emerged as key players in regulating cell migration, invasion, and metastasis. By serving as molecular adaptors that cluster adhesion receptors, signaling molecules, and cell surface receptors; tetraspanins are involved in all steps of the metastatic cascade. They regulate cell proliferation, participate in EMT transition, modulate integrin-mediated cell adhesion, and participate in angiogenesis and invasion processes. Tetraspanins have also been shown to modulate metastasis indirectly through exosomes and by regulating cellular interactions in the immune system. Importantly, targeting individual tetraspanin with antibodies has impacted tumor progression. This review will focus on the contribution of tetraspanins to the metastatic process and their potential as therapeutic tumor targets.

Keywords: cancer, immunotherapy, monoclonal antibodies, bench, bedside

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***Correspondence:**
Shoshana Levy
slevy@stanford.edu

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INTRODUCTION

Malignant transformation of healthy tissues gives rise to cancer, this disease affects millions of people worldwide. Moreover, metastases; the dissemination of cancer cells is still the greatest cause of death. Patients diagnosed with localized disease have a better 5-year survival than patients with distant disease at the time of diagnosis (1). Therefore, treatments that prevent or diminish metastatic lesions are much needed, such as identifying new druggable targets involved in the metastatic cascade.

Monoclonal antibodies (mAbs) are the preferred immunotherapeutic tools to either target the host immune system or to target the tumor (2). The most common tumor targets are cell surface molecules, such as EGFR, HER2, Mesothelin, CD19, or CD20 whose cell membrane localization, and sometimes tumor-specific expression, or overexpression in comparison to healthy tissues, makes them suitable for antibody-based therapy (2). More recent approaches to immunotherapy of cancer do not target antigens expressed on tumor cells—they unleash the host immune checkpoint blockade, and have improved the survival of patients with metastatic cancers (3).

Tetraspanins are a family of conserved proteins in eukaryotic cells that spans the membrane four times. In humans, 33 members have been identified with different tissue distribution. Expression of some tetraspanins such as CD37 and CD53 is restricted to hematopoietic cells, whereas others, such as CD9, CD81, and CD151 are more broadly expressed. Tetraspanins serve as membrane scaffolds that bring together surface molecules, such as integrins and cell-specific receptors, additional growing evidence shows that engagement of tetraspanins leads to recruiting signaling molecules thereby activating downstream pathways (4). This plethora of interacting partners allows tetraspanins to function in different cellular processes under physiological conditions but also in disease. Multiple studies have shown that tetraspanins regulate tumor growth, cell adhesion, invasion, and migration of tumor cells, reviewed in Ref. (5, 6). Importantly, targeting some tetraspanins with mAbs has proven to be efficient in eliminating tumor cells and in preventing metastasis in preclinical models (7). Here, we will give an overview of tetraspanins as prognostic markers in tumors, their role in invasion and metastasis, and discuss recent studies aimed at antibody-based targeting of these molecules in cancer.

TETRASPANINS AS PROGNOSTIC MARKERS OF CANCER PROGRESSION

Among the human tetraspanins, Tspan8 and 12, CD9, CD37, CD63, CD81, CD82, and CD151 play a role in cancer progression (5, 6). Down or upregulation of these tetraspanins on tumors has been correlated with either good or bad prognosis in different types of cancers. Historically, several tetraspanins were identified by studies that compared normal and malignant tissues. Tspan8 was originally identified as a tumor-associated antigen by an antibody (CO-029) (8), CD63 by a melanoma-associated antigen (ME491) (9), CD151 was re-identified by an antimetastatic antibody (10), and CD82/KAI1 as a metastasis suppressor gene (11).

More recent studies have demonstrated that loss of CD82/KAI1 expression is correlated with poor prognosis of several cancers, reviewed in Ref. (12). Interestingly, presence of a specific splice variant of CD82 that removes exon 7 increases tumor progression and invasion (13). Similarly, loss of CD37 expression in patients with diffuse large B-cell lymphoma showed significant correlation with decreased survival after R-CHOP therapy (14). And lack of CD37 in mice increased the development of germinal center derived B cell lymphomas (15).

By contrast, CD151 is expressed in different types of cancer and high expression correlates with poor prognosis (16). It is of note that expression of CD151 in the host contributes to cancer progression—CD151 knockout (KO) mice have fewer skin, melanoma, lung, and prostate cancers than their wild type (WT) counterparts (17–20). Similarly, upregulated CD81 expression in melanoma was found to contribute to an enhanced metastatic phenotype (21, 22). Additionally, expression of CD81 in the host contributes to tumor progression; CD81KO mice have fewer metastases of breast and lung tumors in syngeneic mouse models (23). Importantly, expression of CD81 in immune suppressive cells contributes to tumor progression (24). A recent study showed that expression of CD151 in human is associated with a hyper-proliferative T cell phenotype (25), it would be interesting to know if CD151 expressed in mouse immune cells plays a role in tumor progression. Tspan8 is another tetraspanin whose upregulation is correlated with ovarian cancer progression (26). More recently, the presence of Tspan8 mRNA in the blood was shown to be a sensitive marker for colorectal cancer detection (27). Reduced CD9 expression has been correlated with poor prognosis in several types of cancers, including melanoma, lung, breast, colon, prostate, pancreatic ovarian, and prostate, reviewed in Ref. (28). However, this is not the case for esophageal squamous cell carcinomas that express higher CD9 levels than normal esophageal tissues (29). Lack of CD9 in mice that develop spontaneous prostate cancer mirrors the former human studies, namely, CD9 deficiency increased liver metastases, although it had no effect on onset of primary tumors, nor on lung metastases (30).

Thus, tetraspanins are important players during cancer progression, some tetraspanins are upregulated in certain cancer types while others are downregulated. Clearly, tetraspanins have been used as prognostic markers in cancer.

INTERACTIONS OF TETRASPANINS WITH PARTNER PROTEINS REGULATE INVASION AND METASTASIS

We now know that tetraspanin-enriched microdomains (TEMs) incorporate partner proteins, such as integrins, cell surface receptors, and metalloproteases (MMPs) that contribute to cellular invasion and metastasis (**Figure 1**). Biochemically, very few of these complexes are held together when solubilized by harsh detergents, the majority only withstand mild detergents (31). The association of CD151 with laminin-binding integrins α3β1, α6β1, and α6β4 (32) is strong, stoichiometric, and occurs early in biosynthesis (33). Silencing CD151 in epithelial carcinoma cells disrupts α3β1 association with TEMS and impairs cell migration (34). In addition, CD151 ablation reduces cell migration, invasion, spreading, and signaling in an integrin-dependent manner (35). CD9/CD81 also regulate α3β1 integrin, loss of these two tetraspanins impairs breast cancer spreading, motility, and disrupts its association with PKCα in a CD151-independent manner (36). Tspan8 was also shown to modulate invasion of melanoma *in vitro* and *in vivo* through a β1 integrin by affecting integrin-linked kinase signaling and its downstream target AKT (37).

While integrins serve as receptors to extracellular matrix (ECM) components, matrix metalloproteinases (MMPs) degrade ECM components. Several tetraspanins associate with MMP-14 (MT1-MMP) during biosynthesis and prevent its degradation enabling cell surface expression. And this association also involves another known tetraspanin partner, EWI-2 (38). However, a knockdown of a single tetraspanin, such as CD9 or CD81 had no effect on MMP-14-dependent fibronectin degradation, but a CD9/CD81 double knockdown clearly affected degradation (39). Interestingly, overexpression of CD81 in a human melanoma cell line upregulated MT1-MMP expression and activity with a consequent increased invasion and metastases *in vitro* and *in vivo*

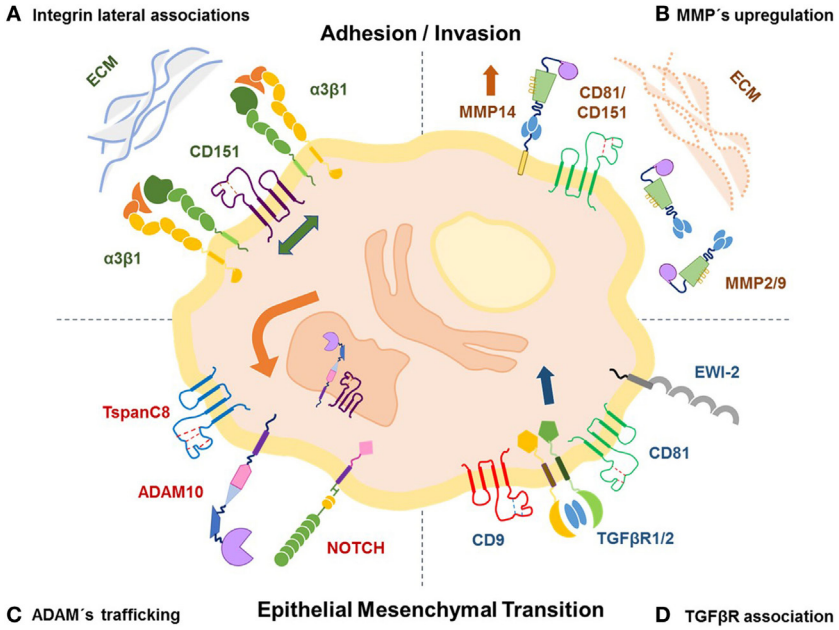

FIGURE 1 | Tetraspanins modulate invasion and metastases by regulating the activity of their partners. **(A)** Tetraspanins form stable lateral association with integrins in tetraspanin-enriched microdomains (TEMS) favoring spreading, cell adhesion, and migration through the extracellular matrix (ECM); on the other hand, **(B)** tetraspanins regulate ECM degradation during cell invasion by modulating expression and activity of metalloproteases, such as MMP-2, MMP-9, and MMP-14. **(C)** The TspanC8 subgroup is known to promote trafficking and cell localization of ADAM10 and its sheddase activity thereby regulating substrates, such as NOTCH receptor, to favor epithelial to mesenchymal transition (EMT). **(D)** Association of CD9/CD81 with EWI-2 affects TGFβ signaling modulating EMT, invasion, and metastases of melanoma.

(21). Similarly, overexpression of CD9 on fibrosarcoma cells increased MMP-9 production and activity, resulting in a more invasive phenotype *in vitro* (40); however, transfection of CD9 into small cell lung cancer cells inhibited transcription of MMP-2 and MMP-14 (41). CD151 was shown to be a link between MMP-14 and integrin α3β1 (42); and to mediate tumor progression by affecting expression and function of MMP-9 in hepatocellular carcinoma (43), melanoma (44), and pancreatic adeno carcinoma (43, 45). Finally, silencing CD63 reduced the levels of β-catenin protein and its downstream target MMP-2 inhibiting metastatic lung colonization of ovarian and melanoma tumors (46).

A disintegrin and metalloproteases (ADAMs) also interact with tetraspanins (47). ADAMs are a family of proteases, classified as sheddases because they can cleave extracellular portions of transmembrane proteins regulating cell functions such as cell invasion and motility. The tetraspanin subgroup TspanC8 (Tspan 5, 10, 14, 15, 17, and 33) mediates trafficking, maturation, and compartmentalization of ADAM10, thereby influencing its function (48, 49). Interestingly, TspanC8 members expressed in *Drosophila* and *C. elegans* were found to regulate ADAM10 levels and to modulate Notch functions that promote epithelial to mesenchymal transition (EMT) in cancer cells (50, 51). Another tetraspanin family member, Tetraspanin-8, is also linked to cancer progression by inducing ADAM12 upregulation in esophageal carcinoma promoting metastases (52).

Transforming growth factor β (TGF-β1) is also regulated by specific tetraspanins. It was shown that absence of CD151 in breast cancer cells affected the compartmentalization of TGFβ receptor 1 thereby disturbing TGF-β1-induced activation of p38, which correlated with reduced lung adhesion and decreased metastases (53). Similarly, CD9/CD81 were shown to regulate TGF-β1 signaling in melanoma by providing critical support for TGFβR2- TGFβR1 association, which in turns favors EMT-like changes, invasion, and metastases (22). However, TGF-β1 signaling is negatively regulated by EWI-motif containing protein 2 (EWI-2), which when present, sequesters CD9/CD81 thereby dissociating EWI-2 interaction with TGF receptors (22). Indeed, lack of EWI-2 in melanoma cells was associated with increased invasion and metastasis *in vitro* and *in vivo* (12).

Tetraspanins clearly regulate tumor progression at different levels by interacting with a plethora of partners, which are implicated in tumor initiation, promotion of an EMT phenotype, invasion, and migration that ultimately lead to metastasis. To better understand the mechanisms of how individual tetraspanin members and their partners contribute to cancer progression *in vivo*, numerous *in vitro* studies have used specific anti-tetraspanins mAbs.

TARGETING TETRASPANINS WITH mAbs

At the cellular level, mAbs that target individual tetraspanin members have been used to study signaling pathways, to disrupt molecular associations, to analyze the dynamics of cell surface partitioning, and to probe the tetraspanin web (54). Because tetraspanins regulate cell adhesion, invasion, and metastases, a strategy that prevents any of these cell functions seems reasonable

(**Figure 2**). Indeed, several anti-tetraspanins' mAbs inhibit tumor cell invasion and migration (7, 55). However, not all antibodies that target the same individual tetraspanin share the same properties, suggesting that engagement of specific epitopes in the tetraspanin molecule could result in different outcomes. Thus, an anti-CD9 mAb (PAINS-13) that disrupts the association of CD9 with β1 integrin (56) inhibited the growth of a human colon carcinoma cell line xenograft more effectively than another anti-CD9 mAb (VJI/20) or integrin mAbs (57). Yet, all tested anti-CD9 mAbs (VJI/10, VJI/20, and GR2110) inhibited the trans-endothelial migration of melanoma cells (58). An additional anti-CD9 antibody (ABL6) (59) induced apoptosis (60) and reduced the growth of a human gastric cancer cell line in a xenograft model (61).

The disruption of the interaction with integrins has been studied even in more details for anti-CD151 mAbs whose epitope map to the QRD sequence in the large extracellular loop (LEL) (33). Anti-CD151 mAbs that disrupt the interaction with integrins prevent prostate cancer metastases, in contrast to anti-CD151 mAbs that bind to integrin-associated CD151 (62–64). Indeed, anti-CD151 mAb (1A5) blocked metastases in SCID mice bearing Hep-3 tumors by inhibiting invasion and migration, although the antibody did not inhibit primary tumor growth (65). However, a study using a different monoclonal antibody (SFA1-2B4) that co-immunoprecipitated CD151 with α3β1 integrin prevented lung metastases of human colon cancer and fibrosarcoma cell lines (66). TEMs in endothelial cell junctions include CD151, CD81, and CD9, a study comparing migration of endothelial cells demonstrated that anti-CD151 and CD81, as well as anti-integrin mAbs inhibited migration (67).

More recent studies have shown that mAb targeting Tspan8 (Ts29.2) inhibited the growth of two human colorectal cancer cell lines when injected into nude mice, interestingly the antibody did not induce direct toxicity nor the inhibition of the previously reported angiogenic properties of Tspan8 (68). Moreover, a mAb reacting with the LEL of Tspan8 inhibited tumor invasion *in vitro* and diminished incidence of ovarian metastases *in vivo* (26, 69). Recently, a radiolabeled anti-Tspan8 mAb, labeled with lutetium-177 was effective against colorectal cancer in a xenograft model, showing a significantly reduced tumor growth (70).

Taken together, anti-tetraspanin mAbs have shown significant anti-tumor effects *in vitro* and in mouse models, but because of expression in both tumor and host, off-target effects are still of major concerns. Strategies to reduce the risk of off-target effects could include the use of bispecific mAbs that target both the individual tetraspanin and its interacting partner, for example, CD81/CD19 in B cells.

EXPLOITING TETRASPANIN FUNCTION FOR IMMUNOTHERAPY

Indeed, a bispecific antibody was engineered to target CD63 on one arm thereby enabling efficient internalization of an anti-HER2 arm that targets the tumor (71). This bispecific construct allows targeting a drug-conjugated tumor binding-antigen, HER2, to the lysosomal pathway by CD63. This novel approach resulted in an improved survival and delayed tumor growth in a xenograft model of ovarian cancer. Mice treated with the bispecific bsHER2xCD63-Duostatin-3 conjugate increased HER2 internalization, this effect was not observed with the monovalent antibodies targeting only HER2 or CD63. This interesting approach of exploiting CD63 for cancer immunotherapy is based on its role in intracellular trafficking and abundance in exosomes (72).

Targeting tetraspanins in exosomes for cargo delivery has been reviewed extensively (5, 73). Exosomes gained attention due to their important function during cellular communication, in addition to tetraspanins they contain a variety of different molecules; receptors, integrins, lipids, mRNAs, and miRNA,

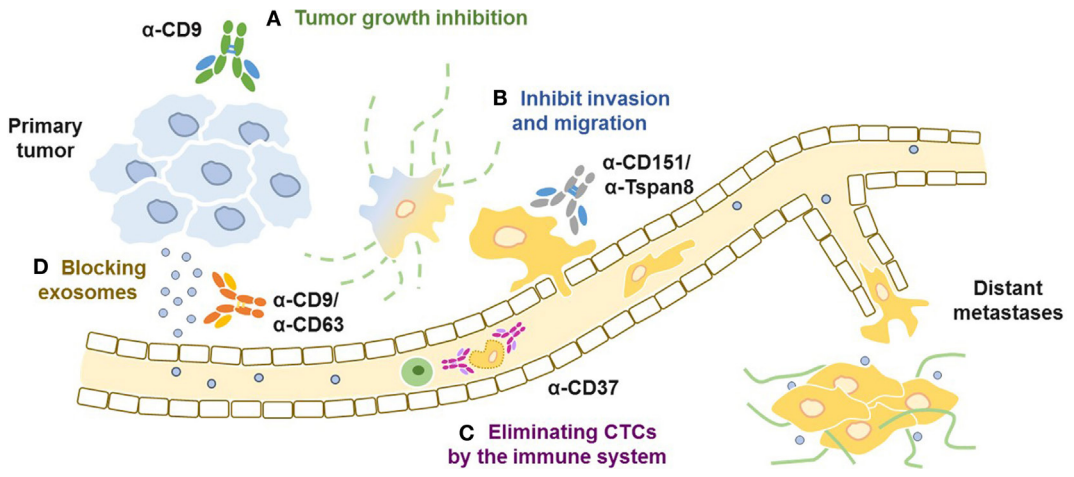

FIGURE 2 | Targeting tetraspanins at different stages of tumor progression. **(A)** Anti-CD9 antibodies have been shown to inhibit proliferation of human gastric tumors **(B)** anti-Tspan8 and anti-CD151 antibodies which inhibit adhesion, migration, and extravasation of tumor cells in different types of cancer *in vitro* and *in vivo*. **(C)** Anti-CD37 antibodies eliminates circulating chronic lymphocytic leukemia cells by recruiting and activating the immune system, **(D)** anti-CD9 and anti-CD63 antibodies block exosomes and enhance their clearance from circulation through macrophage-dependent mechanisms.

all of which have an effect in their target cells (74). Importantly exosomes released from cancer cells have been shown to support metastases. Thus, blocking exosomes from tumor cells with antibodies could be used as a therapeutic strategy to prevent metastases, as shown for cancer stem cells (75). Although tetraspanins (i.e., Tspan8, CD81, and CD63) were originally used as exosome markers it is now clear that they play an active role in exosome cargo-loading and delivery (5, 73). Proteomic analysis using tetraspanins c-terminal domains to pull-down interacting proteins showed a significant overlap with proteins found in exosomes, suggesting that tetraspanins might regulate protein cargo (76). In addition, it has been shown that several tetraspanins regulate protein trafficking to the membrane and intracellular compartments of several receptors (48, 49). Indeed, deleting CD81 revealed a differential protein cargo in exosomes lacking CD81 (76). Moreover, deleting CD81 in endothelial-producing exosome cells but not tetraspanins CD63 or CD82 reduced breast cancer motility and metastasis (77). It is, therefore, likely that individual tetraspanins might regulate protein loading in exosomes, and that such specificity could potentially be exploited to confer selective exosome cargo and/or delivery. For example, the preferential interaction of Tspan8 with α4β4 in exosomes (78). Such preferential interaction could render uptake-specificity of exosome by endothelial and pancreatic cells, and possibly facilitate the use of exosomes for drug delivery, reviewed in Ref. (79).

Proteomic profiling of extracellular vesicles of 60 cancer cell lines (NCI-60) revealed CD81 expression in all 60, while CD9 and CD63 were expressed in about 40 of these cell lines (80). Moreover, clinically relevant exosomes isolated with anti-CD9 or anti-CD63 antibodies and then detected with anti-HER2 revealed a 14–35% tumor-specific exosomes from breast cancer patient serums, which potentially could be used as non-invasive diagnostic method or even used to detect disease progression (81). A study that used anti-CD9 and anti-CD63 antibodies to deplete tumor-derived exosomes in a xenograft model showed a significant reduction in metastases to different organs but had no effect on growth of primary tumors (82). That study showed that exosome depletion from blood was macrophage-mediated. However, in a xenograft model only the tumor tetraspanins are targeted by the antibodies, whereas in human the targeted tetraspanins are expressed both in the host and in the tumors.

FROM BENCH TO BEDSIDE

Several tetraspanins used as therapeutic targets show promise in preclinical models of tumor progression and metastases (7, 28, 55, 83). However, CD37 is the only tetraspanin target that has moved forward into the clinic (84). CD37 is predominantly and abundantly expressed on mature B cell malignancies, but not on solid tumors. B cells serve as especially suitable targets for immunotherapy because of the ability of the antibodies to mediate both direct and indirect immune responses. This therapy is a promising tool, especially in those cases where other immunotherapies have failed. Different anti-CD37 antibodies that better mediate antibody-dependent cell cytotoxicity, improve complement activation, or are conjugated to a cytotoxic drugs (83), have recruited patients to clinical trials (clinicalTrials.gov).

CONCLUDING REMARKS

Tetraspanins regulate cancer progression and metastases; yet, their broad tissue distribution presents an impediment for cancer immunotherapy, due to possible off-target effects. However, with current exponential advances in immunotherapy, these limitations could be overcome. Examples include bispecific antibodies that confer tumor selectivity or shielding antibodies with tumor-specific proteases (85). Lastly, understanding the function of tetraspanins and their molecular partners, both in the tumor and in the host, will ultimately develop new therapies for cancer treatment.

AUTHOR CONTRIBUTIONS

FVC ans SL reviewed literature and wrote the manuscript. FVC prepared the figures. All authors approved the manuscript for publication.

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