Tetraspanins are ubiquitous membrane proteins that induce local membrane curvature and hence co-ordinate cell-to-cell contacts. This review highlights their role in inflammation, which requires control of the nano-architecture of attachment sites between endothelial cells and leukocytes. The active role of endothelial cells in preparing for transmigration of leukocytes and determining the severity of an inflammation is often underscored. A clear hint to endothelial pre-activation is their ability to protrude clustered adhesion proteins upward prior to leukocyte contact. The elevation of molecular adhesive platforms toward the blood stream is crucially dependent on tetraspanins. In addition, leukocytes require tetraspanins for their activation. The example of the B-cell receptor is referenced in some detail here, since it provides deeper insights into the receptor–coreceptor interplay. To lift the role of tetraspanins from an abstract model of inflammation toward a player of clinical significance, two pathologies are analyzed for the known contributions of tetraspanins. The recent publication of the first crystal structure of a full-length tetraspanin revealed a cholesterol-binding site, which provides a strong link to the pathophysiological condition of atherosclerosis. Dysregulation of the inflammatory cascade in autoimmune diseases by endothelial cells is exemplified by the involvement of tetraspanins in multiple sclerosis.

**Introduction**

Cell surface architecture is implicated in virtually all cell-to-cell interactions. The cellular inflammatory response is an example of cell interaction, where circulating leukocytes are recruited to the site of inflammation by resident endothelial cells.

The endothelium is a gatekeeper to the underlying tissues, controlling all flux of nutrients, signaling molecules and leukocytes. Immunological studies often focused on white blood cells, while the perception of the endothelium as a passive filter is just slowly replaced by the viewpoint of endothelia as active immunomodulators.

Endothelial cells prepare actively for a facilitated transmigration before any leukocyte contact [1,2]. Distinct morphological changes are attributed to this activation process, including formation and contraction of actin stress fibers, nanoscale membrane protrusions [3–7] or even transendothelial tunnel formation [8,9].

These pre-contact changes suggest the endothelium to be an active part of the immune system. In the context of infection and inflammation, tetraspanins (TSPs) play an important role [10], potentially by co-ordinating molecular adhesion clusters on the surface [7].

Here, we depict the role of TSPs in leukocyte–endothelial interaction with a focus on functional nano-architecture of endothelial surfaces.
The inflammatory cascade
Blood-patrolling leukocytes adhere and migrate through the vessel wall after tethering and rolling steers them to an inflammation site. A prerequisite for the transmigration step is an arrest (firm adhesion phase), achieved by binding of the leukocytic integrin LFA-1 to the endothelial integrin ICAM-1. This phase is still reversible, i.e. can return into a rolling phase, but the decision-making factors are not clarified yet.

Neither is it clear how a firm adhesion phase turns into an actual diapedesis step. The route of transmigration is nowadays widely accepted to be either the paracellular or the transcellular pathway (directly through a single endothelial cell) [11–16], highlighting the importance of tunnel formation, which can already be provoked by bacterial toxins alone [9].

Since tunnel formation and reclosure would energetically challenge the cell through cytoskeletal remodeling, preformed so-called exit sites in the endothelium would save energy and time demand for the immune response. A functional study on lateral migration of freshly isolated leukocytes on primary human endothelium came to the conclusion that exit channels might explain the reversible arrest and rolling. Extravasation will be successful if leukocyte attachment happens close to pre-deletion sites; otherwise, adhesion will be reversed until the next try [1]. A search for morphological signs to indicate endothelial exit regions unraveled nanoscalar elevation of the molecular adhesion clusters (ICAM-1 clusters). This pre-contact formation of adhesive protrusions was crucially dependent on TSP CD9 [7].

TSPs and cellular morphology
TSPs are a family of transmembrane (TM) proteins found in many multicellular organisms from plants to mammals [17]. They contain four TM helices, which arrange themselves in a conical shape [18].

The superfamily of TSPs may be classified in four different groups, namely CD, CD63, Uroplakin and the RDS family [19,20]. The CD family contains nearly all the vertebrae TSPs, which are the main subject in this review (e.g. CD9, CD81, CD82 and CD151). A flexible extracellular loop (EC2) is responsible for association to various membrane proteins as detailed below [5,21,22]. A link to the cytoskeleton is provided by binding of the C-terminal tail to the Ezrin–Radixin–Moesin proteins [23]. Moreover, TSPs CD9 and CD81 can modulate signaling pathways by interaction with G-protein-coupled receptors [24].

By these interactions, TSPs influence cellular morphology. During diapedesis, the close intertwining of leukocytes and endothelial cells requires dynamic modification of plasma membranes and the cytoskeleton. Both in leukocytes and in endothelial cells, TSPs induced cellular protrusions even before mutual contact. This has been exemplified by high-resolution methods like electron microscopy or atomic force microscopy (AFM) [25]. The TSP-induced subcellular structures, all associated with modulated membrane curvature, comprise nanotubes, microvilli, basal membrane tubes, podocytes, membrane blebs and ridges [26]. The process of membrane bending can even exceed the villous state to finally shed microvesicles into the environment [25,27,28].

Endothelial inflammatory phenotype
When endothelial cells are activated by TNF-α, TSP-enriched molecular domains are observed containing adhesion molecules (like ICAM-1, ICAM-2, PECAM-1 and VCAM-1) [4,5]. Analyzing diffusion kinetics by advanced fluorescence techniques (FRET–FLIM, FRAP), ICAM-1 turned out to affiliate to CD9, while VCAM-1 prefers CD81. The increased diffusion constants of TSPs over other CAMs make them perfect candidates for an initial interaction orchestrator. For example, CD9 also regulates the activity of ADAM17, which is a sheddase (protease) for various inflammatory membrane proteins like ICAM-1 and the TNF-α receptor [29,30].

AFM allows to quantify the protrusion formation after endothelial activation. These protrusions were termed microvilli with respect of the general concept of microvilli, even though their dimensions appear to be mostly smaller than, for example, those of intestinal microvilli (height 160 nm vs. 1–3 μm). Knockdown of TSP CD9 displays the connection of the molecular clusters and the formation of TSP-enriched microvilli [7]. These microvilli are essential in the inflammatory cascade as they present not only adhesion molecules but also membrane-bound chemokines like Ccl-19 or IL-8 [31,32] and therefore facilitate leukocyte adhesion (Figure 1).

Another example of TSP impact on plasma membrane are protrusions called nanopodia, which are longer and thinner than filopodia [33]. In this example, a TSP-like membrane protein ‘Transmembrane-4-L-six-family-1’ (TM4SF1) having low sequence homology with TSPs but sharing TSP
topology \cite{34} is selectively expressed by endothelial cells in vitro and in vivo. TM4SF1 is necessary for the formation of unusually long (up to a 50 \mu m), thin (\sim 100–300 nm wide), F-actin-poor EC cell projections \cite{33}.

**Inflammatory phenotype of leukocytes**

In leukocytes, TSPs have at least two fundamental functional aspects. On the one hand, TSPs modulate the membrane dynamics, as for instance when inducing receptor clustering and microvilli formation. On the other hand, TSPs have an important role regarding the immune synapse. Here, the exchange of TSP-rich extracellular vesicles was reported \cite{35}.

Accordingly, overexpression of CD81 induces the formation of microvilli in peripheral blood mononuclear cells and pre-B cells, whereas a knockout (KO) reduced their number per cell \cite{25}.

Different TSPs can exert opposite effects: while CD81 induces convex curvature, CD82 inhibits the formation of microvilli. Not only is the number of microvilli a result of CD81/CD82 balance, but also their curvature at the very tip \cite{25}.

The triggering of internal signaling cascades by TSPs was demonstrated by externally induced clustering of CD9 with antibodies, which resulted in degranulation of eosinophils \cite{36}.

Heterogeneous association and clustering was investigated in detail in B cells. As visualized by dSTORM microscopy, endogenous IgM- and IgD-B cell receptors exist in preformed nanoscale clusters with the B-cell co-receptor CD19. Disruption of the actin cytoskeleton did not reduce the clustering, but changed the diffusion of the clusters of IgM and IgD. The B-cell co-receptor CD19 diffuses independently of the disruption of the actin cytoskeleton by Latrunculin A. But in B cells of a TSP CD81 KO model, the diffusion was increased three-fold. This behavior of the co-receptor is interestingly independent of the diffusion of the B-cell receptor itself \cite{37}, showing that, for efficient B-cell activation, CD81 is required to organize the interplay of CD19 and the B-cell receptor.

**Membrane curvature**

To communicate with the exterior from a membrane-enclosed compartment by either releasing vesicles with signal molecules or physically palpating the environment, this membrane has to be bent. A major solution of the nature to actively control the shape of the membrane is the introduction of membrane proteins (like BAR, Caveolin, Cathrin, etc.) or specific lipids which minimize the energy toward a more or less curved membrane (\cite{38,39}; for reviews, see refs \cite{40,41}). A part of the mini-review is the special role of TSPs influencing membrane curvature.

**Role of TSPs**

The term 'TSP web' describes the generation of a sub-membrane domain of several TSPs bound to each other by hydrophobic interactions (ganglioside and cholesterol) (\cite{17,42,43}; for review, see ref. \cite{44}). Besides the hydrophobic interaction, a variable extracellular \(\delta\)-domain was found by gSTED to organize CD81 clusters, stabilizing these clusters by dimerization \cite{45,46}.

Some experiments using super-resolution microscopy (STED) revisited the nature of a TSP web and observed only homophilic local clustering between TSPs and heterophilic near proximity clustering between TSPs and their non-TSP-binding partner. These mono-TSP clusters were found to partially overlap with mono-TSP clusters of a different TSP. This leaves the term 'TSP web' to be defined more precisely \cite{47}.

Apart from biophysically and biochemically defined integration of TSPs within the membrane, another explanation of membrane curvature initiation through TSPs would be an indirect option via actin signaling by modifying the TSP-dependent G-protein-coupled receptors (\cite{23,24}; for review, see ref. \cite{26}). However, it could not be unequivocally proved that actin nucleation is upstream of membrane bending.

Given the recent structural insight, an intuitive geometrical model emerges of how membrane bending is induced. The four transmembrane helices are arranged pairwise to form a cone (TM1/TM2 and TM3/TM4), with the tip reaching into the cytoplasm. With this structure given, the convex curvature induced by CD81 could be a simple geometrical consequence of close spatial association of TSPs. Since the sequence structure is evolutionarily conserved, it could also explain the membrane modulations of other TSPs like CD9 \cite{18}. In contrast, the TSP CD82 decreases curvature and the number of protrusions of cells and is known to inhibit actin dynamics by reducing molecular activity of, for example, Rho \cite{48}. The interplay of these competing mechanisms has to be further elucidated.
Direct TSP–lipid interactions

In 2016, Zimmerman et al. reported the first crystal structure of CD81. They discovered a cholesterol-binding pocket between the transmembrane columns. This pocket has two possible configurations, each correlating to a closed or open state of the EC2 domain, with the latter exhibiting higher affinity to TM-binding partners like, for instance, CD19. In the closed state, the EC2 domain is bound by a salt bridge over the two transmembrane columns and cholesterol is bound (Figure 2A) [18].

Tremendous explanatory power resides in the cholesterol-binding pocket, because this finding provides a mechanistic link between TSP structure and the vast knowledge about cholesterol function in endothelial (patho-)physiology like atherosclerosis.

Atherosclerosis

Endothelial inflammation induced by cholesterol

Atherosclerosis is a multifactorial disease of the arteries leading to a thickening of their wall by formation of atheromas. These atheromas initially narrow the lumen of the vessel by accumulating fatty, cholesterol-rich LDL (low-density lipoprotein) particles followed by a chronic inflammation with macrophages. The LDL cholesterol is the main source of cellular cholesterol and distributed after lysosomal processing [49]. The role of cholesterol in endothelium is intensely affiliated to the nanoscale invaginations of the plasma membrane termed caveolae. These membrane domains (50–100 nm) are enriched in cholesterol and harbor a transmembrane protein, which is named after the phenotype: caveolin. Caveolae act as cell signaling scaffolds, they can fission into vesicles or even form transcellular tunnels and are implicated in virtually all cellular functions [50,51]. Cholesterol acts rather pro-inflammatory: adhesion and transendothelial migration of leukocytes are increased. Cholesterol promotes LPS-induced adhesion of THP-1 cells (leukocytes) mediated through the translocation of ICAM-1 from caveolar domains to the plasma membrane [52].
In line with this, a removal of cholesterol using methyl-beta-cyclodextrin (MβCD) diminishes transendothelial migration rates [53]. In this study, a clustering of E-selectin in lipid rafts was the proposed mechanism, but in view of recent data, also a reduced formation of ICAM-1-decorated protrusions might explain the findings [7]. One hypothetical mechanism could be a release of CD9 from CD81, as CD81 retracts its large extracellular loop while it binds cholesterol (Figure 2B) [18].

A physical interaction of TSPs with cholesterol was already demonstrated more than a decade ago. The activation signaling of proteins by tyrosine phosphorylations in lymphoid B cells was decreased by MβCD and induced by MβCD with cholesterol [42]. Recently, MβCD was found to reduce the effect of invasive dissemination mediated by a TSP-like protein TM4SF-5 in hepatocellular carcinoma cells. This was presumably caused by removal of the TM4SF-5 molecular cluster with EGFR from the cellular membrane. The detailed interaction of cholesterol and TSP, e.g. in early endothelial inflammation like atherosclerosis, seems therefore to influence directly the structure and the signaling cascade of TSPs.

In accordance with the findings of Charrin (2003), a role for TSPs in early atherosclerotic plaques has also been shown [54]. Monocyte invasion is correlated to up-regulation of TSP CD81 on both mRNA and protein levels in human vascular tissue. The effect was correlated to increasing the available clusters of ICAM-1 at the cellular surface without a change in the expression level of ICAM-1. These observations are in line with Barreiro’s finding of association of endothelial CD9, CD151 with ICAM-1, VCAM-1 and also CD81 in the docking structures as in the endothelial adhesive platforms [4,5]. This clearly supports a mechanistic model, where TSPs induce membrane protrusions at receptor clustering sites, thereby facilitating interaction with other cells.

The exact interplay between the inflammation-relevant TSPs, CD9, CD81, CD151, VCAM-1 and ICAM-1, and the modulating function of cholesterol, however, is still waiting for full elucidation [54].

Autoimmune diseases

TSPs in multiple sclerosis

Looking at the clinical picture, multiple sclerosis (MS) may illuminate the role of TSPs in immune diseases. The main focus in this context is on oligodendrocytes, which are known to regulate T- and B-cell activity [55–58]. The so far proposed mechanistic models involve clustering of MHC molecules (for reviews, see refs [59,60]).

In this context, it is of note that endothelial cells are key to the development of MS as shown in a murine model of experimental autoimmune encephalomyelitis (EAE) (see MOG-induced EAE [61]), which revealed...
increased severity scores in the case of potassium channel TREK-1 deficiency. These KO mice lack any leukocyte phenotype, but exhibit increased ICAM-1, VCAM-1 and PECAM-1 levels at the brain endothelium [2]. Histological preparations of lesions from MS patients show up-regulated CD9 levels in blood vessels. To complete the evidence, an antibody against CD9, targeting either leukocytes or the endothelium, has been shown to lead to improved barrier function [62]. Preventive usage of CD81 antibodies reduced monocyte immigration and symptoms in EAE [63]. Supporting the relevance of TSP for the maintenance of the blood–brain barrier, even extracellular vesicles of brain endothelial cells contain CD9 and CD81 [64].

Theoretically, involvement of TSPs in autoimmune diseases might also arise from TPSs being the target of autoantibodies. But an investigation concerning CD9, CD81 and CD82 remained inconclusive, stating only a weak immunological reaction [65]. Hence, the overwhelming data on protrusion formation in endothelial cells and in leukocytes are the more likely explanation for TSP involvement in autoimmune disorders [6,7,31,32,66].

**Conclusion**

Cell-to-cell interactions are controlled by ligand–receptor binding. Not only expression levels but also their local distribution and steric accessibility are needed for proper cell communication. The biological solution to this geometrical problem is to either present interaction proteins at the tip of membrane protrusions like microvilli or to store them in caveolae. In endothelial cells and leukocytes, mainly the TSPs CD81, CD9 and CD82 control the local membrane curvature. Via this organization of surface nano-architecture, TSPs pave the way for an efficient immune response. The clinical relevance of TSPs is demonstrated by their involvement in atherosclerosis or autoimmune diseases like MS.

**Abbreviations**

AFM, atomic force microscopy; EAE, experimental autoimmune encephalomyelitis; EC, endothelial cell; EGFR, epidermal growth factor receptor; FRAP, fluorescence recovery after photobleaching; FRET-FLIM, Förster resonance energy transfer-fluorescence lifetime imaging microscopy; ICAM-1, intercellular adhesion molecule 1; KO, knockout; LDL, low-density lipoprotein; LFA-1, lymphocyte function-associated antigen 1; MHC, major histocompatibility complex; MS, multiple sclerosis; MβCD, methyl-beta-cyclodextrin; PECAM-1, platelet endothelial cell adhesion molecule 1; STED, stimulated emission depletion; STORM, stochastic optical reconstruction microscopy; TM, transmembrane; TM4SF1, transmembrane-4-L-six-family-1; TSPs, tetraspanins; VCAM-1, vascular cell adhesion molecule 1.

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**Competing Interests**

The Authors declare that there are no competing interests associated with the manuscript.

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1006