Tspan18 is a novel regulator of thrombo-inflammation

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Abstract
The interplay between thrombosis and inflammation, termed thrombo-inflammation, causes acute organ damage in diseases such as ischaemic stroke and venous thrombosis. We have recently identified tetraspanin Tspan18 as a novel regulator of thrombo-inflammation. The tetraspanins are a family of 33 membrane proteins in humans that regulate the trafficking, clustering, and membrane diffusion of specific partner proteins. Tspan18 partners with the store-operated Ca2+ entry channel Orai1 on endothelial cells. Orai1 appears to be expressed in all cells and is critical in health and disease. Orai1 mutations cause human immunodeficiency, resulting in chronic and often lethal infections, while Orai1-knockout mice die at around the time of birth. Orai1 is a promising drug target in autoimmune and inflammatory diseases, and Orai1 inhibitors are in clinical trials. The focus of this review is our work on Tspan18 and Orai1 in Tspan18-knockout mice and Tspan18-knockdown primary human endothelial cells. Orai1 trafficking to the cell surface is partially impaired in the absence of Tspan18, resulting in impaired Ca2+ signaling and impaired release of the thrombo-inflammatory mediator von Willebrand factor following endothelial stimulation. As a consequence, Tspan18-knockout mice are protected in ischemia–reperfusion and deep vein thrombosis models. We provide new evidence that Tspan18 is relatively highly expressed in endothelial cells, through the analysis of publicly available single-cell transcriptomic data. We also present new data, showing that Tspan18 is required for normal Ca2+ signaling in platelets, but the functional consequences are subtle and restricted to mildly defective platelet aggregation and spreading induced by the platelet collagen receptor GPVI. Finally, we generate structural models of human Tspan18 and Orai1 and hypothesize that Tspan18 regulates Orai1 Ca2+ channel function at the cell surface by promoting its clustering.

Keywords Orai1 · Endothelial cell · Platelet · Store-operated Ca2+ entry · Tetraspanin · Thrombo-inflammation · Tspan18

Introduction
Thrombo-inflammation is the complex interplay between thrombosis and inflammation that can result in acute organ damage [1–3]. The process is best characterized in mouse models of ischaemic stroke and venous thrombosis. Central to thrombo-inflammation is the release of ultra-large adhesive strings of von Willebrand factor (VWF) from activated endothelial cells, in response to inflammatory mediators such as interleukin-1 (IL-1), IL-6, tumour necrosis factor α, thrombin, or histamine [2]. A proportion of the VWF strings remain attached to the endothelial cell surface and recruit platelets and leukocytes. The platelet GPIb-IX-V receptor complex mediates transient rolling of platelets on VWF, which may then firmly adhere if activated to induce adhesion of their αIIbβ3 integrins. Leukocytes similarly roll on VWF using their PSGL-1 receptor followed by stable adhesion via β2 integrins. Alternatively, leukocytes may be captured indirectly by platelets via interactions with platelet GPIb-IX-V or P-selectin [2]. In addition, monocytes can bind to VWF having first bound platelet-derived GPIb-IX-V-positive extracellular vesicles [4]. Together, platelet and leukocyte recruitment lead to further recruitment of inflammatory cells, platelet aggregation, and blockage of vessels causing organ damage [1–3].

VWF is stored by endothelial cells in cigar-shaped vesicles called Weibel–Palade bodies (WPBs). VWF is
constitutively released at a relatively low level by WPB exocytosis, but can be acutely exocytosed in larger quantities via a mechanism involving Ca²⁺ signaling following endothelial cell activation [5]. We have recently discovered that tetraspanin Tspan18 is important for endothelial cell Ca²⁺ signaling, acute VWF release, and thrombo-inflammation, by interacting with the store-operated Ca²⁺ entry channel Orai1 and promoting its trafficking to the cell surface [6]. This review will describe our current knowledge of Tspan18 expression and function, and will speculate on the molecular mechanism by which Tspan18 regulates Orai1.

The store-operated Ca²⁺ entry channel Orai1

Orai1 was identified as the long-sought-after store-operated Ca²⁺ entry channel through three publications in 2006 [7–9]. Ca²⁺ influx through Orai1 channels on the cell surface is termed store-operated Ca²⁺ entry, because it is induced by emptying of Ca²⁺ stores in the endoplasmic reticulum (ER). Orai1 has four transmembrane regions and intracellular N- and C-termini (Fig. 1) [10]. Structural studies suggest that Orai1 functions as a hexamer, forming a channel that mediates a characteristic Ca²⁺ release-activated Ca²⁺ current, termed I_{CRAC}. The I_{CRAC} is distinguished by high Ca²⁺ selectivity, a very low unitary conductance, with an inwardly-rectifying current-to-voltage ratio and a very positive reverse potential. The other Orai family members, Orai2 and Orai3, are approximately 60% identical to Orai1 by protein sequence. They are less well studied, but may form their own Ca²⁺ channels or combine with Orai1 to form heteromeric channels [10].

Orai1 appears to be expressed in all cells and is critical in health and disease processes. Indeed, Orai1-knockout mice die at around the time of birth, and Orai1 mutations in humans cause immunodeficiency, leading to chronic infections that are often lethal, and various non-immunological symptoms [11]. As previously mentioned, Orai1 channel opening is induced by emptying of ER Ca²⁺ stores [10]. Such emptying is the downstream consequence of signaling by cell surface receptors, including G protein-coupled receptors (GPCRs), receptor tyrosine kinases, and immunoreceptors such as B- and T-cell antigen receptors. Their stimulation activates phospholipase C, generating the second messenger IP₃, which induces Ca²⁺ release from IP₃ receptor Ca²⁺ channels on the ER. The release of Ca²⁺ is sensed by ER-localized STIM1 and STIM2 proteins, which undergo conformational change to engage with and activate Orai1 channels on the cell surface in characteristic clusters, or puncta. The influx of Ca²⁺ replenishes ER stores and, importantly, activates a variety of Ca²⁺-responsive signaling proteins to dictate cell function, such as changes in gene expression through activation of NFAT transcription factors [10].

The immunodeficiency observed in the absence of Orai1 has made this protein a promising drug target to treat diseases caused by an over-active immune system, such as autoimmune and inflammatory diseases [12, 13]. Indeed, five inhibitors of store-operated Ca²⁺ entry have recently entered clinical trials for diseases such as acute pancreatitis, asthma, non-Hodgkin’s lymphoma, and the skin disease plaque psoriasis [12, 13]. However, based on the phenotypes of Orai1-deficient humans and mice, there are concerns that inhibition of Orai1 on every cell in the body could lead to toxic side effects, particularly chronic immunosuppression.
and reduced muscle strength and endurance [13]. Hence, there is a need to more fully understand the mechanisms of tissue-specific Orai1 regulation, to explore whether novel, more selective inhibitory strategies might inhibit its function in disease without severe general toxicity.

### Tetrascapins regulate the trafficking and clustering of their partner proteins

The tetraspanins are a superfAMILY of membrane proteins with four transmembrane regions that interact with specific partner proteins, and regulate their trafficking to the cell surface and subsequent lateral mobility and clustering [14, 15]. Tetraspanins appear to exist in all multicellular organisms, from plants to fungi to mammals, and there are 33 tetraspanins in humans [16]. Examples of tetrascapin/partner protein complexes are tetrascapin CD151 and laminin-binding integrins such as α3β1 and α6β1 [17], tetrascapin CD81 and the B-cell receptor co-receptor CD19 [18], and Tspan12 and the Wnt/Norrin receptor Frizzled-4 [19]. The clustering of the partner appears to be the prominent role of CD151 and Tspan12 in these examples, whereas trafficking to the cell surface is the major impact of CD81 on CD19. In each case, tetrascapin deletion or mutation in humans and mice leads to phenotypes that resemble the deletion of the partner [17–19], thus providing genetic evidence for the importance of the interaction. In addition, we and others have shown that a subgroup of six tetrascapins, comprising Tspan5, 10, 14, 15, 17, and 33, and collectively known as the TspanC8s, interact with and regulate trafficking from the endoplasmic reticulum of the ‘molecular scissor’ a disintegrin and metalloprotease 10 (ADAM10) [20–23]. ADAM10 cleaves the extracellular regions from target proteins in a process termed ectodomain shedding. ADAM10 substrates number over 100 and include Notch cell fate regulators, amyloid precursor protein, cadherin adhesion molecules, growth factors, and chemokines. We have proposed a ‘six scissor’ hypothesis, which postulates that ADAM10 is not one scissor, but six different scissors with different substrates depending on which of the six regulatory TspanC8s it is associated with [20–22]. Substantial recent evidence supports this hypothesis [24–32].

Finally, tetrascapins have recently emerged as potential drug targets, following the discovery of the remarkable structure of CD81, the first tetrascapin structure to be solved by X-ray crystallography [33]. CD81 is cone-shaped with a cholesterol-binding cavity and is proposed to undergo a dramatic conformational change if cholesterol is removed [33]. This suggests that tetrascapins might function as ‘molecular switches’ to regulate their partners, but this has yet to be proved. If true, it may be possible to inhibit tetrascapin function using antibodies that lock the tetrascapin in a particular conformation. Alternatively, antibodies could inhibit by down-regulating tetrascapins or disrupting interactions with partners.

#### Tspan18 regulates Orai1/Ca^{2+} signaling on endothelial cells

We recently reported that Orai1 is regulated by Tspan18, a previously uncharacterized tetrascapin in mammalian cells [6]. Our work was initiated by a functional screen for effects of tetrascapins on Ca^{2+} signaling, which found that over-expression of Tspan18, but not other tetrascapins, activates the Ca^{2+}-responsive NFAT transcription factor by 20-fold in an Orai1-dependent manner [6]. Furthermore, Tspan18 co-immunoprecipitates with Orai1, but other tetrascapins do not. In the current absence of an effective Tspan18 antibody, we used RT-PCR to demonstrate Tspan18 expression in human endothelial cells, but not most other cell types. This was supported by our analyses of publicly available RNA-Seq data from mouse lung and brain, which showed that Tspan18 is most highly expressed in endothelial cells of these organs [6]. Analysis of single-cell RNA-Seq data from 20 mouse organs provides further support, since 9 of the 14 most highly Tspan18-expressing cell types are endothelial cells (Fig. 2) [34]. Tspan18 is not entirely restricted to endothelial cells, since there is notable expression in bladder cells and weaker expression in certain other cell types (Fig. 2).

We further showed that Tspan18 knockdown in primary human umbilical vein endothelial cells (HUVECs) impairs Ca^{2+} signaling by 55–70% following stimulation with the GPCR agonists histamine or thrombin, similar to Orai1 knockdown [6]. Orai1 surface localization is 70% reduced following Tspan18 knockdown, suggesting a role for Tspan18 in trafficking Orai1 from the ER. Tspan18-knockout mice are grossly normal and breed as both homozygote knockouts and heterozygotes, the latter yielding offspring at Mendelian ratios. However, the Tspan18-knockout mice display endothelial defects that yield partial protection in two thrombo-inflammatory models: a deep vein thrombosis model (60% reduction in thrombus size) and an ischemia–reperfusion injury heart attack model (50% reduced platelet deposition). Furthermore, there is a 90% and 50% reduction in agonist-induced release of VWF from endothelial cells in vitro and in vivo models, respectively [6]. Together, our data strongly suggest that Tspan18 interacts with Orai1 and regulates its trafficking to the endothelial cell surface, a process which is critical for normal endothelial cell Ca^{2+} signaling and consequent thrombo-inflammatory responses.

In addition to their thrombo-inflammatory phenotypes, Tspan18-knockout mice have a haemostasis defect, as
detected by increased blood loss in a tail bleeding assay [6]. Consistent with a role for Tspan18 in endothelial cells, this bleeding phenotype is the result of Tspan18 deletion from non-haematopoietic cells only; this was determined using wild-type/Tspan18-knockout fetal liver chimeric mice [6]. The underlying mechanism remains unclear, since there is no evidence that an acute release of VWF from endothelial cells following injury is critical for haemostasis. However, we hypothesize that in the context of the tail bleeding assay, activation of endothelial cells proximal to the tail cut would lead to Ca$^{2+}$ entry via Orai1 channels, followed by VWF release and platelet recruitment, such that cessation of bleeding would be quicker than in the absence of such VWF release. The haemostasis defect in endothelial-specific VWF-knockout mice [35, 36] would appear to support this hypothesis. However, interpretation of these data is complicated by the strikingly reduced VWF concentration in the plasma of these mice, which is thought to be responsible for increased bleeding [35, 36]. Plasma VWF concentration is normal in Tspan18-knockout mice [6], and thus, we believe that Tspan18/Orai1/Ca$^{2+}$ signaling is important for acute VWF
release following cell activation, in contrast to the relatively low-level release of VWF that is known to occur constitutively [5]. We propose that acute VWF release has a role in promoting haemostasis, but this will be challenging to test experimentally.

A recent study identifies the Tspan18 orthologue tspan18b as an endothelial-expressed gene in zebrafish, which is required for normal blood vessel development, but its mechanism of action was not investigated [37]. Since VWF is known to promote blood vessel development [38], we propose that impaired Orai1/Ca2+ signaling and impaired VWF release may be responsible for the phenotype in tspan18b-knockdown zebrafish. Thus, the role of Tspan18 in regulating Orai1/Ca2+ signaling in endothelial cells may be conserved across diverse vertebrate species.

It remains to be determined whether blood vessel development may also be impaired in Tspan18-knockout mice, although the grossly normal vasculature in adult kidney, pancreas, and ear [6] suggests that any developmental defect may only be subtle or delayed.

An emerging idea in the Orai1 field is that the extent of clustering (promoted by STIM proteins) affects the nature of the ensuing Ca2+ signal and downstream cellular responses [39, 40]. We hypothesize that Tspan18 provides an additional mechanism of Orai1 clustering in endothelial cells, and that Tspan18/Orai1 complexes may create a Ca2+ channel with membrane dynamics and electrophysiological properties that are distinct from Orai1 alone. A predicted structural model of Tspan18, based on the crystal structure of CD81 [33], is shown alongside the crystal structures of the Orai1 channel in closed and open conformations, to show the comparative dimensions of the proteins (Fig. 1); it has yet to be determined if Tspan18 interacts with higher affinity to the closed or open forms. Mechanistically, Tspan18 may pre-cluster Orai1 channels, resulting in stronger, more rapid Ca2+ signaling in response to agonists, which may be critical for endothelial cell function in blood vessel growth and in thromboinflammation. Furthermore, the proposed function of tetraspanins as molecular switches [33] might enable Tspan18 to modulate Orai1 via conformational change. We believe that Tspan18 is a potential drug target for the treatment of endothelial-driven inflammatory diseases, cancer angiogenesis, and retinal neovascularisation, which is the most common form of vision loss in the western world. Importantly, targeting Tspan18 may enable Orai1 inhibition in a relatively endothelial-specific manner, so avoiding the likely toxicity that would result from inhibiting this important Ca2+ channel on all cells in the body. Targeting tetraspanins is not without precedent, since an antibody to tetraspanin CD37 is in clinical trials for chronic lymphocytic leukaemia [41].

A subtle role for Tspan18 in platelet Ca2+ signaling and aggregation

In addition to the cell types included in Fig. 2, Tspan18 has been detected in human platelets by proteomics [42, 43], and in human and mouse platelets and megakaryocytes at the mRNA level [44–46]. Platelets have a pivotal role in haemostasis, but the bleeding phenotype in the Tspan18-knockout mice is not due to platelet dysfunction, because the experiments with fetal liver chimeras showed that this is a result of Tspan18 deletion in non-haematopoietic cells [6]. Furthermore, Tspan18-knockout platelets aggregate normally in response to the platelet agonists collagen or thrombin [6].

To investigate whether Tspan18 has some role in platelet Ca2+ signaling, platelets were loaded with the Ca2+-sensitive dye Fura-2-AM. Platelets were stimulated with collagen-related peptide, a specific agonist for the collagen receptor GPVI which activates platelets via non-receptor tyrosine kinase signaling. Intracellular Ca2+ concentrations were then monitored with a fluorimeter. The rate of cellular Ca2+ accumulation and the maximal Ca2+ concentration after 10 min were significantly reduced in Tspan18-knockout platelets (Fig. 3a). A similar result was obtained following stimulation with collagen (Fig. 3b). No significant effect was observed following stimulation with thrombin, which activates platelets via a GPCR (Fig. 3c). To assess store-operated Ca2+ entry, platelets were treated in the absence of extracellular Ca2+ with thapsigargin, which inhibits sarco/endoplasmic reticulum Ca2+-ATPase channels to cause Ca2+ store emptying. A reduction in the rate of release and maximal Ca2+ increase was observed in Tspan18-knockout platelets (Fig. 3d). Subsequent addition of extracellular Ca2+ to the platelets, allowing store-operated Ca2+ entry to occur, revealed impaired Ca2+ entry in the absence of Tspan18 (Fig. 3e).

To further explore a potential functional role for Tspan18 in platelets, in vitro aggregation assays were employed. Defective aggregation of Tspan18-knockout platelets was observed in response to the collagen-related peptide at the intermediate concentration (Fig. 4a), which was partially rescued at high concentration (Fig. 4b). However, aggregation induced by the physiological agonist for GPVI, collagen, which also binds α2β1 integrin, was normal for Tspan18-knockout platelets (Fig. 4c, d), as we previously reported [6]. Aggregation was also normal when induced by threshold levels of other platelet agonists, such as anti-CLEC-2 antibody, thrombin, or ADP (Fig. 4e–h). To determine whether this GPVI-specific aggregation defect extends to platelet spreading, platelets were plated on glass coverslips coated with collagen-related peptide or fibrinogen; the latter binds to αIIBβ3, the
Fig. 3 GPVI-induced Ca\(^{2+}\) signaling and store-operated Ca\(^{2+}\) entry is reduced in Tspan18-knockout platelets. Washed platelets from Tspan18\(^{+/+}\) and Tspan18\(^{-/-}\) mice were loaded with the Ca\(^{2+}\) sensitive dye Fura-2 AM and Ca\(^{2+}\) measurements taken using a luminescence spectrophotometer. Collated data were fitted to an exponential one-phase association equation, shown in the left-hand panels, and maximal Ca\(^{2+}\) concentration was calculated, shown in the right-hand column. Platelets were stimulated with a 1 μg/ml collagen-related peptide (CRP), b 3 μg/ml collagen, and c 0.06 units/ml thrombin. d Platelets were treated in the absence of extracellular Ca\(^{2+}\) with thapsigargin (TG) to induce emptying of the intracellular Ca\(^{2+}\) stores. e The experiment in panel (d) was extended by the addition of 1.5 mM Ca\(^{2+}\) to the extracellular buffer. The rate constants were compared using an f test (*** denotes \(P < 0.001\)). Maximal Ca\(^{2+}\) concentrations were analysed by t test (* denotes \(P < 0.05\); ns denotes not significant). Error bars represent the standard error of the mean from 2–6 independent experiments. Detailed methods are available online (https://etheses.bham.ac.uk/id/eprint/5903/)

Additional roles for Tspan18: chick embryonic development and links to schizophrenia

Before our recent characterization of Tspan18, the only functional studies on Tspan18 were in chick embryos, in which Tspan18 is required for neural crest cell migration, albeit without a major effect on embryonic development [50, 51]. Neural crest cells are a population of cells that arise
transiently during vertebrate development from the ectoderm germ layer. They undergo epithelial-to-mesenchymal transition to migrate and differentiate into a diverse range of cell types. Tspan18 mRNA is expressed in pre-migratory neural crest cells, but is down-regulated when the cells migrate, and forced Tspan18 over-expression prevents migration [51]. There is no evidence that Tspan18 is regulating Orai1/Ca\(^{2+}\) signaling in this process. Instead, Tspan18 expression stabilizes the expression of the cell–cell adhesion molecule cadherin 6 at the protein level; down-regulation of cadherin 6-mediated cell–cell interactions is important to allow the cells to disengage from each other and migrate [51]. It has yet to be investigated if Tspan18 regulates cadherin 6 in mammalian cells. Cadherin 6 does not appear to be expressed in endothelial cells in mice [34]. Indeed, cadherin 6 is not expressed by most normal cell types, and is best

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**Fig. 4** Tspan18-deficient platelets have a specific aggregation defect downstream of GPVI. Mouse platelets were isolated from whole blood taken from Tspan18\(^{+/+}\) and Tspan18\(^{-/-}\) mice. Washed platelets were activated with a 3 µg/ml or b 10 µg/ml of the GPVI-specific agonist collagen-related peptide (CRP), c 1 µg/ml or d 3 µg/ml collagen, e 1 µg/ml or f 10 µg/ml CLEC-2 antibody, or g 0.03 units/ml thrombin. h Platelets in PRP were activated with 1 µM ADP. Aggregation was measured via light transmission with stirring. Representative traces are shown in the upper panels and quantitated % aggregation per minute shown in the lower panels. A two-way ANOVA was performed on arcsine-transformed data (* denotes \(P<0.05\), ** denotes \(P<0.01\) and *** denotes \(P<0.001\)). Error bars represent the standard error of the mean from 3–9 pairs of mice. Detailed methods are available online (https://etheses.bham.ac.uk/id/eprint/5903/)
studied for its expression on renal, ovarian, and thyroid cancers [52]. One cell type in which Tspan18 and cadherin 6 are co-expressed is the platelet. Cadherin 6 blocking strategies suggested a subtle role for cadherin 6 in platelet aggregation via interaction with the major platelet integrin αIIbβ3 [53], but a physical and functional interaction between cadherin 6 and Tspan18 on platelets has yet to be explored.

A genome-wide association study on schizophrenia in the Han Chinese population implicated three single-nucleotide polymorphisms in the 5′ untranslated region of Tspan18 [54]. However, this remains controversial, because these polymorphisms were not replicated in all follow-up studies [55–58]. Nevertheless, altered Tspan18 expression in brain endothelial cell or other brain cell types could affect Orai1/Ca$^{2+}$ signaling and impact brain function and neurological disorders such as schizophrenia. For instance, Tspan18 on endothelial cells could be important for brain function, since endothelial dysfunction in the brain and inflammation is linked to neurological disorders [59, 60]. Alternatively, Tspan18 on microglial cells (Fig. 2) could impact on their capacity to mediate synaptic pruning, an excess of which was recently linked to schizophrenia [61].
Résumé and outlook

In summary, Tspan18 is important in endothelial cells for the cell surface localization of Orai1, Ca\(^{2+}\) signaling, and VWF release. As a result, Tspan18 deficiency is protective in thrombo-inflammatory models of venous thrombosis and ischemia–reperfusion injury in the heart. Ca\(^{2+}\) signaling and VWF are both critical to endothelial function in haemostasis, inflammation, blood vessel growth, and angiogenesis [5, 38]. Future work should investigate the importance of Tspan18 in these processes using mouse models of atherosclerosis, ischaemic stroke, and angiogenesis. Generation of Tspan18 monoclonal antibodies will also be important to determine the expression profile of Tspan18 at the protein level. Moreover, such antibodies may have inhibitory activity and, thus, therapeutic potential for thrombo-inflammatory diseases such as deep vein thrombosis, retinal neovascularisation, and cancer angiogenesis to deprive a growing tumour of its blood supply. Finally, it will be interesting to investigate the mechanism by which Tspan18 facilitates Orai1 trafficking to the endothelial cell surface. Once at the cell surface, it will be important to determine whether and how Tspan18 regulates Orai1 clustering and/or channel opening. We may then understand why a process as fundamental as store-operated Ca\(^{2+}\) entry is modified by Tspan18 in cells such as endothelial cells, but not in others.

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Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interest.

Ethical approval Mouse platelet experiments were approved by the United Kingdom Home Office according to the Animals (Scientific Procedures) Act 1986.

![Fig. 6 GPVI-induced protein tyrosine phosphorylation is normal in Tspan18-deficient platelets.](image-url)
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