TNF-induced endothelial barrier disruption: beyond actin and Rho
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Summary
The decrease of endothelial barrier function is central to the long-term inflammatory response. A pathological alteration of the ability of endothelial cells to modulate the passage of cells and solutes across the vessel underlies the development of inflammatory diseases such as atherosclerosis and multiple sclerosis. The inflammatory cytokine tumour necrosis factor (TNF) mediates changes in the barrier properties of the endothelium. TNF activates different Rho GTPases, increases filamentous actin and remodels endothelial cell morphology. However, inhibition of actin-mediated remodelling is insufficient to prevent endothelial barrier disruption in response to TNF, suggesting that additional molecular mechanisms are involved. Here we discuss, first, the pivotal role of Rac-mediated generation of reactive oxygen species (ROS) to regulate the integrity of endothelial cell-cell junctions and, second, the ability of endothelial adhesion receptors such as ICAM-1, VCAM-1 and PECAM-1, involved in leukocyte transendothelial migration, to control endothelial permeability to small molecules, often through ROS generation. These adhesion receptors regulate endothelial barrier function in ways both dependent on and independent of their engagement by immune cells, and orchestrate the crosstalk between leukocyte transendothelial migration and endothelial permeability during inflammation.

Keywords
Endothelial barrier function, permeability, adhesion, transendothelial migration, Rho, Rac, Cdc42, ROCK, reactive oxygen species, Src, Fyn, actin, VE-cadherin, ICAM-1, VCAM-1, JAM, PECAM-1, SHP phosphatases, metalloproteases

Introduction
The inflammatory response is a tightly regulated process involved in pathogen clearance and the repair of damaged tissue. It constitutes an alteration of tissue homeostasis in reaction to stress, which is also detrimental to tissue function. An accurate spatiotemporal restriction of the inflammatory response is therefore crucial for preventing uncontrolled, systemic reactions that would be more harmful than the initial pathological challenges (1).

Blood and lymphatic vessels enable tissue compartmentalisation during the initial stages of the innate and adaptive immune responses and provide spatial constraints required during inflammatory reactions. The basal extravasation of immune cells that survey the body is locally increased in the blood vessels surrounding an inflammatory focus. This increase occurs through stimulation of the endothelium by inflammatory factors secreted by activated, tissue-resident immune cells. Proinflammatory stimulation increases both endothelial permeability to small molecules, such as chemokines and cytokines, and the expression of receptors that mediate the endothelial adherence to immune cells. As a result, the leukocyte transendothelial migration towards the inflamed areas transiently increases. Excessive inflammatory activation of endothelial cells leads to aberrant endothelial barrier disruption, inducing local swelling and edema formation. Chronic, unresolved endothelial inflammation that prolongs hyperpermeability underlies the origin of diseases such as atherosclerosis and multiple sclerosis, which adds further interest to the study of the mechanisms regulating endothelial barriers.

Among the plethora of inflammatory mediators that contribute to endothelial barrier disruption, the tumour necrosis factor (TNF) is central to the initiation and termination of long-term inflammatory responses (2). TNF signals to the actin cytoskeleton by promoting a notable morphological remodelling of endothelial cells, which has been extensively investigated but whose inhibition is not enough to prevent the progressive loss of endothelial barrier properties in response to this cytokine. Here we review additional molecular pathways, emerging as critical mediators of the transient disruption of endothelial barriers in response to TNF. They include the disruption of cell-cell junctions by reactive oxygen species (ROS) and the signalling by adhesion receptors involved in leukocyte-endothelial interactions. This signalling occurs partly through ROS generation and reveals a remarkable molecular...
The cytokine TNF in the inflammatory response

TNF, also known as TNFα, is a cytokine with pleiotropic effects in many cell types that is expressed as a type II transmembrane protein mainly by macrophages, dendritic cells and T-cells (3, 4). Upon arrival at the plasma membrane, some of the TNF molecules are shed by the metalloproteinase TNFα-converting enzyme (TACE), also known as a disintegrin and metalloproteinase-17 (ADAM-17), and released as a soluble homotrimer of 17 KD (5, 6). TNF binds to two type I transmembrane receptors, TNFR1 and TNFR2, which are homologous in their extracellular domain but quite divergent with respect to the cytoplasmic segments that recruit different signalling machinery. TNFR1 is ubiquitously expressed whereas TNFR2 is expressed only in immune cells and endothelium (7). Both receptors lack intrinsic catalytic activity and require assembly of supramolecular complexes of cytosolic proteins to transduce signals. A detailed description of the signalosome recruited by both receptors upon TNF interaction and the subsequently elicited signalling pathways, has recently been reviewed elsewhere (7-10). Briefly, the activation of inhibitor of kappa-B (IkB) kinases (IKKs) and their association with TNFR signalling complexes are amongst the most important events of the TNF signalling cascade. IKKs are the kinases that phosphorylate the IkBα, leading to its ubiquitination and degradation. The absence of IkBα exposes the nuclear translocation sequence of the p65 subunit of the nuclear factor (NF)-κB transcription factor, which enters the nucleus and regulates a plethora of genes associated with long-term inflammatory responses (11). TNF signalling complexes also activate the stress activated protein (SAP) kinases, p38 MAP kinase and the Jun N-terminal kinase (JNK), which phosphorylate c-Jun. Phosphorylated c-Jun is a component of the heterotrimeric transcription factor AP-1, which becomes activated and regulates another set of genes (12). In addition, TNFR1 engagement can induce apoptosis by activating Caspase8, and necrosis by assembling a multiprotein complex called necrosome (13). The variation in composition of the TNFR signalosomes determines the relative induction of each pathway and the final outcome of TNF-mediated signalling. As a consequence, depending on the cell type and the physiological context, TNF activates different subsets of target genes that promote diverse cellular effects such as survival, apoptosis, necrosis, migration, proliferation, barrier disruption and cell–to-cell adhesion.

TNF signals to the actin cytoskeleton and remodels cell-cell junctions through Rho GTPases in endothelial cells

In addition to the stimulation of these canonical pathways that lead to gene expression changes, TNF signals to the actin cytoskeleton by activating Rho GTPases. Rho GTPases cycle between a GTP-loaded active form and a GDP-bound inactive conformation (14). Rho GTPases are activated by guanine nucleotide exchange factors (GEFs) and inactivated by GTPase-activating proteins (GAPs) and GDP dissociation inhibitors (GDIs). GDIs form an inactive complex with the Rho GTPase in the cytosol. The local combination of these regulators spatially confines and confers specificity on Rho-mediated signalling. Due to the central role that this family plays in regulating cellular barriers, the TNF-mediated signalling to Rho GTPases has been of great interest in the study of the endothelial inflammatory response. So far, it has been established that TNF activates RhoA, Rac and Cdc42, the three best known of the 22 members of the Rho family and the master regulators of filamentous actin (15, 16, 17-22). The molecular connection between TNFR engagement and Rho GTPase activation is still poorly understood. Various GEFs have been reported to be activated downstream of TNF, mostly in non-endothelial cell types, although the underlying molecular interactions still need to be explored. In addition, taking into account the similarities and functional cooperation found between Rho family proteins in different physiological scenarios, the involvement of other members of the family is quite probable and should be investigated in detail in the future.

TNF induces actin stress fibres through RhoA and ROCK

One of the main effects of TNF in endothelial cells is their morphological remodelling through the reorganisation of actin cytoskeleton, which has been observed in vivo (23) and in vitro (20, 24, 25). In response to this cytokine, the endothelium turns from a pavement-like monolayer, containing a peripheral actin belt close to stable cell-cell junctions, to a monolayer formed by elongated cells enriched in actin stress fibres (20, 24, 25). The formation of stress fibres is a paradigmatic outcome of RhoA-mediated signalling to Rho kinase (ROCK) (26, 27). ROCK phosphorylates and inhibits myosin light chain phosphatase, which promotes myosin light chain (MLC) phosphorylation, which causes the assembly of these filaments and actomyosin contractility (28). ROCK can directly phosphorylate MLC, at least in in vitro assays (29). Since actomyosin contraction is a major mechanism of cell barrier disruption, it was hypothesised early on that this signalling pathway was pivotal to TNF-mediated endothelial morphological reorganisation and long-term barrier alteration. However, experimental evidence has confirmed this only for the case of endothelial morphological remodelling. As we shall see below, the central role of Rho-ROCK in long-term barrier function decrease in response to TNF is unclear.
Stress fibres are connected to adherens junctions in a ROCK-dependent manner in TNF-stimulated endothelial cells

TNF transiently activates RhoA and its main effector ROCK in different cell types, including macrovascular and microvascular endothelial cells (17, 22, 30-32). Following gene silencing with siRNA, Mong and Wang (33) demonstrated in microvascular endothelial cells that ROCKI and ROCKII isozymes have an additive role in increasing MLC phosphorylation in response to TNF. However, ROCKII is the major Rho kinase activating actomyosin during long-term cytokine stimulation. Short-term pharmacological inhibition of ROCK or Rho has confirmed the critical role of ROCK in incrementing stress fibres in response to TNF (17, 30, 34).

Endothelial adherens junctions (AJs) are multiprotein junc-tional complexes formed by the vascular endothelial (VE)-cadherin. VE-cadherin complexes establish cell-cell homotypic interactions that are fundamental for maintaining endothelial barrier integrity (35). The VE-cadherin cytoplasmic tail nucleates a protein complex formed by p120-catenin, β-catenin and α-catenin, the latter linking the complex to actin filaments. Actin binding is required to stabilise cell-cell junctions, but also regulates AJ dynamics in response to soluble mediators or transendothelial cellular migration. The most detailed analysis of AJ dynamics comes from in vitro studies. In resting microvascular and macrovascular endothelial cells, AJs exhibit a heterogeneous morpholory that includes areas apparently devoid of F-actin (22). In contrast stress fibres induced by TNF remodel AJs, which become discontinuous and aligned with these actin microfilaments (Figure 1). As expected, the formation of discontinuous AJs requires ROCK activity. Moreover, discontinuous AJs often connect stress fibre ends coming from adjacent endothelial cells (34). Intriguingly, the stress fibre increase occurring in response to TNF is not accompanied by a comparable rise in the number of focal adhesions (FAs), the adhesive protein complexes that stabilise and attach these filaments to the plasma membrane and the extracellular matrix. Indeed, abrogating AJs through VE-cadherin knockdown does not significantly diminish the number of stress fibres, but is sufficient to increase the number of FAs and alter cell morphology in confluent, TNF-stimulated endothelial cells (34). These data suggest that discontinuous AJ stabilise tension-bearing, force-generating stress fibres, independently of FAs, in TNF-stimulated endothelial monolayers (34, 36). This connection participates in the actin-mediated morphological remodelling of endothelial cells upon TNF exposure.

Rac regulates TNF-mediated actin and junction remodelling

Rac is involved in endothelial barrier stabilisation and mediates the barrier-protective effects of various soluble mediators. Rac GTPase controls F-actin and often plays a role antagonistic to that of RhoA. RhoA and Rac activities are inversely correlated throughout TNF stimulation. Rac activity increases during the first minutes of TNF activation (21, 37) but decreases during long-term stimulation (32, 38). Moreover, TNF-mediated Rac activation probably depends on the stimulatory conditions and the vascular cells analysed, since other authors have convincingly reported sustained activation of this GTPase up to 20 hours post-stimulation (39). The F-actin increase caused by TNF and the subsequent redistribution of cell-cell junctions is altered by the expression of a constitutive active mutant of Rac1, but also by the expression of a dominant-negative form (20). In addition, Rac1 gene silencing significantly diminishes TNF-mediated cell elongation, one of the main morphological traits of the endothelial response to this cytokine (40). Collectively, these results suggest a role for this GTPase as a mediator of endothelial cell remodelling upon TNF beyond that of being a mere antagonist of RhoA function.

Cdc42 activity contributes to F-actin induction upon TNF stimulation

The contribution of Cdc42 to TNF-mediated signalling has been less extensively investigated than that of RhoA and Rac. Cdc42 is activated a few minutes after TNF exposure (21, 39) and mediates the prosurvival signalling (41) and the cytoskeletal remodelling induced by this cytokine (19, 20). Cdc42 is considered to be a stabilising mediator of endothelial barriers and to play an important role in maintaining endothelial AJs (42), although its role in cell-cell junction remodelling in the context of TNF stimulation remains to be established.

TNF-mediated increase of endothelial permeability: beyond actin and Rho

Experiments performed in macrovascular endothelial cells with specific siRNA or inhibitors reveal a cooperative role of ROCKI and ROCKII in the homeostatic maintenance of the endothelial barrier, which is disrupted upon double ROCK knockdown in the absence of inflammation (43). There is also evidence from the use of inhibitors, expression of dominant-negative mutants and siRNA that demonstrates the essential role of the Rho-ROCK pathway during acute contraction induced by mediators such as thrombin and histamine (43-45). However, the function of Rho and ROCK-mediated actin remodelling in TNF-induced barrier disruption is more controversial. As mentioned above, the endothelial cytoskeletal and junctional reorganisation induced by TNF is mediated by Rho, ROCK and Rac. However, inhibiting such reorganisation is not always sufficient to prevent endothelial barrier disruption. ROCK inhibition reduces the permeability increase within the first minutes of TNF-stimulation in microvascular endothelial cells (33). Several reports have clearly shown that inhibition of actin polymerisation through Rho-ROCK and of myosin is not enough to impair microvascular and macrovascular barrier disruption after several hours of TNF exposure (17, 22, 32, 33, 46). This is in agreement with the fact that stress fibres generated by long-term TNF stimulation have little myosin activity, suggesting that these filaments became stabilised but generate little tension in the ab-
sence of further stimulation (17). Similarly, single Rac1 knock-down promotes a clear reduction of endothelial cell elongation in response to TNF, but has a minor effect on permeability increase and leukocyte transendothelial migration (40). Hence, the lack of correlation between actin-mediated remodelling and alteration of barrier function clearly suggests that, in addition to actomyosin-mediated contractility, there must be mechanisms that promote long-term barrier disruption in response to this cytokine. The research focus on endothelial permeability has been directed towards the actin-independent roles of Rac and the expression or re-localisation of adhesion receptors involved in leukocyte transmigration. The latter emerge as central players in the molecular mechanisms mediating endothelial permeability increase upon TNF stimulation.

**RAC and ROS in TNF-induced endothelial permeability**

In contrast to the minor effect that Rac1 knockdown has on endothelial permeability, expression of the Rac dominant negative mutant Rac-T17N or incubation with the Rac inhibitor
NSC-23766 completely abrogates the TNF-mediated decrease of TEER in human lung microvascular endothelial cells (47, 48, 49). Mutants and inhibitors, both alter the function of at least Rac1, Rac2 and Rac3, the closely related and members of the Rac GTPIase subfamily. This suggests that whereas Rac1 significantly contributes to TNF-induced endothelial actin and morphological remodelling (40), it overlaps in regulating barrier function through additional mechanisms with the other two members of the subfamily, which are also expressed in endothelial cells. Upstream of Rac, the Rac GEFs Vav, Tiam-1, Trio and P-Rex, are activated in response to TNF (37, 39, 40). Vav activation regulates canonical signalling pathways mediated by this cytokine and has only been reported in non-endothelial cell types (21). T cell lymphoma invasion and metastasis (Tiam)-1 associates with VE-cadherin upon TNF stimulation (40). In other stimulatory contexts, Tiam-1 is essential, together with Vav-2, for Rac-mediated regulation of endothelial barrier function (50-53). Nonetheless, the roles of Tiam-1 and Vav-2 in TNF-induced barrier modulation still need to be addressed. Trio expression and association with active Rac are increased in response to TNF. Unlike Tiam-1, Trio does not specifically regulate endothelial barrier function but instead has a role in activating Ets-2, a transcription factor that induces VCAM-1 and ICAM-1 expression (39). As detailed below, VCAM-1 and ICAM-1 regulate endothelial permeability, so it is likely that Trio regulates endothelial barrier properties via these receptors. Finally, phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger (P-Rex)-1 also plays a general role in TNF stimulation regulating not only barrier function but also NFkB signalling and adhesion receptor expression. However, to date, there has been no comparative study of the relative contribution of each of these GEFs to TNF-induced endothelial barrier disturbance (37, 40).

Rac GTPIases play an important role as a component of the active NADPH oxidase (NOX) complex, which is the main source of ROS in endothelial cells. The endothelium expresses various NOX complexes, namely those formed by NOX1, NOX2, NOX4 and NOX5 (54). In HUVECs, the most widely used human endothelial cell system, NOX2 and NOX4 are the most abundant complexes (55). The phagocyte NOX2 complex is the best characterised endothelial NOX and is formed by the catalytic gp91phox (NOX2) subunit associated with the regulatory p22phox. The cytosolic subunits p40phox, p47phox and p67phox, and Rac form the active complex that transfers electrons from NADPH to oxygen to form superoxide (O2-) (54, 56, 57). Rac also binds to and regulates the NOX1 complex, which contains different cytosolic subunits, but does not regulate either NOX4, which is constitutively active (58), or NOX5, which is regulated by calcium (59). The TNFR1 signalosome recruits the NOX1 complex, including active Rac, upon TNF interaction, which mediates sustained Jnk activation and necrosis in non-endothelial cells (60). Alternatively, ROS can be directly activated by the TNF signalosome component TRAF4, which binds to p70phox and is required for the full TNF-mediated ROS induction in murine microvascular endothelial cells (61). In addition, TNF increases the membrane localisation of NOX2 and Rac1 in HUVECs (55). In human colon adenocarcinoma cells, the mixed-kinase domain-like (MLKL) (62) is associated with the necrosome complex and is also required for ROS induction and late Jnk activation in response to TNF. However, neither the role of MLKL in ROS production, nor the possible contribution of Rac to TRAF4- or MLKL-mediated ROS formation, have yet been addressed in endothelial cells.

In human endothelial cells, P-Rex knockdown, expression of dominant-negative Rac and specific Rac inhibitors abrogate ROS production in response to TNF (37). The expression of a constitutively active mutant of Rac is also sufficient to increase ROS production. One of the main effects of elevated ROS production is the oxidation of several residues from phosphatases and kinases, thereby controlling the main system of cellular signalling (63). ROS oxidise various amino acids such as those with aromatic residues, large and hydrophobic, which often causes conformational changes in the proteins. ROS also induce irreversible carboxylation and/or racemisation of various amino acids that direct proteins for degradation (63). However, the sulfur-containing amino acids cysteine and methionine are the most prone to oxidation. Importantly, their oxidation is reversible, which makes this modification important in signal transduction. In Src-family kinase members (64, 65), elevated ROS induces two cysteine residues, Cys245 and Cys487 in the case of Src kinase (66), to form intramolecular disulfide bridges upon oxidation, which lock the kinase in an active conformation taken it to a super activation state. The nucleophilic chain of cysteines can be oxidised in three states sulfenic (SOH), sulfinic (SO2H) and sulfonic (SO3H). The sulfenic acid state is able to form a disulfide bond with other cysteine residue in the vicinity. The cellular antioxidants glutathione or thioredoxin can reverse this state of cysteine oxidation, but further oxidative stress can lead to the formation of sulfonic or sulfonylic states that become irreversible (63). Hyperoxidation of Src kinases cause enzyme inactivation and results in their final degradation (63). Indeed, oxidation of Src Cys277, which causes the formation of homodimers and results in Src inhibition, has also been described (67). ROS can also indirectly control Src kinases by oxidising phosphatases such as SH2-containing protein tyrosine phosphatase-1 and -2 (SHP-1 and SHP-2), upstream regulators of Src (64). It is of note that the effect of TNF on ROS-mediated oxidation of Src at molecular level has not been studied yet. Rac-induced ROS regulate endothelial permeability by elevating the tyrosine phosphorylation of VE-cadherin (68), which prevents the binding of β-catenin and p120-catenin and results in inhibition of endothelial barrier function (37, 69, 70). Exposure to tyrosine kinase inhibitors blocks TNF-induced endothelial permeability, illustrating the importance of tyrosine phosphorylation in this process (70, 71).

Src-family kinases can phosphorylate not only VE-cadherin, but also β-catenin, p120-catenin and other junctional molecules such as PECAM-1 (40, 71, 72, 37, 54, 63). Amongst the Src kinase members, some authors propose that Fyn, but not Src or Yes, are fundamental to TNF-mediated barrier disruption and phosphorylation of VE-cadherin, γ-catenin and p120-catenin, at least in human lung microvascular endothelial cells (71). Alternatively, ROS-regulated tyrosine kinases other than Src, such as Pyk2 (73),...
also associate with and regulate the phosphorylation of VE-cadherin and AJ disruption in response to TNF (40). The closest relative of Pyk2, FAK, is also activated by TNF in non-endothelial cell types (74-76). FAK regulates endothelial barriers in response to other soluble mediators such as thrombin, SIP or VEGF (77, 78, 79) making plausible its involvement in TNF-mediated increase in permeability. In addition, FAK and Pyk2 autophosphorylation recruit Src kinases (80, 81), so both families of kinases may have a cooperative effect on cell-cell junctions. Together, these data imply that whereas an increase in Rho-ROCK activity and F-actin promotes morphological changes in the endothelium upon TNF exposure, the Rac-ROS-Src/Pyk2 kinase axis is responsible for the loss of barrier function through the phosphorylation and destabilisation of cell-cell junction complexes (Figure 2).

Control of endothelial permeability by TNF-regulated adhesion receptors

The molecular mechanisms that regulate the passage of immune cells through the endothelium have been thoroughly investigated in the last 25 years, although the differences found between heterogeneous vascular beds and leukocyte subsets, as well as between different inflammatory challenges, are still not fully understood (82-84). In the absence of inflammation, leukocyte TEM is tightly controlled to the extent that only a small subset of immune cells are able to extravasate, acting as “immune guards” and helping to transmit information to the adaptive immune system. In contrast, in inflammatory foci, TNF secretion activates a transcriptional program in the neighbouring endothelium that includes a dramatic increase of chemokine release and the expression of adhesion receptors. These receptors include the lectin E-selectin (85) and the immunoglobulin superfamily members ICAM-1 and VCAM-1, which are the ligands of β2 and β1 integrins, respectively (86-88). These expression changes mediate the local increase in leukocyte adhesion to endothelial cells and the subsequent leukocyte infiltration of the inflamed tissues. It is of note that TNF not only increases the expression of some receptors, but also changes the localisation of some others. For example, TNF favours initial endothelial cell–leukocyte interactions, called tethering and rolling, not only by increasing E-selectin, but also by inducing the translocation of P-selectin from the Weibel–Palade bodies to the plasma membrane (85, 89). These initial contacts and the interaction with surface chemokines mature leukocyte adhesions through changes in the activation state of the mentioned integrins, which adhere firmly to VCAM-1 and ICAM-1. Arrested immune cells then crawl on the endothelium and transmigrate between two endothelial cells (paracellular diapedesis) or through transcellular channels away from cell-cell junctions (transcellular diapedesis) (90). In the paracellular diapedesis, leukocytes encounter another subset of adhesion receptors, such as PECAM-1, CD99 and junctional adhesion molecules (JAMs) that guide cells across the endothelium. Again, TNF does not modulate the expression of these junctional receptors, but determines their localisation, at least in the cases of PECAM-1 (91, 22) and JAM-A (92). In addition to their role in paracellular diapedesis, the adhesion receptors ICAM-1 and PECAM-1 help guide cells that follow transcellular diapedesis (93-95). Thus, TNF changes expression and localisation of receptors that participate in the different steps of the cascade of interactions between immune cells and the endothelium. The rise in endothelial permeability to small molecules parallels leukocyte TEM during the inflammatory course. In recent years, evidence has accumulated that points to an additional role for adhesion receptors in controlling endothelial permeability, a role that, it is important

Figure 2: TNF induces endothelial barrier disruption through Rac activation and ROS generation. TNF activates Rac and generates reactive oxygen species (ROS) through NOX complexes. Rac and ROS activates different tyrosine kinases and phosphatases that contribute to phosphorylate and destabilise adherens junctions. Rac also mediates the increase of adhesion receptor expression via Ets-2 and NF-κB.
to note, is both dependent on and independent of their interactions with leukocyte counterreceptors.

**Intercellular adhesion molecule-1 (ICAM-1) and endothelial barrier regulation in response to TNF**

TNF increases ICAM-1 expression more than 15-fold in human umbilical vein endothelial cells (HUVECs) *in vitro* (96). In endothelial cells, TNF-1 induces ICAM-1 expression mainly via TNFR1 (97, 98). The DNA region upstream the ICAM-1 translation start site, involved in the translational increase of ICAM-1 mediated by TNF and other cytokines, has been well mapped and consist on variant NF-kB binding site flanked by other regulatory sequences (99). NF-kB/Rel complexes can be composed of different subunits that in turn can bind other regulatory factors. The transcriptional regulation of ICAM-1 in different cell types through NF-kB have been extensively reviewed elsewhere (99, 100). In HUVECs, the RelA homodimer and NF-kB1/RelA heterodimer bind to the NF-kB binding site of ICAM-1 (101). Over expression of RelA activated ICAM-1 promoter, whereas NF-kB1 overexpression reduces such transactivation (101). In human lung microvascular endothelial cells, TNF also regulates ICAM-1 expression post-transcriptionally by controlling mRNA stability through the p38 mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MK2) pathway (102, 103).

ICAM-1 is the counter receptor of leukocyte β2 integrins. ICAM-1 is a type I transmembrane receptor of the immunoglobulin (Ig) superfamily that contains five extracellular Ig domains. In TNF-stimulated endothelium, ICAM-1 localises in plasma membrane microvilli and cell-cell junctions (104-106). The ICAM-1 cytoplasmic tail interacts with proteins that connect the receptor to subcortical filamentous actin. Artificial overexpression by retroviral transduction of ICAM-1 at levels comparable to those induced by TNF is sufficient to increase endothelial permeability significantly. Interestingly, these levels of expression do not affect the ratio of filamentous to globular actin and exert a moderate effect on tight junctions, suggesting an actin-independent effect of the receptor on permeability (107). Higher levels of ICAM-1 completely disrupt the endothelial barrier, increase F-actin staining and fragment the linear staining of adherens and tight junctions. In accordance, ICAM-1 silencing partially reduces the reorganisation of cell-cell junctions occurring in response to TNF (107). It is of note that overexpression of a truncated form of ICAM-1 lacking the cytoplasmic segment is also sufficient to induce endothelial permeability (107). This, together with the uncoupled effects of ICAM-1 overexpression on F-actin remodelling and permeability, suggests that the transmembrane and extracellular domains of the ICAM-1 contribute significantly to endothelial permeability in the absence of any receptor engagement (107).

ERM proteins are connectors of subcortical F-actin with cytoplasmic domains of membrane-associated proteins, including endothelial ICAM-1 (90, 108). The association of ERM proteins with ICAM-1 determines its localisation on surface F-actin-rich microvilli (109). Interestingly, TNF-induced endothelial barrier disruption is mediated by the activation of ERM proteins and cell-cell junctions (110). The contribution of ICAM-1 to this effect has not been analysed to date. However, in the absence of TNF stimulation, knockdown of a raft-protein of unreported function, known as myeloid-associated differentiation marker (MYADM), induces a proinflammatory phenotype that features hallmarks of TNF stimulation such as raised levels of endothelial permeability, activation of ERM proteins and ICAM-1 expression (96). Barrier disruption caused by MYADM depletion requires ICAM-1 expression, which in turn depends on ERK activation (96). Collectively, these results strongly suggest a role for MYADM as a negative regulator of the inflammatory response and permeability through the control of ICAM-1 expression. However, the possible participation of MYADM and ICAM-1 in TNF-induced barrier disruption has not been tested yet. TNF exposure is not sufficient to reduce MYADM (96), and further studies are required to place this novel protein within any known inflammatory signalosome.

The molecular bases underlying a ligand-independent role of ICAM-1 on endothelial permeability have not been well characterized to date. In the different cellular context of the blood-testis barrier, ICAM-1 coimmunoprecipitates and colocalises with ZO-1, claudin, N-cadherin and β-catenin at cell-cell contacts (111). In contrast to what was observed in dermal endothelial cells (107), overexpression of full ICAM-1 reduced epithelial permeability, whereas a truncated form containing ICAM-1 extracellular domain increased permeability and reduced the expression of proteins involved in the formation of cell-cell junctions. Although the components of cell-cell junctions certainly differ between blood-testis and endothelial barriers, a role for ICAM-1 as a constituent stabiliser/distabiliser of junctional complexes in the endothelium is a possibility worth investigating in the future.

**ICAM-1-mediated endothelial barrier disruption upon β2-integrin engagement**

Based on their findings with the ICAM-KO mice, the laboratory of Sarelius has proposed a thought-provoking alternative, in which the increase in vascular permeability to solutes is mediated by ICAM-1-dependent signalling upon leukocyte interaction (112, 113). Endothelial cell remodelling in response to TNF would thus be secondary for barrier control. This increase in permeability would depend almost exclusively on the transcriptional regulation of adhesion receptors that bind leukocyte ligands and signal to increase overall vascular leakiness. Sumagin el al. (112, 113) showed that antibody-mediated engagement of ICAM-1 is sufficient to increase vascular permeability *in vivo*, whereas blocking antibodies against β2 integrins dramatically decreased permeability in venules and arterioles. Consistent with this, vascular permeability in ICAM-1 KO and CD18 KO mice was also reduced with respect to control mice (113). Preventing leukocyte rolling by blocking P-selectin, an early step in the cascade of leukocyte-endothelial interactions, also strongly diminished *in vivo* vascular permeability in unstimulated and TNF-stimulated vessels (112). Nonetheless, the authors also pointed out that in unstimulated arterioles and venules, leukocyte-endothelial cell interactions are negligible while permeability was largely reduced in ICAM-1 KO mice. Moreover,
the effect of the absence of ICAM-1 on permeability in unstimulated and TNF-stimulated animals was much stronger than that caused by the absence or the inhibition of CD18. Together, these results imply the existence of an engagement-independent contribution of ICAM-1 to the control of vascular permeability also in vivo. Indeed, whereas ICAM-1 KO mice showed a marked reduction in permeability in all the vascular beds analysed, antibody-mediated engagement of the receptor induced permeability only in arterioles but not in venules. This suggests the co-existence, in vivo, of ligand-dependent and ligand-independent roles for ICAM-1 in regulating barrier function, whose relative importance depends on the type of vessel analysed (113).

ICAM-1 signals to AJs upon engagement

What are the molecular mechanisms underlying the effect of ICAM-1 engagement on barrier function? Data from in vitro experimental approaches highlight the importance of ICAM-1 signalling to F-actin upon clustering. From its C-terminal segment, ICAM-1 interacts with proteins such as filamin, α-actinin, or ERM proteins, which bind F-actin (90). Antibody-mediated clustering induces ICAM-1 alignment with moesin and actin stress fibres (93, 114), which are anchored by their ends to cell–cell junctions (34). Clustered ICAM-1 not only aligns with these actin filaments but also activates RhoA, generates more stress fibres and eventually moves to the periphery of the cell, close to cell–cell junctions, where the receptor may generate tension and transduce signals to junctional protein complexes (93, 115). Thus, TNF would assemble stress fibres that do not generate tension by themselves, but that would work as platforms where ICAM-1 rapidly clusters and induces transient contraction at cell borders. Indeed, as occurs with TNF, ICAM-1 engagement is sufficient to induce VE-cadherin phosphorylation in tyrosine residues and increase paracellular permeability (104, 115). Further, ICAM-1 crosslinking activates Trio and Rac (116) and produces ROS (117, 118), although the signalling pathways that lead VE-cadherin phosphorylation have not been fully elucidated for this adhesion receptor. For instance, they may (104), or may not involve (115) the activation of Src and Pyk2, in response to receptor clustering (117, 104, 119). Although the effect of ICAM-1-induced tyrosine phosphorylation of VE-cadherin in endothelial permeability has not yet been examined, it is well known that inhibition of Src-regulated tyrosine phosphorylation of VE-cadherin greatly impairs the increase in endothelial permeability in response to TNF (71), LPS (120) and VEGF (121). In addition, the modulation that the different phosphorylated tyrosine residues exert on VE-cadherin and AJs also remains to be investigated in detail. For instance, VE-cadherin phosphorylation in response to ICAM-1 does not cause significant catenin dissociation (115), but VE-cadherin mutants that prevent tyrosine phosphorylation in two cytoplasmic tyrosine residues dampen permeability induction and p120-catenin dissociation in response to VEGF (68). The picture is complicated by the fact that, depending on the stimulus and vascular bed analysed, different kinases from the Src family are activated and different VE-cadherin tyrosine residues are phosphorylated (115, 71, 121, 68, 122). A clear example of the role of different tyrosine residues of VE-cadherin function comes from a recent report demonstrating the different roles of Y685 and Y731 in murine VE-cadherin. TNF and other inflammatory mediators increase Y685 phosphorylation to induce permeability, whereas T-cells increase Y685 phosphorylation and promote Y732 dephosphorylation, the latter being necessary for neutrophil extravasation (123). Finally, it should be taken into account that the local increase in actomyosin-mediated tension provoked by ICAM-1 activation of RhoA may open intercellular gaps solely due to the abrupt changes in cell morphology with no active, signalling-mediated disruption of junctional complexes. This may provide an alternative mechanism for barrier rupture in response to receptor signalling (Figure 3) (114, 124, 125).

A significant proportion of antibody-crosslinked ICAM-1 is translocated to peripheral domains enriched in caveolae, where actin filaments converge and where the receptor can undergo transcytosis (93). In addition to forming part of the channels that mediate leukocyte transcellular diapedesis (93, 95, 126), caveolae mediate transcytosis of plasma solutes such as albumin (127). Consistent with this, ICAM-1 signals to caveolae and mediates the phosphorylation of caveolin-1 by Src, which produces endothelial hyperpermeability that is dependent on the caveolar vesicular system (128). It is not clear, however, whether this hyperpermeability involves the modulation of transcytosis only or of both transcytosis and paracellular permeability, as shown for other soluble mediators (Figure 3) (129).

VCAM-1 signals to AJs upon engagement through ROS generation

Another adhesion receptor dramatically increased in the endothelium by TNF is the vascular cell adhesion molecule-1 (VCAM-1). TNF-mediated increase of VCAM-1 also occurs through TNFRI activation of the NF-κB pathway (97, 98). Two NF-κB sites proximal to VCAM-1 promoter are necessary for its transcription (100, 130, 131). In HUVECs, the NF-κB/RelA heterodimer bind to the NF-κB binding site of VCAM-1 gene and controls its translation (130). Like ICAM-1, VCAM-1 also associates with ERM (106), aligns with and increases stress fibres upon crosslinking (93, 132). However, parallel crosslinking of ICAM-1 and VCAM-1 produces differential outcomes, mostly depending on the experimental system being examined. Using bone marrow endothelial cells, van Buul et al. (133) demonstrated that VCAM-1 crosslinking increased permeability whereas ICAM-1 crosslinking did not. In contrast, in vivo crosslinking of these two receptors revealed a role for ICAM-1 in permeability induction in unstimulated venules and arterioles, but not for VCAM-1 (112, 113). None of these reports addressed the relative expression of the two receptors, and it is not possible to determine whether receptor expression or the different experimental settings determines the prevalence of one or other receptor in signal transduction. However, using antibody-coated beads as surrogate leukocytes that uniquely bind ICAM-1 or VCAM-1 has revealed that single receptor engagement can induce co-clustering of the other receptor (134, 135). This strongly suggests that, in a physiological context, leukocytes interacting...
Rac1 GTPase is activated downstream of crosslinked ICAM-1 and VCAM-1 (132, 136). However, the effect of endothelial Rac1 activation upon receptor clustering has been characterised only for VCAM-1. Rac1 inhibition prevents intercellular gaps opening in response to VCAM-1 crosslinking (137). Further, Rac activity is necessary for the VCAM-1-mediated generation of ROS (137), which are produced at similar levels by leukocyte adhesion to endothelium. Importantly, dampening ROS levels with ROS scavengers inhibits the effect of active Rac on endothelial permeability (132). Like TNF, VCAM-1-induced ROS may activate Src family kinases and trigger disruption of cell-cell junctions in a manner similar to TNF. However, some additional players have been identified in the VCAM-1-mediated signalling pathway. First, an additional molecular mechanism for VCAM-1 and ROS regulation of endothelial cell–cell junctions has been recently proposed (138). Vockel et al. showed that VCAM-1-induced Rac activity and ROS generation promotes dissociation between VE-cadherin and VE-PTP, leading to the disruption of AJs. VE-PTP is a receptor-type protein tyrosine phosphatase expressed in endothelium that associates with VE-cadherin and maintains AJs (139). Antibody engagement of VCAM-1 or lymphocyte adhesion clearly disrupts the interaction between these two proteins, leading to VE-cadherin hyperphosphorylation through the tyrosine phosphatase Pyk2 (140). These findings therefore suggest that immune cells transiently regulate endothelial barrier function during TEM through VCAM-1-mediated transient disruption of the VE-PTP-VE-cadherin complex (Figure 3).

Second, VCAM-1-induced ROS also stimulate matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases with structural homologies that regulate a plethora of events related to inflammation, cancer and tissue remodelling (141). In particular, VCAM-1 stimulates MMP2 and MMP9, as determined by zymography (142). The real contribution of MMPs to endothelial permeability during leukocyte transmigration has not yet been determined (143, 144, 145, 146). However, in a broader context, it has been shown that MMP inhibition impairs TNF-induced VE-cadherin shedding in HUVECs (147), suggesting a role for these proteases in regulating cell-cell junctions.

**TNF and junctional adhesion molecules (JAMs)**

JAMs are a paradigm of proteins playing a double function in regulating permeability and leukocyte transmigration. JAMs establish cell-cell junctional complexes that regulate permeability in epithelium and endothelium (148, 149). JAM-family members contain two extracellular immunoglobulin-like domains, a single transmembrane segment and a short cytoplasmic tail with a PDZ-domain-binding motif. They can establish homophilic and heterophilic interactions in cis and trans (148). JAM-A, JAM-B and

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**Figure 3: Endothelial adhesion receptors regulated by TNF induce endothelial barrier disruption.** TNF induces a long-term inflammatory response that implies generating stress fibres and expression of adhesion receptors, namely ICAM-1 and VCAM-1, involved in leukocyte adhesion and transendothelial migration. Inhibition of TNF-induced actin remodelling through RhoA or Rac is not sufficient to prevent permeability increase. Hence, additional mechanisms are likely to contribute to barrier disruption. Upregulated ICAM-1 and VCAM-1 associate to F-actin and promote actomyosin tension and activation of connectors between plasma membrane and F-actin, such as ERM proteins, which induce cell contraction and barrier function decrease. Clustered ICAM-1 is internalized in caveolae and increases caveolin-1-mediated albumin permeability. TNF also causes dispersion from junctions and from the parajunctional LBRC of proteins playing a dual function as adhesion receptors and cell-cell junction components such as JAMs and PECAM-1. ICAM-1 and VCAM-1 activate Rac and generate reactive oxygen species (ROS) that destabilise adherens junctions through various signalling pathways.
JAM-C are expressed in endothelial cells, localised in tight junctions and associated with tight junction components, such as ZO-1 (150-152), and with polarity proteins such as PAR3 (152, 153). In addition, JAM-A and JAM-C interact with β2 integrins LFA-1 (154) and Mac-1 (155), respectively, whereas JAM-B is a counter-receptor of the β1 integrin VLA-4 (156). Furthermore, JAM-B is a counter-receptor of JAM-C (157), and JAM-C expression is required for an efficient JAM-B-VLA-4 interaction (156). Although there is sound evidence that JAM proteins help regulate epithelial barrier function (158-160), the research on endothelial JAM proteins has mainly focused on their role in TEM of immune cells and their function in maintaining endothelial permeability has not been elucidated for all of them. Only the role of JAM-C as a negative regulator of endothelial barrier function has been addressed in some detail (161, 162).

Thus, the JAMs are all prime candidates for regulating the crosstalk between immune cell adhesion and permeability in the endothelium during inflammation, and they may also contribute to the TNF-mediated changes of endothelial barrier features. The expression of JAM-A and JAM-B is regulated by TNF in cultured aortic or lymphatic endothelial cells (163-166). A significant increase in JAM-B expression has been found in arterioles within inflammatory foci in lung with chronic bronchopneumonia, suggesting that its expression may be regulated by some inflammatory mediators (165, 167). As mentioned, TNF induces the cleavage and shedding of JAM-A in HUVECs. However, other researchers working on the same cellular model did not find a significant decrease in total or surface JAM-A levels (17, 92, 164, 166). Instead, they detected significant dispersion of this molecule from junctions (92). The molecular events mediating JAM-A relocation or shedding and the contribution of JAMs to TNF-mediated barrier disruption in vascular endothelium have not been yet investigated.

TNF regulates endothelial barrier function through platelet-endothelial cell adhesion molecule-1 (PECAM-1)

PECAM-1 is a transmembrane receptor of the immunoglobulin superfamily that forms homophilic interactions between adjacent endothelial cells or between endothelial cells and leukocytes. It is important for efficient leukocyte diapedesis, being involved in its paracellular and transcellular routes (94, 168). In the last decade it has become clear that PECAM-1 is a regulator of endothelial permeability. PECAM-1 KO mice exhibit hyperpermeability in the blood brain barrier, an early onset of experimental autoimmune encephalomyelitis (EAE), the experimental animal model of multiple sclerosis. The lack of this receptor also causes a prolonged increase in permeability in response to acute contraction caused by histamine (169, 170) and thrombin (171). The cytoplasmic segment of PECAM-1 is a scaffold for phosphatases and components of cell-cell junctions. PECAM-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM), with two tyrosine residues (Y663 and Y686) that become phosphorylated by Src, Csk and Fer kinases in response to different stimuli (172, 173, 174). This phosphorylated ITIM recruits SH2 domain-containing protein tyrosine phosphatases (SHPs), of which SHP-2 is the best characterised in endothelial cells (169, 175). On the other hand, PECAM-1 also binds to β-catenin (176) and γ-catenin (177) in endothelial cells. γ-catenin binds the region coded by exon 13, which overlaps with the ITIM motif, whereas β-catenin binds a region closer to the protein C-terminus coded by the exon 15. The association of these two catenins with PECAM-1 depends on PECAM-1 tail phosphorylation status. Reciprocally, PECAM-1 negatively regulates vascular β-catenin phosphorylation in tyrosine residues in response to acute contraction provoked by histamine. This phosphorylation is mediated by the recruitment of SHP-2 to PECAM-1, which dephosphorylates β-catenin in the proximity of the receptor. Accordingly, histamine induces association of β-catenin to PECAM-1 over a period consistent with the process of endothelial barrier reformation. In the absence of PECAM-1, SHP-2 is not available at junctions and β-catenin is not dephosphorylated. This impairs β-catenin association to VE-cadherin during junctional reannealing, thereby debilitating intercellular junctions (169, 176, 177). In addition, SHP-2 not only interacts with and phosphorylates β-catenin during barrier formation (178, 179), but also may target VE-cadherin, since SHP-2 inhibition increases both β-catenin and VE-cadherin tyrosine phosphorylation in pulmonary endothelial cells (180). Finally, similar to SHP-2, SHP-1 is associated with PECAM-1 in non-endothelial cell types (172, 181), which suggests that it may also play a role regulating endothelial PECAM-1 and cell-cell junctions.

The detailed analysis of PECAM-1 localisation has provided important information about the spatiotemporal relationship between PECAM-1 and AJs and its role in barrier function. It has been reported that PECAM-1 is localised in a subjunctional compartment known as the lateral border recycling compartment (LBRC), from where it constitutively recycles to the junctional surface (168) (Figure 3). This localisation is necessary for PECAM-1 function in leukocyte diapedesis, although the nature and organisation of this compartment are still unclear. Importantly, PECAM-1 at the LBRC crosstalks with AJs, which form a junctional reticulum in resting cells. Analysis by superresolution confocal microscopy showed that the distributions of PECAM-1 and AJ overlap at these reticular borders, but form different compartments (22). Indeed, experiments expressing p120-catenin tagged to two different fluorescent proteins in two adjacent endothelial cells demonstrated that reticular AJs are exclusively distributed in the areas of contact between two adjacent cells, which suggests that they are surface, intercellular complexes, but not an intracellular compartment. Reticular AJs, however, determine the localisation of the internal LBRC (22).

TNF increases PECAM-1 phosphorylation, implying a stronger association with SH2-containing proteins, and disperses PECAM-1 from cell borders over the entire cell plasma membrane (72, 91). Localisation studies suggest that perijunctional JAM-A is also a component of the LBRC (182) and, as mentioned above, is also dispersed in response to TNF, suggesting that this cytokine may disrupt the whole LBRC rather than dispersing individual receptors. It is not yet clear how TNF disrupts the LBRC. The distribution of the LBRC is controlled by reticular AJs, which in turn...
are distributed at junctional regions with low F-actin content (22). TNF increases stress fibres that become associated from their ends to AJs (34), decreasing reticular organisation of cell-cell junctions which may thereby lead to LBRC dispersion (22). However, reduction of F-actin increase by the ROCK inhibitor Y27632 does not prevent the disappearance of reticular AJ, just as it likewise does not prevent permeability increase. This implies that additional mechanisms may regulate PECAM-1 dispersion from cell–cell borders during long-term inflammation. SHP phosphatases are candidates for regulating PECAM-1 localisation in response to TNF because they are involved in TNF-mediated signalling: SHP-2 is associated with IKK and mediates NF-κB activation in response to TNF (183); SHP-1 and SHP-2 associate with the death domain of TNFR1 in response to TNF, thereby modulating cytokine-mediated survival (184). TNF also induces SHP-2 activity in HUVECs (185) as well a moderate increment in SHP-1 expression and activity (186, 187). To date, however, the effect of TNF-mediated activation of SHPs on the localisation of PECAM-1 has not been investigated.

Finally, PECAM-1 inhibition with blocking antibodies or by gene silencing mimics the effect of TNF on cell-cell junctions and endothelial barrier dysfunction, but no other TNF-related features such as actin remodelling or ICAM-1 expression (22). Collectively, these data suggest that PECAM-1 dispersion is, indeed, what disrupts low actin reticular junctions and not vice versa, strongly supporting PECAM-1 and, probably, LBRC dispersion as an additional, actin-independent mechanism that contributes to permeability increase during TNF stimulation (▶Figure 1 and ▶Figure 3).

Concluding remarks

The fact that inhibiting actin remodelling through Rho GTPases has less effect than expected on endothelial barrier disruption upon TNF exposure indicates the existence of additional mechanisms involved in this process. In recent years, the Rac GTPase subfamily emerges as a main regulator of endothelial barrier function, not only through the direct regulation of actin filaments, but also as part of the protein complexes that elevate ROS production and modulate cell-cell junctions in response to cytokines or leukocyte adhesion. However, if actin stress fibres are not essential for increasing endothelial permeability in response to TNF in the absence of immune cells, they may play other roles in the endothelial inflammatory response. In this review we have presented evidence that leukocyte-endothelial cell interactions are of fundamental importance for barrier disruption during inflammation in experimental in vivo models. As mentioned above, stress fibres become connected to engaged adhesion receptors such as ICAM-1 and VCAM-1 (114, 93) and to discontinuous AJs from their ends (34). This type of AJs contains vinculin, a protein involved in transmitting mechanical forces that is required for TNF-induced junction remodelling (34, 36, 188). The identification of a mechanosensory protein in the junctional structures that hold stress fibres, suggest that these actin filaments are able to withstand mechanical stress, like that generated by leukocyte interactions during TEM. Rather than a cytoskeletal network responsible for the gradual and prolonged barrier function decline that TNF induces, stress fibres could instead be a prerequisite for enabling immune cells to signal efficiently to endothelial cell-cell junctions. Such signalling would involve generating actomyosin-mediated tension and ROS to produce successful paracellular diapedesis (and subsequent hyperpermeability). On the other hand, at least for ICAM-1 and PECAM-1, a role in modulating endothelial barrier function independent of their encounter with immune cells is also emerging from recent reports. Nevertheless, many questions regarding the contribution of these and other receptors to TNF-mediated barrier disruption remain unanswered, but it seems clear that such a contribution largely depends on their relative expression levels, on the vascular beds in which they are expressed and on their molecular crosstalk to cell-cell junctional proteins. A deeper knowledge of human endothelial and vascular heterogeneity seems essential if we want to fully elucidate the molecular mechanisms that contribute to vessel leakiness during long-term physiological and pathological inflammation.

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Conflicts of interest

None declared.

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