Breaching multiple barriers: leukocyte motility through venular walls and the interstitium

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Abstract | The shuttling of leukocytes between the bloodstream and interstitial tissues involves different locomotion strategies that are governed by locally presented soluble and cell-bound signals. Recent studies have furthered our understanding of the rapidly advancing field of leukocyte migration, particularly regarding cellular and subcellular events at the level of the venular wall. Furthermore, emerging cellular models are now addressing the transition from an adherent mode to a non-adherent state, incorporating mechanisms that support an efficient migratory profile of leukocytes in the interstitial tissue beyond the venular wall.

Haptotactic gradient
A molecular gradient that mediates directional cell migration by surface-bound adhesive molecules.

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Rapid locomotion of leukocytes is fundamental to the efficient operation of the immune system. In response to infection or injury, locally presented stimulating factors, such as chemokines and cytokines, induce circulating leukocytes to establish a sequence of adhesive contacts with endothelial cells that line blood vessel walls[1]. This response begins with the capture of free-flowing leukocytes from the circulation and adhesion onto the stimulated endothelium (as induced by certain cytokines and bacterium-derived endotoxins), leading to leukocyte rolling along the vasculature. Stimulation of rolling leukocytes through the ligation of adhesion molecules (for example, selectins) and through endothelial cell-presented chemokines induces firm leukocyte attachment, spreading and, through chemotactic and/or haptotactic gradients, supports intravascular crawling. These responses are mediated by the activation of leukocyte integrins (mostly β1 and β2 integrins) and are required for leukocyte migration through venular walls. Great progress has been made in understanding the mechanisms that collectively mediate leukocyte adhesion and activation in the vascular lumen (reviewed in REF. 1) but less is known about the subsequent events that mediate leukocyte migration into and within the extravascular tissue. Venules, small blood vessels that drain capillary beds, are the primary site of leukocyte transmigration *in vivo*. This response, as guided by tissue chemotactic factors (including host- and bacterium-derived molecules), involves penetrating multiple barriers: endothelial cells, pericytes and a basement membrane that is generated by both of these cell types (FIG. 1). Once through the endothelium, leukocytes migrate below the endothelial cells and extend lateral protrusions to find permissive sites in the vascular basement membrane and possibly the pericyte sheath (for example, gaps between adjacent cells). This sequence of events facilitates emigration into the extravascular tissue, where leukocytes move through the interstitium towards sites of inflammation.

Attachment to venular walls and migration through and beyond the vasculature were first described in elegant intravital microscopy studies more than 100 years ago by pathologists such as Waller and Cohnheim[2,3]. More recently (~ 50 years ago), pioneering electron microscopists described the morphological changes that are observed in leukocytes during transmigration and the alterations that this process induces to the vascular wall (for examples see REFS 4,5). Such investigations led to the first descriptions of the key events of extravasation. These include: the formation of leukocyte membrane protrusions during transmigration; breaching the endothelial cell barrier (transendothelial migration; TEM) through junctions between adjacent cells (the paracellular route) and through the endothelial cell body (the transcellular route); the role of the endothelial cell basement membrane as an important barrier to emigrating cells; and the ability of transmigrating leukocytes to render the endothelial cell basement membrane transiently permeable to macromolecules (FIG. 1). The molecular basis of these processes has only just begun to emerge, and continued progress in cell and molecular biology techniques coupled with advanced *in vitro* and *in vivo* imaging have opened many new avenues in this field.

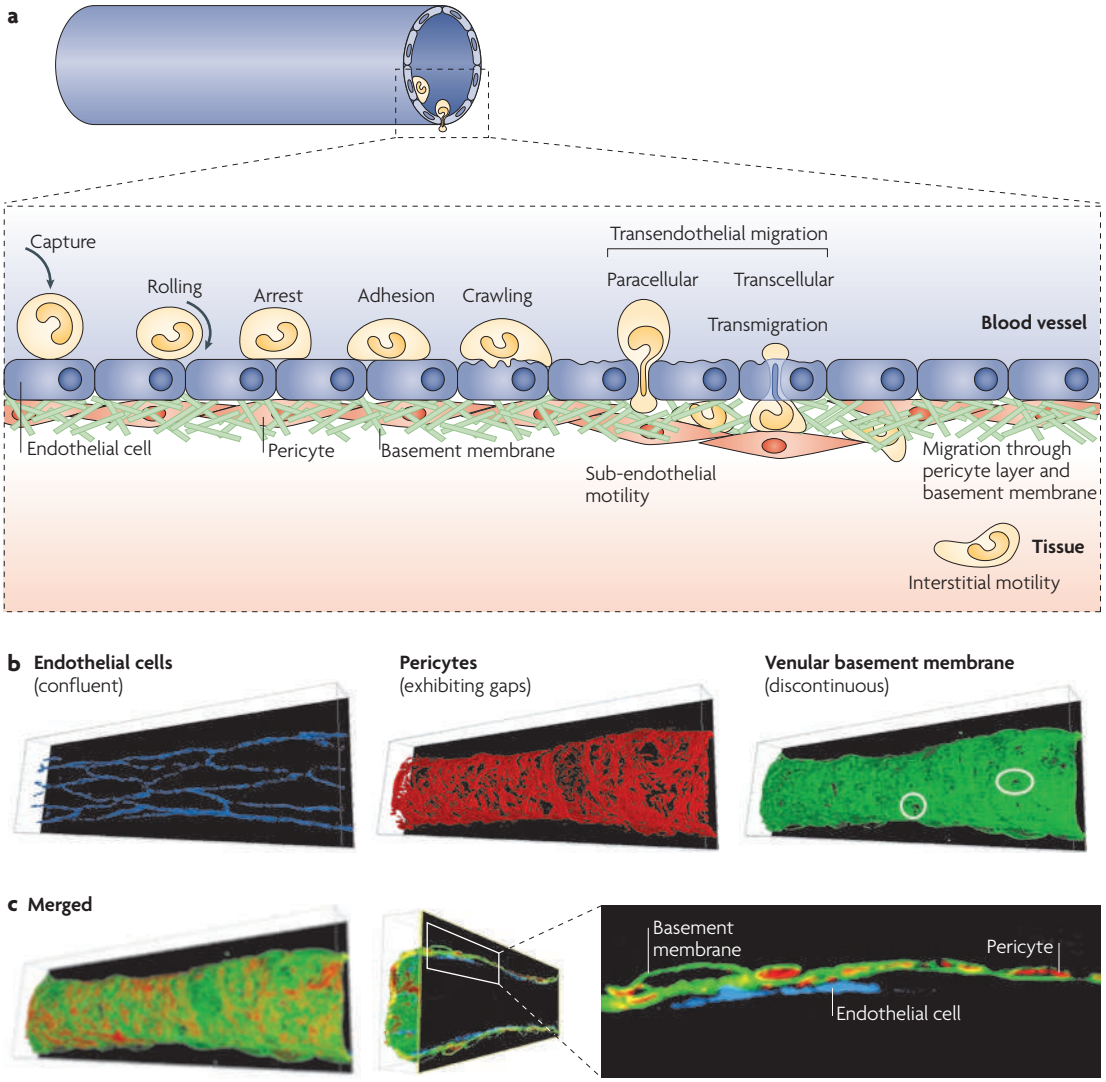

Figure 1 | **Leukocyte migration through different components of venular walls. a** | A simplified illustration of the leukocyte adhesion cascade indicating the key cellular events involved in leukocyte migration through different components of the venular wall. Leukocyte rolling, firm attachment and intravascular crawling are considered as prerequisites to the transmigration response. Migration through endothelial cells (whether by the paracellular or transcellular routes) involves the generation of leukocyte and/or endothelial cell membrane protrusions. Once through the endothelial cell barrier, leukocytes exhibit sub-endothelial cell motility and extension of ventral membrane protrusions to seek permissive sites in the pericyte sheath and venular basement membrane for their continued migration through venular walls. In the interstitial tissue, leukocytes migrate in an amoeboid manner that is largely autonomous from the molecular composition of the extracellular environment. **b** | Confocal microscopy images acquired from a cremasteric venule that is triple immunofluorescently stained for different components of the venular wall: endothelial cells (labelled for platelet endothelial cell adhesion molecule (PECAM1)), pericytes (labelled for α-SMA (also known as ACTA2)) and the venular basement membrane (labelled for laminin). The images show the different expression profiles of these structures: endothelial cells (which are confluent), pericytes (which exhibit gaps between adjacent cells) and a heterogeneous expression profile of basement membrane matrix protein (with low-expression regions; examples indicated with white circles). **c** | Analysis of the triple-stained venular wall cross-section illustrates the relative localization of the vessel wall components: endothelial cells line the lumen and pericytes are embedded in the venular basement membrane that is generated as a result of the combined deposition of matrix proteins by both endothelial cells and pericytes.

Basement membrane
A thin sheet of interconnecting macromolecules, including laminins and collagen IV, that are interconnected by other proteins, such as nidogens and perlecan, generated by cells such as epithelial cells, Schwann cells and cells of the vascular wall (endothelial cells and pericytes). The basement membrane provides structural support and signalling properties to its neighbouring cell.

Interstitium
The structure outside blood vessels and between extravascular cells that is formed largely by macromolecules such as fibrillar collagen.

Intravital microscopy
A microscopy technique used for the observation of biological responses, such as leukocyte–endothelial cell interactions, in living tissues in real time. Translucent tissues are commonly used, such as the mesentery or cremaster muscle, which can be easily exteriorized for microscopic observation.

This Review discusses developments in leukocyte migration through venular walls and key concepts that regulate leukocyte motility in the extravascular tissue, highlighting common and distinct mechanisms that mediate these responses.

Morphological changes that support TEM

The integrin-mediated adhesion of leukocytes to venular walls is associated with substantial morphological changes in both leukocytes and endothelial cells, which together support leukocyte migration through

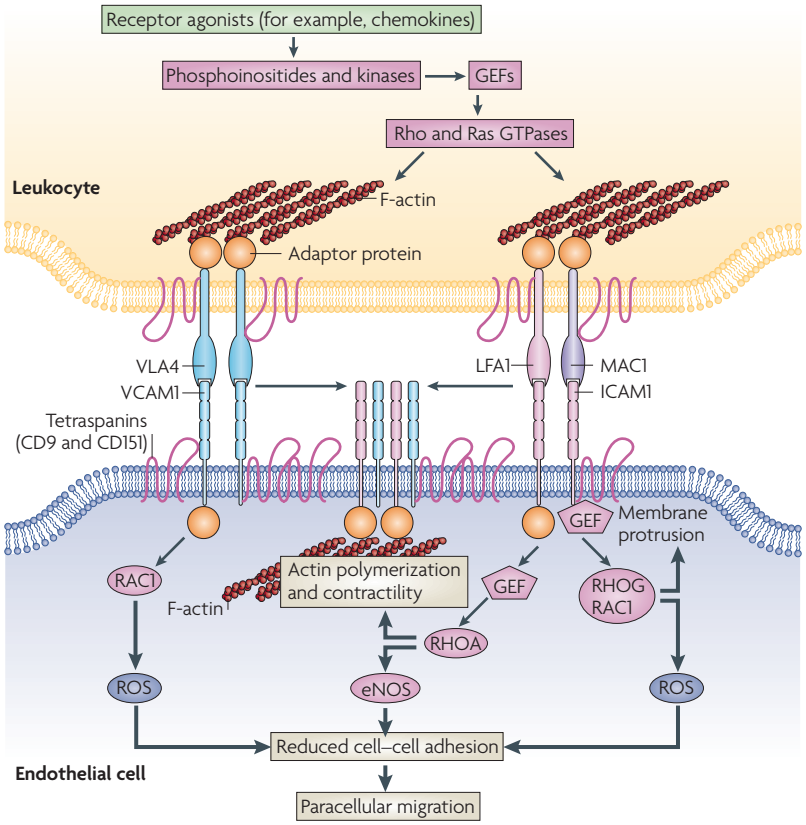

Figure 2 | **Endothelial signalling in TEM.** Activation of leukocytes by stimuli such as chemokines induces intracellular signalling, which comprises the activation of guanine nucleotide exchange factors (GEFs) through local phosphoinositide production and kinase activity. The GEFs activate Rho and Ras GTPases, which orchestrate cytoskeletal rearrangements in conjunction with inside–out signalling towards integrins. These will then be able to bind avidly to their cognate ligands on endothelial cells. In this case, tetraspanin (for example, CD9 and CD151)-dependent clustering of the integrin ligands vascular cell adhesion molecule 1 (VCAM1) or intercellular adhesion molecule 1 (ICAM1), following leukocyte binding through a β1 integrin (for example, VLA4 (also known as α4β1 integrin) or a β2 integrin (for example, leukocyte function-associated antigen 1 (LFA1; also known as αLβ2 integrin) and MAC1), recruits adaptor proteins that link these clustered membrane proteins to the cortical actin cytoskeleton. Subsequent activation of different Rho-like GTPases (such as RHOA) further contributes to VCAM1 and ICAM1 clustering through their control of cortical actin polymerization. This part of the pathway exerts positive feedback as both adaptors and filamentous actin (F-actin) dynamics are required for efficient VCAM1 and ICAM1 clustering and leukocyte binding. Moreover, the RHOG GTPase, possibly through RAC1, mediates membrane protrusion and formation of endothelial cell docking structures. In parallel, RhoGTPases initiate downstream signalling, including the activation of kinases and endothelial NO synthase (eNOS) and the production of reactive oxygen species (ROS), which can reduce cell–cell contact and facilitate paracellular migration. This pathway also requires myosin-based contractility, which targets the vascular–endothelial cell cadherin (VE-cadherin) complex at cell–cell junctions (not shown).

the endothelium. Below, we describe principal morphological changes and the molecular events that induce them.

Changes induced in leukocytes. Following adhesion to blood vessel walls, the shape of leukocytes changes markedly from a mostly spherical to a flattened phenotype. This change is associated with a reorganization of the actin cytoskeleton and is coupled with polarization, during which the migrating leukocyte forms a protrusive leading edge and a contractile rear — the uropod[6,7]. Polarization involves a redistribution of intracellular signalling proteins, surface receptors and adhesion molecules in the front and back of the cell, driving persistent directional motility over the endothelial cell surface. Furthermore, leukocytes develop ventral membrane protrusions (various types of which have been identified[6]) while crawling in the vascular lumen to probe for permissive sites on the endothelial surface[8–11] (FIG. 1). Shear stress can stimulate protrusion formation in neutrophils, and protrusions cause endothelial cells to develop invaginations that facilitate TEM by both the paracellular and transcellular routes (see below)[8]. By contrast, the formation of invasive protrusions in cultured lymphocytes migrating under static conditions correlates primarily with transcellular migration[9]. In the lymphocyte study, protrusions had a filamentous actin (F-actin)-rich core surrounded by an adhesive ring expressing leukocyte function-associated antigen 1 (LFA1; also known as αLβ2 integrin) and the cytoskeletal protein talin 1. These protrusions are structurally related to podosomes[9,12], and are similarly dependent on the protein Tyr kinase Src and the cytoskeletal regulator Wiskott–Aldrich syndrome protein (WASP). The potential role of podosomes in leukocyte migration *in vivo* has yet to be clearly shown.

The generation of podosomes and other protrusions such as pseudopodia may be assisted by the redistribution of high affinity LFA1 to the leading edge. This would facilitate the interaction of LFA1 with its ligand, intercellular adhesion molecule 1 (ICAM1), on the surface of endothelial cells, thus supporting leukocyte attachment and subsequent motility on the venular wall[6,13,14]. Chemokines and shear stress, in addition to podosomes and pseudopodia, promote the formation of filopodia, which are membrane protrusions composed of parallel bundles of actin filaments and actin-associated proteins[15]. It is suggested that lymphocytes use these LFA1-expressing adhesive filopodia to crawl in a millipede-like manner, scanning the surface of endothelial cells for sites of emigration. Studies to date indicate that different β2 integrins mediate the crawling of different leukocyte subtypes, with LFA1 and MAC1 (also known as αMβ2 integrin) supporting lymphocyte[11] and neutrophil crawling[16], respectively.

Changes induced in endothelial cells. In recent years, our knowledge of the molecular events at the leukocyte–endothelial cell interface has increased substantially and as a result it is now generally accepted that the vascular endothelium actively participates in leukocyte TEM (FIG. 2). Activation of the endothelium by inflammatory cytokines such as tumour necrosis factor (TNF) and interleukin-1β (IL-1β) induces the increased expression of selectins (for example, E-selectin) and integrin ligands (for example, ICAM1 and vascular cell adhesion molecule 1 (VCAM1)) on the luminal endothelial cell surface. As was recently shown for ICAM1 and VCAM1, these are part of higher-order clusters that aggregate at the apical membrane following binding to leukocyte integrins, forming rings around the bound leukocyte[17]. This aggregation is also due to the coalescence of pre-existing membrane microdomains that contain members

Shear stress
A mechanical force created by blood flow through a vessel that impinges on the endothelium by virtue of its unique location in the vessel wall.

Box 1 | **Organization of adhesions: different structures, same principles?**

Prior to leukocyte transendothelial migration (TEM), heterotypic cell–cell contact is mediated by various types of adhesion molecule, including leukocyte integrins, such as leukocyte function-associated antigen 1 (LFA1; also known as αLβ2 integrin) and VLA4 (also known as α4β1 integrin), and their ligands on the endothelium, such as intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1). These interactions stimulate the formation of protrusions of ICAM1- and VCAM1-expressing membrane sheets in endothelial cells, forming docking structures (see also FIG. 2). Endothelial cell adhesion molecules also associate with actin-binding adaptor proteins such as cortactin, α-actinin and filamin. This secures anchorage to the endothelial cell cortical cytoskeleton, resulting in the formation of a force-transduction platform (also known as a diapedesis synapse), which promotes strong adhesive interactions with the leukocyte.

A substantial portion of the intracellular endothelial proteins that bind to E-selectin and ICAM1 are also found in focal adhesions[42] (filamentous actin (F-actin)-linked integrin clusters that mediate cell–extracellular matrix adhesion). Proteins that are found in both focal adhesions and docking structures include α-actinin, filamin, vinculin, paxillin and focal adhesion kinase. The higher-order clustering of endothelial ICAM1 depends on RHOA, the actin cytoskeleton and its association with actin-binding proteins such as filamin[44,59]. Similarly, focal adhesion formation and integrin clustering requires RHOA-mediated actomyosin-based contractility[125], the actin cytoskeleton and association to actin-binding proteins such as α-actinin and paxillin (reviewed in REF. 126). Thus, because the same (or similar) sets of proteins are involved in the clustering and adhesive functions of integrins and their ligands, these processes seem to be controlled by comparable inside–out signalling events, which might regulate the different types of adhesion through similar principles. Given the relevance of TEM for inflammatory disorders, further characterization of the mechanisms that control efficient leukocyte–endothelial cell interactions is important. Only recently have studies started to identify such events at the level of endothelial integrin ligands. The analogy to the well-characterized control of integrin function may help to identify the relevant events and signalling pathways more efficiently.

of the tetraspanin family of integral membrane proteins[18]. In human umbilical vein endothelial cells (HUVECs), the tetraspanins CD9 and CD151, and possibly CD81, promote the clustering of ICAM1 and VCAM1 (REFS 18,19). Interestingly, leukocyte-induced clustering of endothelial ICAM1 results in the recruitment of VCAM1 into membrane microdomains that are enriched for tetraspanins and ICAM1, and vice versa[18]. This suggests that the clustering of integrin ligands at the leukocyte–endothelial cell interface primarily serves to generate membrane platforms for firm attachment.

Integrin ligand clustering can be accompanied by the protrusion of the apical endothelial membrane, forming 'docking structures' on endothelial cells *in vitro*[20,21] (FIG. 2). Electron microscopy and real-time imaging of inflamed tissues has provided some *in vivo* evidence for the existence of endothelial cell 'dome-like' structures, potentially similar to the docking structures identified *in vitro*, that seem to encapsulate adherent neutrophils during TEM[22,23]. It is still unclear whether these structures occur ubiquitously during TEM of different leukocyte subtypes[24]. It is, however, accepted that the clustering of ICAM1 and VCAM1 induces the recruitment of regulatory and structural components such as F-actin in the endothelial cell. This promotes the local connection of the integrin ligands with the endothelial cortical actin cytoskeleton (see below), resulting in the formation of force-transduction platforms (that is, surfaces that facilitate migration through force generation) (BOX 1) that support leukocyte TEM.

Podosome
A small (~ 0.5 μm diameter) cylindrical structure, containing typical focal adhesion proteins such as vinculin and paxillin, that is a distinct form of cellular protrusion implicated in establishing close contact with the ECM. Podosomes have been reported in many cell types, including various malignant cells, macrophages and smooth muscle cells, where they are consistently associated with motile and/or invasive responses.

Pseudopodium
A temporary projection of the cytoplasm of certain cells, such as neutrophils, or of certain unicellular organisms, especially amoebae, that functions in locomotion.

Filopodium
A thin (~ 1 μm in diameter and up to 5 μm in length) and highly dynamic actin cellular protrusion that is formed by the elongation of bundled actin filaments in its core.

TEM

The leukocyte and endothelial cell morphological changes detailed above are required for leukocyte TEM. For this to occur, the leukocyte cell body follows membrane protrusions towards the basal side of the endothelium and then attaches to and migrates over the basal endothelial membrane and/or the endothelial cell basement membrane while retracting its rear to complete the TEM process. This step is associated with and depends on signalling events in both leukocytes and endothelial cells that regulate the leukocyte–endothelial cell interaction and adhesion between adjacent endothelial cells.

Subcellular signalling events in leukocytes. The main leukocyte signalling pathways underlying chemokine-stimulated integrin-dependent adhesion, directional motility, protrusion formation and TEM involve Rho and Ras small GTPases, which have important roles in regulating cell shape, adhesion and motility[25]. These are activated by guanine nucleotide exchange factors (GEFs)[26]. The best studied GEFs in leukocytes are T lymphoma invasion and metastasis-inducing protein 1 (TIAM1), VAV1, VAV2 and VAV3, as well as the atypical RhoGEF dedicator of cytokinesis protein 2 (DOCK2) for Rho GTPases, and C3G (also known as RAPGEF1) and exchange factor directly activated by cAMP1 (EPAC; also known as RAPGEF3) for Ras-related Rap GTPases. These GEFs, in turn, are targeted and activated by different upstream signals, including Src-mediated Tyr phosphorylation[27] and the synthesis of specific phosphoinositides or phosphatidic acid[28] (or cyclic AMP in the case of EPAC)[26]. The contribution of these GEFs to leukocyte TEM varies: Vav deficiency mainly impairs leukocyte adhesion and consequent TEM, whereas loss of DOCK2 in T cells impairs Rac activation and actin polymerization[29] as well as lateral mobility over activated endothelium, but does not affect the initial arrest and TEM[30]. TIAM1-deficient T cells show an even more subtle phenotype as they exhibit normal arrest on the endothelium but have a crawling defect and a concomitant shift towards transcellular migration[31].

Following their activation by GEFs, Ras and Rho GTPases regulate adhesion, cytoskeletal dynamics and cell polarization, for example through the atypical protein kinase Cζ (PKCζ)–partitioning defective 3 homologue (PAR3; also known as PARD3) and PKCζ–PAR6 (also known as PARD6A) complexes [32]. The classical view has been that the Rho GTPase RAC1 controls polarized actin polymerization in the leading edge by stimulating the actin-related protein 2/3 (ARP2/3) complex, which nucleates actin filaments[33], and RHOA controls myosin-based contraction of the uropod, which is crucial for the completion of the TEM response[34,35]. The use of sophisticated GTPase biosensors has shown that this model is probably too simplistic because, for example, RHOA activity was also detected in the leading edge of polarized fibroblasts[36]. Furthermore, Cdc42 and RAP1A have also been shown to control lymphocyte polarity by acting through the PKCζ–PAR3 and PKCζ–PAR6 polarity complexes[6,37]. Thus, it is now thought that these GTPases act in concert to determine leukocyte polarity, adhesion and TEM.

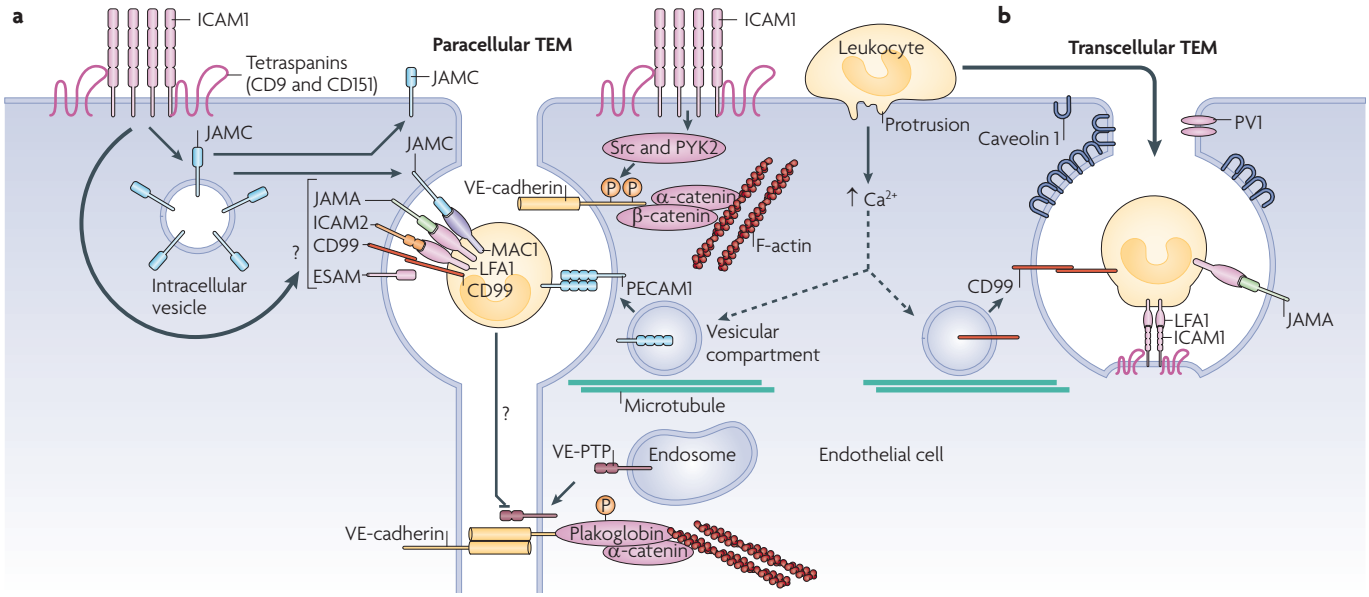

Figure 3 | **Paracellular and transcellular TEM. a** | Intercellular adhesion molecule 1 (ICAM1) clustering induces protein Tyr kinase activity, impairing vascular–endothelial cell cadherin (VE-cadherin) function. In addition, VE-cadherin is constitutively internalized and recycled, but evidence that this has a role in paracellular transendothelial migration (TEM) is currently lacking. Platelet endothelial cell adhesion molecule (PECAM1) is recruited to sites of transmigration from a vesicular compartment, specifically the lateral border recycling compartment (LBRC), in a process that depends on microtubules and kinesin. Additional vesicular compartments, such as vesiculovacuolar organelles (VVOs), are also important and may even be closely related to the LBRC. Other adhesion molecules (CD99, and junctional adhesion molecules JAMA and JAMC) are also stored in and maybe recycled from vesicular compartments. The VE-cadherin-stabilizing Tyr phosphatase VE-PTP (also known as PTPRB) is recruited from endosomes following the formation of a confluent endothelial monolayer (not shown). VE-PTP dissociates from VE-cadherin during TEM, which allows plakoglobin (also known as γ-catenin) phosphorylation, leading to loss of VE-cadherin function and endothelial integrity. **b** | Increased levels of intracellular Ca^{2+} is a well-established endothelial response to leukocyte binding. This signal is important for contractility as well as fusion and transport of intracellular vesicles and may also regulate vesicles containing adhesion molecules such as CD99 or PECAM1 (shown by the dashed arrows), which are involved in regulating TEM by the transcellular route. The LBRCs and VVOs might also contribute to this process. In addition, transcellular migration may require endothelial caveolin 1, which in some studies has been linked to the formation of the transcellular pore, similar to the transmembrane protein plasmalemma vesicle protein 1 (PV1). Leukocyte protrusions, formed by adherent cells, have been proposed to induce pore formation, but the underlying molecular mechanism remains to be identified. ESAM, endothelial cell-selective adhesion molecule; F-actin, filamentous actin; LFA1, leukocyte function-associated antigen 1.

The recent development of mice lacking key components of these pathways (for example, RAC1, Cdc42 and Vav proteins) has provided new insights into their functions *in vivo*[38]. For example, VAV1 and VAV3 were shown to have a role in the regulation of intravascular leukocyte–endothelial cell interactions[39,40] and post-recruitment neutrophil cytotoxicity[41]. Despite increasing knowledge of the roles of GTPases and their regulators in leukocyte motility (see below), their specific involvement in TEM *in vivo* requires further investigation.

Subcellular signalling events in endothelial cells. Luminal leukocyte–endothelial cell interactions trigger a cascade of signalling events in endothelial cells that may support TEM. For example, leukocyte crawling can trigger signalling events that induce the transient weakening of endothelial cell junctions or the formation of intracellular pores, which are crucial for paracellular and transcellular TEM, respectively (FIG. 3). This concept is supported by findings that the association of clustered ICAM1 with the cortical actin cytoskeleton causes rearrangement of cytoskeletal and possibly junctional molecules in the endothelium. The ICAM1–cytoskeleton interaction is mediated by actin-binding adaptor proteins, many of which have been found to interact with both ICAM1 and E-selectin. Depending on the type of endothelial cell, these adaptor proteins include α-actinin, filamin, vinculin, paxillin, cortactin and the ERMs (ezrin, radixin and moesin)[20,42–44]. It is currently unclear why so many different adaptor proteins are biochemically and functionally linked to leukocyte adhesion and TEM, although there is some evidence that they may carry out different functions. For example, filamin efficiently cross links filaments in orthogonal actin networks and is integral for the formation of membrane protrusions[45], and α-actinin is a Ca^{2+}-regulated antiparallel cross-linker of actin filaments that has been implicated in the maturation of small, nascent adhesions[46]. Purified filamin and α-actinin cooperatively increase the stiffness of actin filament networks[47] and, when in close proximity in endothelial cells, these proteins could enhance the stability of docking structures. Whether this also occurs downstream of ICAM1-mediated signalling remains to be investigated.

Tetraspanin
One of a family of proteins that span the membrane four times with two exoplasmic loops and that can be found at the cell surface. Some are highly restricted to specific tissues, whereas others are widely distributed. Members of this family have been implicated in cell activation, proliferation, adhesion, motility, differentiation and cancer.

In addition to stabilizing membrane protrusions, actin cross-linkers control the functional connection between the endothelial cell surface and the intracellular signalling machinery. For example, filamin binds small Rho and Ral GTPases, GTPase activators, such as the GEF TRIO, and signalling mediators, such as caveolin 1, facilitating localized signal transduction (reviewed in REF. 45). Clustering E-selectin or integrin ligands on the endothelial cell surface induces a series of intracellular signalling events, including the release of intracellular Ca^{2+} and the activation of Src kinases, mitogen-activated protein kinases (MAPKs) and RHOA, RAC1 and RHOG GTPases[48–53] (FIG. 2). RHOA and RAC1 promote actomyosin-based contractility, and RAC1 and RHOG have been linked to the formation of docking structures through the induction of apical membrane protrusions[51]. RAC1 drives the activation of an endothelial NADPH oxidase (for example, NOX2 (also known as CYBB)), which generates reactive oxygen species (ROS), such as superoxide anions. ROS in turn can regulate the activity of protein kinases and phosphatases, such as protein kinase C, PTP1B (also known as PTPN1) and PYK2 (also known as PTK2B)[54–56], that can control the phosphorylation of junctional proteins such as vascular–endothelial cell cadherin (VE-cadherin; a key regulator of endothelial cell junctions), leading to vascular permeability (see below)[57]. Indeed, recently, an ICAM1-activated, endothelial NO synthase (eNOS)-dependent pathway was identified in microvascular endothelial cells that promotes lymphocyte TEM by regulating VE-cadherin phosphorylation[58]. Thus, clustering of E-selectin and integrin ligands initiates various signalling events that control cytoskeletal dynamics and protein phosphorylation, which seem to converge at the level of endothelial junctions, impairing VE-cadherin-mediated cell–cell adhesion (see below).

Accumulating evidence indicates that, as in most signalling systems, feedback loops constitute an important part of leukocyte-induced signalling in endothelial cells. RHOA is activated following ICAM1 clustering but is in turn also required for the clustering of ICAM1, E-selectin and VCAM1 (REFS 59,60). Similarly, the ICAM1-binding proteins cortactin and filamin, which are recruited following ICAM1 binding to leukocyte integrins, are required for further, efficient ICAM1 clustering[43,44,61]. These findings support the notion that the adhesive function of leukocyte ligands is controlled by endothelial inside–out signalling (BOX 1). Observations indicating that receptor agonists such as TNF[62] and vascular endothelial growth factor (VEGF)[63] also promote ICAM1 and E-selectin clustering add another layer of complexity, but the associated molecular pathways remain to be identified.

Another endothelial cell-associated signalling molecule that has been implicated in leukocyte TEM is the F-actin-binding intracellular phosphoprotein lymphocyte-specific protein 1 (LSP1), a p38 MAPK substrate that is expressed by leukocytes and endothelial cells. LSP1-deficient mice exhibit a defect in neutrophil transmigration through cremasteric venules, which was linked to LSP1 deficiency in endothelial cells in this model[64]. The mechanism by which endothelial cell LSP1 mediates neutrophil transmigration is unclear, but its role in this response seems to depend on the inflammatory model used[64], suggesting a potential heterogeneity in its function and/or expression in different vascular beds.

Junctional events regulating TEM. Numerous molecules expressed at high levels at junctions between adjacent endothelial cells support leukocyte TEM. These include platelet endothelial cell adhesion molecule (PECAM1), ICAM2, members of the junctional adhesion molecule family (JAMA, JAMB and JAMC), CD99 and endothelial cell-selective adhesion molecule (ESAM). Their role in leukocyte TEM is well established and has been comprehensively reviewed[1]. Recent evidence suggests that some of these molecules are involved in the TEM of specific leukocyte subtypes or in response to specific stimuli[1,65,66]. For example, there is now clear evidence that in many mouse strains, PECAM1, ICAM2 and JAMA[67] mediate neutrophil TEM through cremasteric venules that is induced by IL-1β but not TEM elicited by direct leukocyte stimulating agents such as TNF or chemokines[67]. Furthermore, ESAM-deficient mice have defective neutrophil but not lymphocyte infiltration into sites of inflammation[68]. PECAM1, ICAM2 and JAMA are rate-limiting for distinct phases of the emigration process, acting in sequence to mediate neutrophil TEM through venular walls[67]. A role for specific endothelial cell junctional molecules in the regulation of TEM in different vascular beds and/or in different phases of inflammatory responses, for example in acute or chronic inflammatory reactions, is also plausible but has not been conclusively shown. To complicate matters further, JAMC was found to mediate directed leukocyte emigration, as its blockade enhances reverse TEM of monocytes *in vitro*[69], and there is a growing body of evidence to indicate that endothelial cell junctional molecules can also mediate leukocyte emigration by the transcellular route[9,70], suggesting a more intricate relationship between the two modes of TEM than originally anticipated (FIG. 3).

The contact between endothelial cells crucially depends on VE-cadherin, an endothelial specific homotypic adhesion molecule. VE-cadherin is regulated by cytoplasmic adaptor proteins such as β-catenin and plakoglobin (also known as γ-catenin). These mediate the functional interaction between VE-cadherin and the cortical actin cytoskeleton, which includes bidirectional signalling between the cytoskeleton and the cell surface, as for ICAM1. Paracellular leukocyte TEM requires a transient loss of VE-cadherin function and thus of junctional integrity. This can be induced by a combination of actomyosin-based cytoskeletal contractility triggered by the activation of the small GTPase RHOA[59,60] and by Tyr phosphorylation of VE-cadherin, β-catenin and plakoglobin, which negatively affects cell–cell contact. Indeed, interfering with either pathway reduces the efficiency of leukocyte TEM.

Tyr phosphorylation of VE-cadherin in the context of TEM is thought to be regulated by the Src and PYK2 protein Tyr kinases, and the Tyr phosphatase VE-PTP (also known as PTPRB). Src kinases and PYK2 are activated by ICAM1 clustering, and inhibition of each of these kinases is sufficient to block VE-cadherin phosphorylation, which

Inside–out signalling
The process by which intracellular signalling mechanisms result in the activation of a cell surface receptor, such as integrins. By contrast, outside–in signalling is the process by which ligation of a cell surface receptor activates signalling pathways inside the cell.

reduces neutrophil TEM across HUVECs[53,57]. VE-PTP is constitutively associated with VE-cadherin and positively regulates VE-cadherin function[71]. VE-PTP dissociates from VE-cadherin following neutrophil adhesion to TNF-activated endothelium, which leads to increased endothelial permeability and increased neutrophil TEM. VE-PTP promotes the association of VE-cadherin and plakoglobin by binding Tyr-phosphorylated plakoglobin rather than β-catenin or VE-cadherin. The recruitment of VE-PTP and plakoglobin to cell–cell contacts is thought to depend on the differentiation state of endothelial cells, which suggests that this is another variable that determines the contribution of specific pathways and surface proteins to leukocyte TEM.

Endothelial cell vesicular traffic. Recent findings have revealed an important role for endothelial cell vesicular traffic in leukocyte TEM. The most extensively studied vesicular systems in endothelial cells are caveolae and vesiculovacuolar organelles (VVOs). Caveolae are located close to the luminal or abluminal membrane, whereas VVOs, which are found in venular endothelium, constitute an elaborate network of vesicles that regulate transcellular transport of solutes and macromolecules, as well as the transcellular migration of leukocytes and platelets[72]. VVOs, which are often located close to intercellular junctions, are positive for the membrane-associated adaptor caveolin 1 and might be related to, or form by the fusion of, caveolae. VVOs are also positive for vesicle-associated membrane protein (VAMP), which regulates vesicular traffic and has a role in lymphocyte transcellular migration[9]. Although the importance of caveolin 1 in TEM remains controversial[9,70], the available evidence indicates that there is a close structural and functional link between caveolin 1, plasmalemma vesicle-associated protein 1 and VAMP and structural components such as caveolae and VVOs in the regulation of transcellular migration.

Recent data support the idea that vesicular traffic of endothelial cell junctional proteins is another important aspect of TEM. VE-PTP is recruited to endothelial junctions from an endosomal compartment[71]. Similarly, VE-cadherin associates with endosomes following clathrin-dependent internalization[73]. Although VE-cadherin internalization seems to be constitutive, the process is stimulated by VEGF[74] but inhibited by the association of VE-cadherin with p120 catenin[73]. Another junctional molecule, PECAM1, traffics to and from a lateral border recycling compartment (LBRC) in HUVECs during leukocyte TEM in a Src-, kinesin- and microtubule-dependent manner[24,75]. Through this recycling, unligated PECAM1 becomes specifically recruited to sites of paracellular transmigration of monocytes, possibly to provide sufficient endothelial PECAM1 for leukocyte binding. Recent studies have revealed that PECAM1 Tyr663 is necessary both for efficient PECAM1 recycling through the LBRC and for monocyte TEM, further linking the two processes[76]. Furthermore, JAMA and CD99 also seem to be components of the LBRC[70]. Whether LBRCs and VVOs are distinct or the same structural entities *in vivo* requires further clarification.

JAMC has also been detected in endothelial cell intracellular compartments. In tissues that have sustained ischaemia–reperfusion injury, endothelial cell JAMC was redistributed from cytoplasmic vesicles and endothelial cell junctional sites to non-junctional plasma membranes, a response that seems to account for the unexpected role of JAMC in both leukocyte adhesion and TEM under conditions of ischaemia–reperfusion injury[77]. By contrast, using the same model of ischaemia–reperfusion injury, JAMA-deficient mice exhibited a defect in leukocyte TEM only[78]. Although the nature of the JAMC-containing endothelial cell vesicles remains to be determined, together these studies show that the mobilization of intracellular stores of endothelial cell junctional proteins in inflammation represents an exciting aspect of leukocyte TEM that is likely to receive increased attention in the coming years.

Vascular basement membrane and pericytes

In line with other stages of the leukocyte adhesion cascade, in which one step triggers events that mediate the subsequent step[1], TEM may facilitate the continued migration of leukocytes into sites of inflammation. Indeed, there is evidence that TEM events mediate changes in leukocyte chemokine receptor, integrin and protease expression and/or activation, which might aid the interactions of leukocytes with components of the vessel wall beyond the endothelium and leukocyte motility in the extravascular tissue (reviewed in REFS 1,79).

Following migration through endothelial cells, leukocytes breach the basement membrane and the pericyte coverage of venular walls to enter the interstitial space (BOX 2). Owing to complexities associated with the generation of physiologically relevant basement membranes and difficulties in isolating and studying primary pericytes *in vitro*, this phase of leukocyte emigration is not well understood, although recent findings have provided some mechanistic insights. Pioneering electron microscopists noted that emigrating leukocytes are more often seen between the endothelium and its basement membrane than in the process of passing between endothelial cells. This led to the conclusion that the venular basement membrane offers more resistance to emigrating cells than the much thicker endothelium[4]. Basement membranes are composed of tightly packed networks of laminins and collagen type IV that are interconnected with molecular bridges involving other glycoproteins such as perlecan and nidogens[80]. Although its tightly packed nature indicates that cellular passage requires strategies to either locally displace or disassemble it, there is currently no unifying concept regarding how leukocytes may migrate through the vascular basement membrane. The following aspects, some interrelated, seem to influence the process.

Migration through physically permissive sites. To continue their migration beyond the endothelium, leukocytes probably exhibit motility on the abluminal side while seeking preferred sites for their maintained migration through the venular wall. Little is known about

Ischaemia–reperfusion injury
Damage caused to tissues when blood supply is restored to the tissue after a period of vascular occlusion. The absence of oxygen and nutrients from blood creates a condition in which the restoration of blood supply results in leukocyte and endothelial cell activation through the induction of oxidative stress.

Laminin
A member of a family of the principal non-collagenous secreted glycoproteins that are an integral part of the structural scaffolding of basement membranes in all tissues. Structurally they contain three subunits (α-, β- and γ-subunits) connected in the shape of a cross.

Box 2 | **Leukocyte transmigration beyond the endothelium**

Pericytes. In addition to endothelial cells, pericytes are a cellular component of capillaries and post-capillary and collecting venules, and closely associate with the underlying endothelium through their many long protrusions[127] (FIG. 1). They exhibit morphological and phenotypical differences depending on vessel type, vascular bed, developmental stage, species and the pathological conditions under which they are found[127]; this has made their study difficult. In contrast to endothelial cells, which form a confluent polarized monolayer that is anchored away from the vessel lumen to its basement membrane, in most tissues the pericyte network is loosely distributed around endothelial cells[127] and is embedded in the venular basement membrane (FIG. 1). The extent of pericyte coverage around microvessels varies substantially between different organs, suggesting different levels of barrier function in different tissues. Leukocyte migration through the pericyte sheath can occur by both paracellular and transcellular routes[22,81,82], but details of the associated mechanisms and the potential role of pericytes in the regulation of leukocyte transmigration remain unclear.

Venular basement membrane. The venular basement membrane is generated by both endothelial cells and pericytes (FIG. 1). Basement membranes provide an adhesive and structural support for the generating cell, and in venules provide a substantial barrier to migrating cells[80]. Leukocyte migration through this structure occurs in several ways (see main text for details), including through biochemically permissive sites. For example, regions expressing laminin 511 are proposed to be anti-migratory, whereas laminin 411 reportedly promotes migration[92]. Specifically, mice lacking the laminin α4 chain (a subunit of laminin 411) show compensatory upregulation of laminin α5 (a subunit of laminit 511) and display reduced extravasation of T cells into the inflamed central nervous system (CNS) and reduced rates of neutrophil infiltration into inflamed peritoneum[128,129]. Reduction of CNS infiltration was linked to deficiency in laminin α4 expression (and the resultant increase in laminin α5 expression) by the endothelium, and reduced peritoneal infiltration was associated with deficiency in laminin α4 in leukocytes[128,129]. By contrast, *in vitro* studies have indicated that laminin 511 shows a greater ability to promote human lymphocyte migration than other laminin isoforms (including laminin 411 (REF. 130)), and in mouse cremasteric venules, leukocyte permissive regions are deficient in both laminin 411 and laminin 511 (REF. 82). Such inconsistencies and divergent reports suggest that the potential role of biochemical differences in the composition of the vascular basement membrane in the regulation of leukocyte migration may be leukocyte specific and/or may be governed by the species, inflammatory stimuli or model being studied.

the mechanisms associated with leukocyte migration through the pericyte network, but recent *in vivo* studies have shown that in response to multiple inflammatory stimuli (IL-1β, TNF, CC-chemokine ligand 2 (CCL2), lipopolysaccharide and ischaemia–reperfusion injury), neutrophils migrate across the pericyte sheath through gaps between adjacent cells[81,82]. Emigration through pericytes by the transcellular route has also been noted in strongly activated tissues[22], indicating that pericytes can provide a true barrier to transmigrating leukocytes.

Pericytes also seem to govern the existence and expression profile (for example size and localization) of leukocyte-permissive sites in the venular basement membrane[81–83]. Specifically, as pericytes together with endothelial cells contribute to the generation of the venular basement membrane[84], the loose net-like pericyte coverage yields a combined basement membrane that shows a discontinuous expression of laminins and collagen IV[81–83] (FIG. 1). These regions, which have been termed low expression regions (LERs), are present in many tissues and act as gates for migrating leukocytes in a diverse range of inflammatory reactions[82]. Furthermore, there is evidence that neutrophils and monocytes identify these permissive regions by projecting ventral membrane protrusions[83].

Biochemical remodelling of the vascular basement membrane. Neutrophil migration is associated with a transient enlargement of LERs[81–83]. The precise mechanism by which this occurs is currently unclear, but the detection of laminin-expressing (but not collagen-expressing) neutrophils in the extravascular tissue suggests local disassembly of the basement membrane. In the mouse model used in these studies, the laminin on transmigrated neutrophils was not derived from the neutrophils themselves and was associated with α6β1 integrin, suggesting a key role for this integrin in neutrophil interactions with the venular basement membrane[81]. However, a role for proteases in leukocyte extravasation is still debated. The controversies stem from differences in the models used to test this, including differences in cell types and inflammatory stimuli, redundancies in proteases and different experimental approaches. Despite this, numerous proteases have been implicated in neutrophil transmigration and LER remodelling, including matrix metalloproteinase 8 (MMP8), MMP9 and neutrophil elastase[81,85,86]. Although conceptually the potential role of proteases is linked to leukocyte-derived enzymes, endothelial cell-associated enzymes can also have a role in regulating leukocyte transmigration[80]. Indeed, there is evidence for the involvement of endothelial cell MMPs in VCAM1-dependent lymphocyte TEM[87]. Furthermore, neutrophil elastase cleaves the laminin β1 chain (the common β-subunit of the venular basement membrane-specific laminins, laminin 511 and laminin 411), resulting in the release of laminin fragments, which can induce leukocyte chemotaxis[88,89]. Hence, leukocyte-associated or derived proteases such as neutrophil elastase may facilitate the penetration of the basement membrane by degrading laminin (but not type IV collagen). This may enlarge laminin LERs and simultaneously generate chemotactic laminin fragments that could enhance neutrophil motility.

Although some traversing leukocytes can remodel basement membranes, this does not seem to be essential for all cell types. Specifically, although both neutrophils and monocytes use LERs as preferred sites for penetrating the vascular basement membrane, only neutrophils cause enlargement of these regions and, in fact, monocytes squeeze through LERs without remodelling the basement membrane[83] through an unknown mechanism. Of interest, septin deficiency is associated with loss of cortical stability and the ability of T cells to migrate through small pores, suggesting an important role for the septin cytoskeleton in cortical rigidity and cell migration through confined tissues[90].

Biochemically or biophysically permissive sites. The biochemical (BOX 2) or biophysical composition of the basement membrane could also contribute to the transmigration of leukocytes. The biophysical composition could be determined by the degree of covalently cross-linked collagen IV[80,91] and the ability of laminin isoforms to build networks. For example, the laminin α4 chain (but not the laminin α5 chain) is truncated, which probably leads to reduced cross-linking of collagen IV[92]. A low degree of cross-linking might allow penetration of the basement membrane without digestion and facilitate

Septin cytoskeleton
A polymeric protein scaffold comprising the septins, which assemble in rings and gauzes. In mammals, the septin cytoskeleton typically colocalizes with actin stress fibres and its function is poorly characterized.

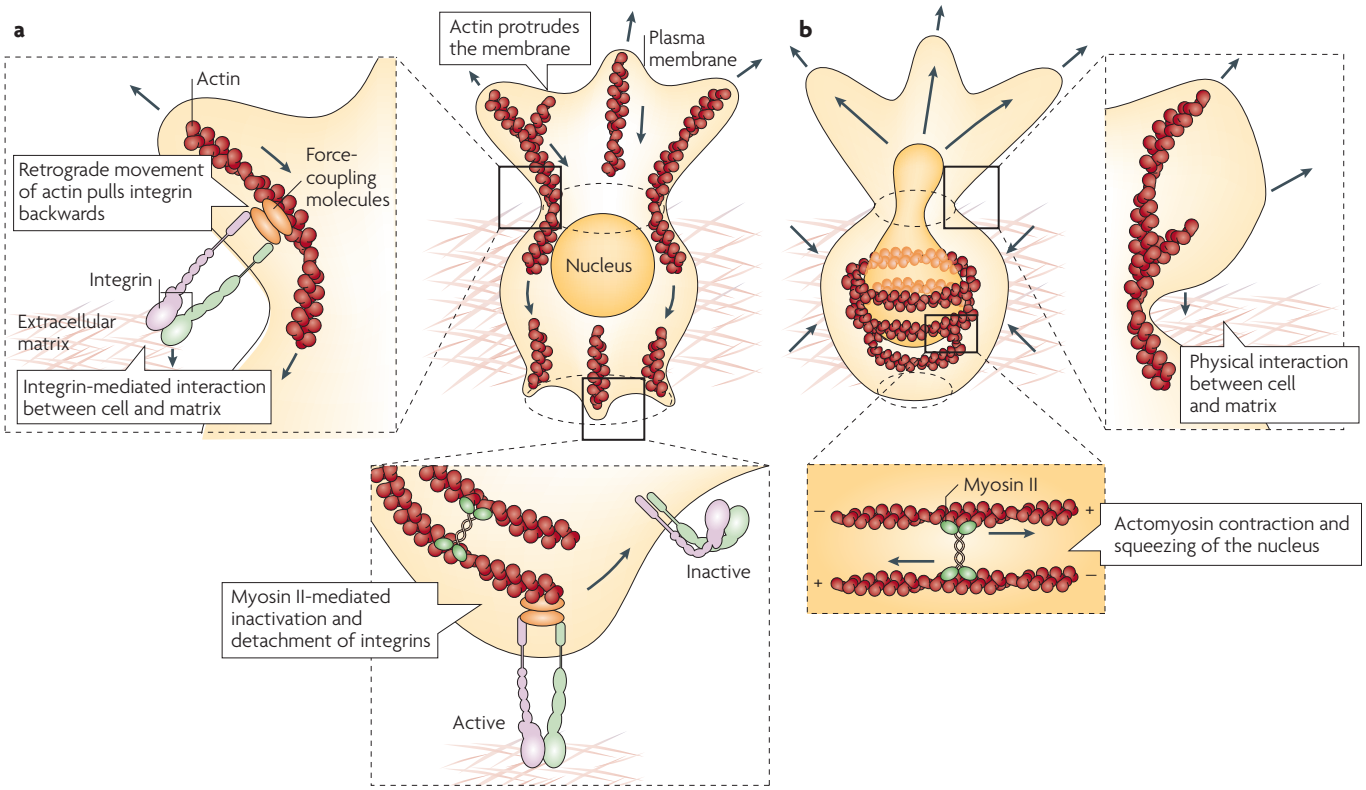

Figure 4 | **Protrusive and hydrostatic principles cooperate to propel leukocytes through a porous interstitium.** **a** | Cell protrusion through a pore in the interstitium, using the force of actin polymerization to bulge out the leading membrane. Pushing against the leading membrane drives the membrane forwards while actin filaments slide towards the trailing edge. If integrins successfully interact at the contact site between cell and substrate, the retrograde sliding of actin is turned into a force that pulls the substrate backwards and thereby the cell body forwards. At the trailing edge, myosin II slides antiparallel actin filaments against each other, leading to the contraction of the actin cortex and subsequent retraction of the tail. Simultaneously, active myosin II causes physical and biochemical inactivation and thus detachment of the integrins. **b** | Cell protrusion through a pore in the interstitium that is too narrow to allow passage of the rigid nucleus. In this case, actomyosin contraction at the trailing edge leads to shrinkage of the actin cortex and thereby squeezes and deforms the nucleus and propels it through the pore. Actomyosin contraction also creates hydrostatic pressure that aids in protruding the membrane or might even protrude the membrane by itself by inducing the formation of actin-free membrane blebs (not shown). If integrin receptors are absent or unable to bind to the substrate, the cell can physically interact with the substrate as the actin cortex braces the membrane and thereby pushes against the substrate, providing the counter-force that is required to advance the cell body.

immediate resealing[80]. Although an entirely biophysical mode of basement membrane penetration is certainly attractive, direct experimental evidence for this possibility is not yet available.

Leukocyte migration in the interstitial tissue

Once detached from the perivascular basement membrane, leukocytes approach their final destination by crawling within the three-dimensional (3D) interstitial space, which can either be a fibrillar network or a cell-packed environment such as organ parenchymas and lymphatic tissues. There is accumulating evidence that the mechanism of leukocyte interstitial migration resembles that of single-celled eukaryotes more than that of most other metazoan cells. Such 'amoeboid' movement, in which cells undergo rapid shape change during locomotion[93], is surprisingly independent of the molecular composition of the extracellular environment (FIG. 4). To what extent leukocytes use extracellular guidance structures as migration tracks remains unclear (BOX 3), but it seems that the extracellular matrix (ECM) modulates rather than strictly determines migration patterns. This mode of locomotion differs grossly from the extravasation steps described above and also from epithelial and mesenchymal movements, during which cells remain tightly integrated in a tissue context through cell–cell or cell–ECM adhesions. Regarding the guidance cues, it is well established that chemokines and lipid mediators maintain the positioning and motile state of leukocytes. However, it is still largely unknown how these mediators are distributed in the tissue and to what extent this distribution leads to random motility (chemokinesis) or directed migration (chemotaxis) in the interstitium.

Intracellular forces mediating interstitial migration. In leukocytes (in contrast to, for example, neurons), force to actively deform the cell is almost exclusively generated by the actomyosin cytoskeleton (FIG. 4). The two principal

Box 3 | **The role of guidance structures in interstitial migration**

Epithelial or mesenchymal cells that use the haptokinetic principle of locomotion require extracellular guidance structures. These cellular or extracellular matrix substrates are bound by integrins and thereby deterministically define the migratory path and at the same time provide an essential survival signal for the cells. Peripheral leukocytes do not depend on anchorage to cellular or extracellular matrix substrates for their survival and can also move independently of integrins[106,116], raising the question of whether they use preformed guidance structures. In lymph node and splenic white pulp, lymphocytes have been shown to migrate along the scaffold of the non-haematopoietic fibroblastic reticular cell network[131]. In the dermis, effector lymphocytes preferentially migrate along thick collagen bundles[132] and in the inflammatory brain parenchyma, a reticular network of undefined nature has been described that serves as a guidance structure for infiltrating lymphocytes[133]. However, in all cases, the cells could also detach from their substrate, which suggests that prominent guidance structures are not required for movement as long as the cells are embedded in a three-dimensional tissue context. It is not known whether the cells, particularly those in peripheral tissues, follow the structures owing to spatial constraints or because the structures are adhesive for the cells. A further possibility, which was suggested for migration in lymphatic organs, is that the guidance structures are decorated with immobilized chemokines that keep the cells motile[131] and eventually induce adhesion to the surface.

types of force that mediate shape change during migration are network extension (actin polymerization) and network shrinkage (actomyosin contraction). F-actin polymerization can directly push against and thereby protrude the plasma membrane to create actin-rich pseudopodia. In leukocytes, polymerization is mediated by Rac, which activates members of the WAVE (WASP-family verprolin homologue; also known as Scar) family, the function of which is to activate the actin polymerizing complex ARP2/3. Indeed, simultaneous deletion of RAC1 and RAC2 in dendritic cells and neutrophils leads to spherical cells that cannot migrate *in vitro* and *in vivo*[94,95]. By contrast, macrophages in which RAC1 and RAC2 were deleted could revert to an alternative mode of migration[96] that seems less dependent on high polymerization rates.

Although polymerization-driven protrusion seems to be integral during interstitial migration of leukocytes, it is difficult to study this response in isolation *in vivo* as it also plays a key part in leukocyte migration through venular walls, and, as a result, cells with severe defects in polymerization do not even reach the interstitium. Most reported defects in interstitial migration result from interference with either upstream regulators or downstream effectors of Rac activity. Indeed, depletion of Rac activators such as the GEFs switch-associated protein 70 (SWAP70)[97], Vav proteins[98] and DOCKs)[99] caused impaired interstitial motility. With respect to downstream effectors, studies to date have mainly focused on members of the WASP family. Interfering with its members WAVE1 (also known as WASF1) and WASP[100] caused reduced but not completely stalled migration, suggesting limited redundancy. In mice with a nonsense mutation in HEM1 (also known as NCKAP1L), which is essential for the stability of WAVE proteins in haematopoietic cells, Rac activity was maintained but locomotion of lymphocytes *in vitro* and *in vivo* was completely blocked, revealing the crucial importance of WAVE proteins[101]. Furthermore, molecules that modulate ARP2/3 activity at the cell's leading edge, such as the haematopoietic cortactin homologue haematopoietic lineage cell-specific protein (HS1)[102] and coronin[103,104], partially affect interstitial migration.

Contraction is mainly mediated by members of the non-muscle myosin II family of motor proteins that have actin cross-linking and contractile (network shrinking) functions. Myosin II activity is controlled by RHOA, which triggers myosin light-chain phosphorylation and activation of contractile motor activity through myosin light chain kinase (MLCK) and Rho-associated protein kinases (ROCKs). Contractility serves two mechanical purposes in leukocyte locomotion: it detaches adhesions at the trailing edge[105] and it generates hydrostatic pressure at the back to squeeze and propel cytoplasmic matter forward. Accordingly, myosin II is distributed in a polarized manner, increasing from the front to the back of the cell[106,107]. As deformability supports squeezing locomotion, the septin cytoskeleton that regulates cortical rigidity greatly affects the ability of lymphocytes to pass through narrow pores[90].

Unlike actin polymerization, contractility is not essential for leukocyte motility[106,108], especially when the pore size of the interstitium is large. This implies that leukocytes are in a low adhesive state during interstitial movement, which eliminates the requirement for contractility to detach the cell posterior (see below; FIG. 4). However, posterior contractility is required during the switch from extravasation to interstitial motility to detach the cell from the abluminal side of the blood vessel[109]. It is currently unclear which extracellular signals trigger the switch to interstitial movement. The absence of shear stress[110], steep local gradients of factors such as sphingosine-1-phosphate[111] and differences in chemokine presentation (soluble versus immobilized) may all have roles in this context.

Force transduction to the environment. Contraction and protrusion by actin polymerization can deform the cell cortex, but only transmitting these forces to the extracellular environment creates the retrograde traction forces that are necessary to move the cell body. How do retrograde forces arise? The common model is based on transmembrane force coupling. In this model, actin filaments that polymerize against the leading membrane push themselves backwards, and myosin II-driven contraction in more posterior regions enhances this actin flow by also pulling the cortex backwards. As a consequence, the whole actin cortex slides towards the posterior pole, where actin filaments depolymerize. Once this retrograde actin flow is coupled across the membrane it causes the phenomenon of receptor capping, during which transmembrane receptors that are externally cross-linked on polarized but non-adherent lymphoblasts are rapidly dragged towards the posterior cell pole, where they accumulate[112]. When these transmembrane receptors bind to an immobilized substrate, the same forces that drive capping lead to the generation of traction forces and thereby cause locomotion of the cell. Importantly, traction forces act in parallel, rather than perpendicularly, to the membrane, meaning that a cell does not necessarily have to tightly adhere to a substrate to move on it. It is only on 2D surfaces that adhesion is a prerequisite for migration because the cells cannot

maintain contact with the surface. This is different in tight 3D environments, where cells are squeezed between surfaces. Accordingly, it was shown that cells that are non-adherent and therefore unable to migrate on 2D surfaces can become highly motile once embedded in a 3D environment, where the contact between cell and substrate is enforced[106,113]. As many leukocytes are either weakly adherent or non-adherent, this explains their entirely different behaviour in 2D versus 3D environments.

Although the force-coupling receptors that mediate traction in leukocytes seem to be exclusively integrins[114], these are not essential for leukocyte motility: the migration of neutrophils in the interstitium *in vivo* only partially depends on integrins[115], and *in vitro* antibody blocking and knockout studies suggest that lymphocyte and neutrophil migration is integrin independent[106,116]. Furthermore, a recent genetic study showed that integrins are not required for interstitial migration of dendritic cells *in vivo*[106]. By visualizing the actin cortex in chemotactic dendritic cells it was subsequently shown that these cells can fully compensate for the loss of integrin-mediated adhesion and the resulting slippage of the cytoskeleton through increased actin polymerization rates[114]. These findings suggest that leukocytes can use alternative mechanisms to generate traction in 3D environments, which may not depend on cytoskeletal coupling to the outside but instead may rely on actin-driven deformations of the cell body. Indeed, recent theoretical models showed that the deformability of an actin network alone can be sufficient to drive locomotion in confined environments[117]. These data and studies localizing intermediate rather than high-affinity integrins to the leading edge of migrating lymphoblasts[118], and findings showing that no specific actin-rich adhesion structures assemble in leukocytes[116], suggest that intermediate-affinity integrins might generate traction for interstitial locomotion. In the absence of integrins or at sites where no integrin ligands are present, leukocytes can switch to a mode of motility that is independent of transmembrane force coupling. High-affinity integrins seem to immobilize leukocytes, and this is supported by findings that genetically altered integrins that are locked in a high-affinity state lead to reduced migration rates owing to impaired detachment of the trailing edge[119,120].

Collectively, leukocyte migration in the interstitium is driven by actin protrusion at the leading edge and occasionally supported by actomyosin contraction at the trailing edge. The cytoskeletal forces can be transduced onto the environment either through integrins or by direct physical interaction of the cell body with the extracellular environment. This flexible mode of migration renders leukocytes largely independent of the molecular composition of the interstitium.

Conclusions

Targeting leukocyte migration remains a principal strategy aimed at developing new therapies for conditions in which there is a need for an enhanced or dampened immune response[121,122]. The slow fruition of therapeutics interfering with the functions of established leukocyte migration targets, such as leukocyte chemokine receptors and adhesion molecules, suggests that new complex experimental disease models are required that more closely mimic human disorders for pre-clinical assessments of drug efficacy and potential adverse effects. In line with this, despite the significant benefits of natalizumab, a monoclonal antibody specific for α4β1 and α4β7 integrins, in the treatment of multiple sclerosis, there is concern regarding the small but increased occurrence of the deadly demyelinating disease progressive multifocal leukoencephalopathy (PML) in patients treated with natalizumab[123].

A better understanding of the mechanisms of action of established anti-inflammatory therapies could also pave the way to a wider use of clinically tested drugs. For example, statins, a class of drugs used for their cholesterol-lowering effects, are now known to also possess broad immunomodulatory and anti-inflammatory properties that seem to include effects on expression and function of key pathways that are involved in leukocyte motility[124]. In addition, a more in-depth understanding of the common and distinct mechanisms involved in regulating leukocyte motility at different stages of their emigration from the vascular lumen to the extravascular tissue and beyond will contribute to the identification of more specific molecular targets and target functions that can be manipulated under different disease conditions without compromising host defence. For example, new targets involved in leukocyte motility through venular walls (such as pathways regulating endothelial cell vesicular trafficking) may lead to the development of strategies for a more selective suppression of leukocyte transmigration that could benefit the treatment of inflammatory disorders such as atherosclerosis. Moreover, manipulating the mechanisms that mediate leukocyte motility in the interstitial tissue (such as pathways involved in the regulation of leukocyte actin networks) with the aim of enhancing tissue infiltration and/or the leukocyte's surveillance and molecular recognition properties could enhance effector functions and thereby improve the treatment of disorders such as cancer and infectious pathologies[121,122].

Finally, as current and emerging data suggest the existence of tissue-, leukocyte- and stimulus-specific mechanisms of TEM, investigations of cellular and subcellular events in different vascular beds using *in vivo* models will be a challenging component of future studies.

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Competing interests statement

The authors declare no competing financial interests.

DATABASES

UniProtKB: http://www.uniprot.org
C3G | CD9 | CD81 | CD151 | DOCK2 | EPAC | ICAM1 | ICAM2 | IL-1β | JAMA | JAMB | JAMC | LSP1 | NOX2 | PAR3 | PAR6 | PECAM1 | PKCζ | RAC1 | RAP1A | RHOA | TIAM1 | TNF | TRIO | VAV1 | VAV2 | VAV3 | VCAM1 | VE-PTP | WASP

FURTHER INFORMATION

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